

Genes Involved in Anaerobic Metabolism of Phenol in the Bacterium *Thauera aromatica*

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Genes involved in the anaerobic metabolism of phenol in the denitrifying bacterium *Thauera aromatica* have been studied. The first two committed steps in this metabolism appear to be phosphorylation of phenol to phenylphosphate by an unknown phosphoryl donor (“phenylphosphate synthase”) and subsequent carboxylation of phenylphosphate to 4-hydroxybenzoate under release of phosphate (“phenylphosphate carboxylase”). Both enzyme activities are strictly phenol induced. Two-dimensional gel electrophoresis allowed identification of several phenol-induced proteins. Based on N-terminal and internal amino acid sequences of such proteins, degenerate oligonucleotides were designed to identify the corresponding genes. A chromosomal DNA segment of about 14 kbp was sequenced which contained 10 genes transcribed in the same direction. These are organized in two adjacent gene clusters and include the genes coding for five identified phenol-induced proteins. Comparison with sequences in the databases revealed the following similarities: the gene products of two open reading frames (ORFs) are each similar to either the central part and N-terminal part of phosphoenolpyruvate synthases. We propose that these ORFs are components of the phenylphosphate synthase system. Three ORFs showed similarity to the *ubiD* gene product, 3-octaprenyl-4-hydroxybenzoate carboxy lyase; UbiD catalyzes the decarboxylation of a 4-hydroxybenzoate analogue in ubiquinone biosynthesis. Another ORF was similar to the *ubiX* gene product, an isoenzyme of UbiD. We propose that (some of) these four proteins are involved in the carboxylation of phenylphosphate. A 700-bp PCR product derived from one of these ORFs cross-hybridized with DNA from different *Thauera* and *Azoarcus* strains, even from those which have not been reported to grow with phenol. One ORF showed similarity to the *mutT* gene product, and three ORFs showed no strong similarities to sequences in the databases. Upstream of the first gene cluster, an ORF which is transcribed in the opposite direction codes for a protein highly similar to the DmpR regulatory protein of *Pseudomonas putida*. DmpR controls transcription of the genes of aerobic phenol metabolism, suggesting a similar regulation of anaerobic phenol metabolism by the putative regulator.

Phenolic compounds are important plant constituents. For example, lignin represents about 20 to 30% of the biomass of higher plants and consists of phenylpropane units with various hydroxyl or methoxyl groups. Phenol is formed from lignin and several other natural or synthetic substrates during microbial degradation. The best-known phenol-generating enzyme is tyrosine phenol lyase, whose activity is used in bacterial taxonomic studies. Because of its toxic effects on cells, phenol was used as an antiseptic in clinical applications for a long time. In the chemical industry, phenol is a basic substance for the production of phenolic compounds from coal. Because of its broad application in industry, phenolic compounds are among the most prominent man-made groundwater contaminants derived from coal treatment processes, such as gasifying coal.

The aerobic metabolism of phenol has been studied extensively; in all known aerobic pathways, mono-oxygenases initiate the degradation of phenol by hydroxylation to catechol (for a recent review on phenol *ortho* hydroxylases, see reference 60). Among *ortho*-hydroxylating phenol hydroxylases, one-component flavoproteins or multicomponent enzyme systems are recognized. The following ring cleavage is a key reaction in microbial degradation of aromatic compounds. Catechol is oxygenolytically cleaved by dioxygenases by either *ortho* or *meta* cleavage.

Anaerobic growth on phenol has been observed for various bacteria (4, 24, 37, 55, 59, 62, 63, 64, 68). In all cases studied, phenol appeared to be carboxylated to 4-hydroxybenzoate and growth on phenol was dependent on the presence of CO₂ (62). Consortia of fermenting bacteria convert phenol to benzoate (24, 68) and decarboxylate 4-hydroxybenzoate to phenol (24, 68). They also catalyze an isotope exchange between D₂O and the proton at C-4 of the aromatic ring of phenol (23).

Phenol carboxylation to 4-hydroxybenzoate is a paradigm for a new type of biological carboxylation reaction. The process has been studied in the denitrifying bacterium *Thauera aromatica* (3, 32–34, 63) (Fig. 1). The gram-negative, rod-shaped bacterium belongs to the β -group of the proteobacteria and was enriched and isolated under denitrifying conditions with phenol as substrate (1, 62; for similar strains, see references 61 and 64). It has a broad substrate spectrum and is able to grow anaerobically on many aromatic compounds. Cells grown with phenol were simultaneously adapted to growth with 4-hydroxybenzoate, whereas 4-hydroxybenzoate-grown cells did not metabolize phenol. Induction of the capacity to metabolize phenol required several hours.

Phenol carboxylation proceeds in two steps and seems to involve formation of phenylphosphate as the first intermediate (equation 1) (32, 33). An enzyme activity catalyzing an isotope exchange between [¹⁴C]phenol and the phenyl moiety of phenylphosphate was identified in extracts of phenol-grown cells (equation 3) and was lacking in 4-hydroxybenzoate-grown cells. [³²P]Phosphate did not exchange with phenylphosphate. This suggests a phosphorylating enzyme, E₁, with a ping-pong

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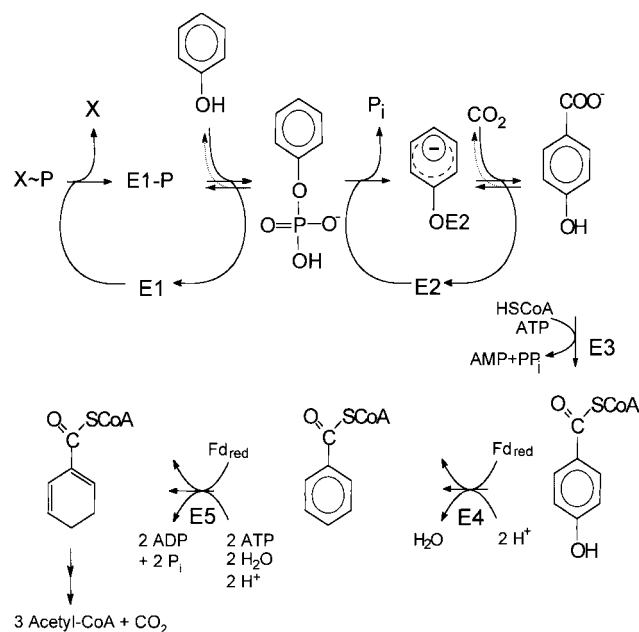
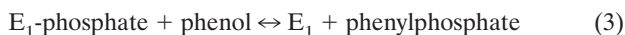
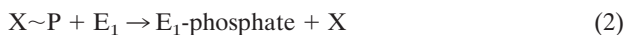
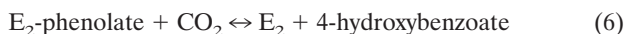
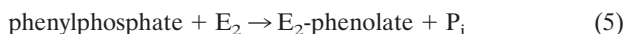
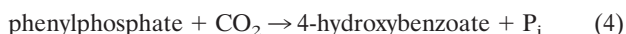


FIG. 1. Postulated pathway of anaerobic phenol metabolism in the denitrifying bacterium *T. aromatica*. E1, phenylphosphate synthase; E2, phenylphosphate carboxylase (the mechanism by which the phenolate anion is bound to the enzyme E2 is unknown so far); E3, 4-hydroxybenzoate-CoA ligase; E4, 4-hydroxybenzoyl-CoA reductase (dehydroxylating, ferredoxin dependent); E5, benzoyl-CoA reductase (dearomatizing, ferredoxin dependent). For further metabolism of benzoyl-CoA to three acetyl-CoA, one CO₂, and six H molecules, see reference 25. Fd, ferredoxin. X~P, phosphoryl donor, unknown so far.

mechanism which becomes phosphorylated in an essentially irreversible step (equations 2 and 3). The phosphorylated enzyme E₁ is postulated to transform phenol to phenylphosphate in a reversible reaction (equation 3). The whole reaction is understood as the sum of equations 2 and 3. Unfortunately, the phosphoryl donor X~P is unknown so far. The enzyme E₁ was termed "phenylphosphate synthase."



Phenylphosphate is postulated to be the substrate of a second enzyme, E₂ (32). It requires Mn²⁺ and catalyzes the carboxylation of phenylphosphate to 4-hydroxybenzoate (equation 4). An enzyme activity catalyzing an isotope exchange between ¹⁴CO₂ and the carboxyl group of 4-hydroxybenzoate (equation 6) was present in phenol-grown cells; [¹⁴C]phenol did not exchange with 4-hydroxybenzoate. It was assumed that the carboxylation reaction (equation 4) and the CO₂ isotope-exchange reaction (equation 6) are catalyzed by the same enzyme, reaction 6 being a partial reaction of reaction 4. The CO₂ isotope-exchange activity again suggests a ping-pong mechanism for enzyme E₂ (equations 5 and 6), involving an enzyme E₂-phenolate intermediate which is formed in a (presumably) exergonic reaction (equation 5) followed by a reversible carboxylation (equation 6).



The actual substrate is CO₂ rather than bicarbonate (as is the case in biotin-dependent carboxylases), and the carboxylating enzyme was not inhibited by avidin, a potent inhibitor of biotin-dependent carboxylases. Both results suggest that biotin is not involved in carboxylation. The enzyme E₂ was termed "phenylphosphate carboxylase." Synthesis of both the phosphorylating and the carboxylating enzymes is strictly regulated. All activities were only present in cells grown anaerobically on phenol and were lacking, e.g., in 4-hydroxybenzoate-grown cells (29, 34). The phenol-carboxylating enzyme in *T. aromatica* does not belong to any group of the studied carboxylases, as it seems to proceed via a phosphorylated free intermediate, it is extremely oxygen sensitive and sensitive to radical-trapping agents (S. Breinig, M. Hügler, and G. Fuchs, unpublished results), and it is not dependent on biotin or thiamine diphosphate. It differs from most known carboxylases by using carbon dioxide as substrate and by the requirement of a metal as cocatalyst.

Further metabolism of 4-hydroxybenzoate proceeds in two steps to the intermediate benzoyl coenzyme A (CoA) (Fig. 1). A specific CoA ligase forms 4-hydroxybenzoyl-CoA (8), which becomes reductively dehydroxylated to benzoyl-CoA by a molybdo-flavo-iron-sulfur protein, 4-hydroxybenzoyl-CoA reductase (11, 14). A 2 [4Fe-4S] ferredoxin serves as electron donor for this reaction (14). The ligase and reductase are both induced in phenol- and in 4-hydroxybenzoate-grown cells, but not in benzoate-grown cells (29). Benzoyl-CoA is a common intermediate in the metabolism of many aromatic compounds. It is reductively dearomatized (9, 10) and further metabolized to three acetyl-CoA molecules and one CO₂ molecule (note that one CO₂ was originally introduced into phenol) (reviewed in reference 28). The anaerobic metabolism of phenol is shown in Fig. 1.

Nothing was known so far about the molecular background of anaerobic phenol metabolism. Based on the N-terminal and internal peptide sequence information of five phenol-induced proteins, it was our aim to find the genes coding for phenol-induced proteins. Analysis of the DNA sequences should provide new insights into the function of the phenol-induced proteins and into the regulation of anaerobic phenol metabolism.

MATERIALS AND METHODS

Materials. Chemicals were reagent grade and purchased from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), or Sigma-Aldrich (Deisenhofen, Germany). Biochemicals were from Boehringer (Mannheim, Germany). Scintillation cocktail and acrylamide stock solution were purchased from Roth (Karlsruhe, Germany). [¹⁴C]Na₂CO₃ (2 GBq mmol⁻¹) was purchased from American Radiolabeled Chemicals Inc./Biotrend Chemikalien GmbH (Cologne, Germany). Nitrogen, helium, and a N₂-H₂ gas mixture (95% nitrogen, 5% hydrogen) were purchased from Linde (Höllriegelskreuth, Germany).

Bacterial strains, growth conditions, cell harvesting, and storage. Strains of *T. aromatica*, K172 (1, 62), S100 (62), SP (54), LG356 (unpublished results), and T1 (61), and *Azoarcus evansii* KB740 (12) were cultured anaerobically at 30°C in a mineral salt medium with 0.5 mM phenol, 10 mM bicarbonate, and 2 mM nitrate or with 5 mM 4-hydroxybenzoate and 15 mM NaNO₃ as described elsewhere (11, 62). Growth was measured by following the optical density at 578 nm (OD₅₇₈). An OD₅₇₈ of 1.0 corresponded to a cell concentration of approximately 0.3 g of dry cell mass per liter. The harvested cells were immediately frozen and stored in liquid nitrogen. Growth conditions of *Escherichia coli* strains (XL1-blue MRF' [$\Delta(\text{mcrA})183 \Delta(\text{mcrCB-hsdSMR-mrr})173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI}^{\text{q}} \Delta\text{Z}\Delta\text{M15 Tn10 (Tet}^{\text{r}}\text{)]}$]; K38 [hfrC phoA4 pit-10 tonA22 ompF627 relA1 spoT⁻]; P2392 [E14⁻ (McrA⁻) hsdR514 supE44 supF58 lacY1 or $\Delta(\text{lacIZY})6 \text{ galK2 galT22 metB1 trpR55 (P2 lysogen)}$]) and XL0LR [$\Delta(\text{mcrA}) 183 \Delta(\text{mcrCB-hsdSMR-mrr})173 \text{ endA1 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI}^{\text{q}} \Delta\text{Z}\Delta\text{M15 Tn10 (Tet}^{\text{r}}\text{)]}$] Su⁻ (nonsuppressing) λ^{I} (lambda resistant)) were as previously described (50).

Preparation of cell extract. All steps were performed under strictly anaerobic conditions. Frozen cells (20 g) were suspended in 20 ml of 20 mM imidazole-HCl (pH 6.5), 10% glycerol, 0.5 mM dithionite, and traces of DNase I, disrupted by a passage through a French press (137 MPa), and ultracentrifuged (1.5 h at

100,000 × g and 4°C). The supernatant containing the soluble protein fraction (55 mg of protein/ml) was used immediately or stored at -20°C for later experiments. The soluble protein fraction (cell extract) contained all 4-hydroxybenzoate: ¹⁴C₂ exchange and phenylphosphate carboxylase activity.

Enzymatic tests. All tests were conducted at 30°C under strictly anaerobic conditions. The assays used 1 ml of assay mixture in a stoppered vial with a gas phase of approximately 250 μl. The assays for ¹⁴C₂:4-hydroxybenzoate isotope exchange and carboxylation of phenylphosphate were performed as described elsewhere (32, 43). The amount of radioactivity in acid-stable products (4-hydroxybenzoate) in a sample of 250 μl was analyzed by liquid scintillation counting. The amount of labeled 4-hydroxybenzoate formed was calculated from the amount of fixed radioactivity, taking into account the known specific radioactivity of bicarbonate in the assay (80 Bq/nmol).

Partial purification of three dominant phenol-induced proteins, F1, F2, and F3. All chromatographic steps were performed under anaerobic conditions in a glove box under an N₂-H₂ atmosphere (95%/5% [vol/vol]) at 4°C. All buffers were filtered and degassed and then supplemented with dithionite to a final concentration of 0.5 mM. Generally used buffers were as follows. Buffer A contained 20 mM imidazole-HCl (pH 6.8), 20 mM KCl, 0.5 mM MnCl₂, and 10% glycerol. Buffer B contained buffer A plus 500 mM KCl. The purification was started with extract (approximately 20 ml) from 20 g of frozen cells grown on phenol and nitrate.

Ion-exchange chromatography 1. Cell extract (20 ml) was applied on a DEAE-Sepharose fast-flow column (35 ml; Pharmacia), which was run at a flow rate of 0.5 ml/min. The column was washed with 50 ml of buffer A, followed by a flat linear gradient from 20 mM KCl (100% buffer A) to 200 mM KCl (60% buffer A, 40% buffer B) in 100 ml and a steeper gradient from 200 mM KCl (60% buffer A, 40% buffer B) to 500 mM KCl (100% buffer B) in another 100 ml. Fractions of 2.5 ml were collected, and 6-μl samples of every second fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Compared with extract of 4-hydroxybenzoate-grown cells, three dominant phenol-induced proteins, F1 (60 kDa), F2 (58 kDa), and F3 (67 kDa), were separated. F1 eluted between 60 and 100 mM KCl, F2 eluted between 140 and 190 mM KCl, and F3 eluted between 250 and 350 mM KCl. Fractions containing F1, F2, and F3 were pooled and tested for phenylphosphate carboxylase and CO₂ isotope exchange activities.

Ion-exchange chromatography 2. Two milliliters of the pooled fractions of F1, F2, and F3 after DEAE-Sepharose fast-flow chromatography was applied to a high-performance liquid chromatography (HPLC) system equipped with a MonoQ HR5/5 column (1 ml; Pharmacia), which was run at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected in each run, and aliquots of 10 μl were analyzed by SDS-PAGE. For F1 purification, the column was equilibrated with buffer A containing 60 mM KCl and eluted with this buffer until the UV absorption baseline at 280 nm was stable. Samples of every fraction were tested for both activities and analyzed by SDS-PAGE. F1 eluted after 5 to 10 column volumes. For F2 purification, the column was equilibrated with buffer A containing 150 mM KCl. The column was washed with this buffer until the UV absorption baseline at 280 nm was stable. F2 eluted after 10 to 14 column volumes. For F3 purification, the column was equilibrated with buffer A containing 260 mM KCl and washed until the baseline at 280 nm was stable. Then, a linear gradient of 260 to 400 mM KCl in buffer A (8 column volumes) was applied. F3 eluted early in the gradient between 260 and 330 mM KCl.

Affinity chromatography on blue Sepharose. Ten milliliters of the F2 pool after chromatography on DEAE was loaded on a blue Sepharose column (48 ml; Pharmacia), equilibrated with 20 mM Tris-HCl, pH 7.5, at a flow rate of 2 ml/min. Because of the instability of the color matrix in the presence of reducing agents, no dithionite was added. The column was washed with 1 volume of the same buffer, and then F2 was eluted with 10 mM phenylphosphate in 20 mM Tris-HCl, pH 7.5, in 3 volumes. Fractions of 5 ml were collected, and 100-μl samples of each fraction were analyzed by SDS-PAGE (10% acrylamide).

SDS-PAGE. Polyacrylamide gels (normally 11.5% acrylamide) were prepared as previously described (35). Staining of the proteins was done according to a previously described method (67).

Two-dimensional gel electrophoresis. The first dimension (isoelectric focusing) was done with cell extract (120 μg of protein) according to Görg et al. (25) by using the Immobiline Dry Strip system (linear pH gradient 3 to 10; Pharmacia) according to the manufacturer's protocol. The second dimension (SDS-PAGE) was performed as described above. Comparison of extracts of cells grown on phenol and 4-hydroxybenzoate allowed the identification of phenol-induced proteins.

Protein transfer onto polyvinylidene difluoride membranes. Proteins of cell extract and of fractions after chromatography were electrophoretically separated on SDS-polyacrylamide gels and were then transferred to an Immobilon-P⁹⁴ transfer membrane (Millipore, Bedford, Mass.) for N-terminal sequencing of the phenol-induced proteins by using the Nova Blot System (Multiphor II; Pharmacia LKB, Freiburg, Germany) according to the manufacturer's protocol.

Amino acid sequencing and tryptic digest. Protein spots from cell extract of phenol-grown cells obtained after two-dimensional electrophoresis and fractions containing the phenol-induced proteins F1 to F3 after chromatography on MonoQ were transferred to a polyvinylidene difluoride membrane. Induced proteins were excised and sequenced using an Applied Biosystems 473A sequencer or an automated gas phase sequencer. The sequencing was performed

according to the Edman method. The separated amino acid derivatives were analyzed by C₈ reversed-phase HPLC. For peptide sequencing, the pooled fractions containing F2 after chromatography on blue Sepharose were digested with modified trypsin (Promega, Mannheim, Germany) as follows: 500 μg of protein in 200 μl of 20 mM Tris-HCl, pH 7.5, was adjusted to pH 8 with 3 μl of triethylamine, and 10 μg of trypsin in 10 μl of H₂O (Promega sequencing grade modified) was added. The digest was carried out at 37°C for 4 h. The reaction was stopped by heating for 5 min to 100°C. After centrifugation, volumes of 5, 70, and 100 μl were sequentially applied to the HPLC. The peptides generated were separated on a reversed-phase C₁₈ Superpac-Sephasil HPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden).

Cloning, DNA sequencing, and nucleic acid manipulations. Plasmids used for transformation of *E. coli* strains were pBK-CMV phagemid [f1(-)ori lacZ CMV SV40 poly(A) colE1 ori Neo^r Kan^r], pBluescript II KS/SK(±) [f1(±)ori lacZ colE1 ori Amp^r], and pGP1-2 [Kan^r T7 Gen1]. Oligonucleotides used for amplification of the probes for screening via PCR were as follows (>, forward; <, reverse; all in 5' to 3' direction): fubrei p374 (>CGGGCGCTTTCCGGCACA), fubrei 375 (<CGGACTTCGCGCATCTTGT), F2H (>ATGGAYCTSCGSTACTTCATC), F243R (<CATSAGGAAYTCSGCCTGCTG), λ15H (>TCGC CGGCGACGACGCCG), and λ15R (<CCGCGCGCTGCGCCGCCG). PCR products used as probes were labeled with [α-³²P]dCTP (Amersham). Standard protocols were used for DNA cloning, transformation, amplification, and purification (50). Recombinant plasmids were generated in pBluescript vectors and were maintained in *E. coli* XL1-blue. *T. aromatica* DNA has a high GC content (67%). This made sequencing difficult because of the compression phenomenon. Three methods were applied. First, sequencing of plasmid DNA by the Sanger method was performed with ³⁵S-dATP. Second, sequencing of a PCR product with internal Cy5-dATP labeling, 0.5 μg of template/kbp, was performed according to the manufacturer's protocol and analyzed with an ALF express (Pharmacia). Third, cycle sequencing of PCR DNA according to the manufacturer's protocol (Pharmacia) was performed. Sequences were analyzed with an ALF express (Pharmacia). Part of the sequencing was performed with an ALF Automated Sequencer (Pharmacia).

Cloning strategy. Based on the N-terminal amino acid sequences of F2 and of two internal fragments, degenerate oligonucleotides were designed according to the codon usage of *T. aromatica*. A 500-bp PCR product (F2 forward and F2T6 reverse primers) of the N-terminal sequence of F2 was labeled with [³²P]dCTP and used as a probe for screening the λ EMBL3 gene library. One phage hybridized with the probe. From the positive λ clone, two adjacent *Bam*HI fragments of 2.7 and 4.0 kbp and an *Eco*RI fragment of 5.25 kbp were subcloned in pBluescript and sequenced (for restriction sites, see Fig. 5). The boundary between the *Bam*HI fragments was verified by amplification and sequencing of an overlapping DNA fragment. A double-stranded contiguous sequence of ca. 9 kbp was obtained from the insert of this λ clone. To obtain sequence information of the 5' and 3' flanking regions of the first λ clone, gene libraries of *T. aromatica* were rescreened. A 500-bp probe of the 5' boundary of the known sequence was amplified and used to screen the λ EMBL3 library again. Two positive clones were obtained whose inserts were subcloned in pBluescript. Plasmids containing parts of the known sequence were identified by PCR assays and used for DNA sequencing. A total of 6.2 kbp of DNA was sequenced from two overlapping subclones containing either a 3.7-kbp *Pst*I fragment or a 9-kbp *Bam*HI fragment. A 600-bp probe of the 3' boundary of the known sequence was amplified and used to screen a λ ZAP library. One positive phage was detected and converted to a phagemid as recommended by the supplier (Stratagene). It contained an insert of 8 kbp which overlapped with the known sequence and was used to sequence a further 400 bp of the 3' flanking region.

Southern hybridization. DNA fragments resolved by agarose gel electrophoresis were partially hydrolyzed by gently rinsing in 0.25 M HCl for about 20 min, followed by denaturing in 0.5 M NaOH-1.5 M NaCl for another 20 min. Finally, the gel was neutralized by excessively rinsing it in 1 M Tris-HCl-3 M NaCl, pH 7, three times for 10 min each. Then, the DNA was vacuum blotted onto a nylon/NC membrane with the help of a vacuum gel dryer system (Slab Gel Dryer 2003; Pharmacia LKB). After the blot procedure, the slots of the gel were marked on the membrane and the membrane was allowed to dry at room temperature. Fixation of the DNA on the membrane was achieved by incubating for 2 h at 80°C. In addition, chromosomal DNA was also blotted by capillary blotting, which guaranteed a better transfer of the large fragments (50).

Screening of gene banks of *T. aromatica*. A λEMBL3 gene bank (Stratagene) and a λZAPExpress gene bank (Stratagene) were prepared and screened according to the manufacturer's protocol.

PCR. PCR conditions were as follows: 100 ng of target DNA, 200 nM concentrations of each primer, 200 μM (each) dATP, dCTP, dTTP, and dGTP, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 1 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The assay was overlaid with 75 μl of paraffin oil. PCR parameters were as follows: 30 s at 95°C, 1 min at 40°C, 2.5 min at 72°C, 30 cycles. For amplification of fragments larger than 3 kbp, *Taq* DNA polymerase from Qiagen (Hilden, Germany) was used under the following conditions (*Taq* PCR kit from Qiagen): 100 ng of target DNA, a 200 nM concentration of each primer, 200 μM (each) dATP, dCTP, dTTP, dGTP, (NH₄)₂SO₄, and KCl, 4.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.7), 1 × Q solution, and 1 U of *Taq* DNA polymerase (Qiagen). PCR parameters were as follows: 30 s at 95°C, 1 min at 45°C, 2.5 min at 72°C, 30 cycles.

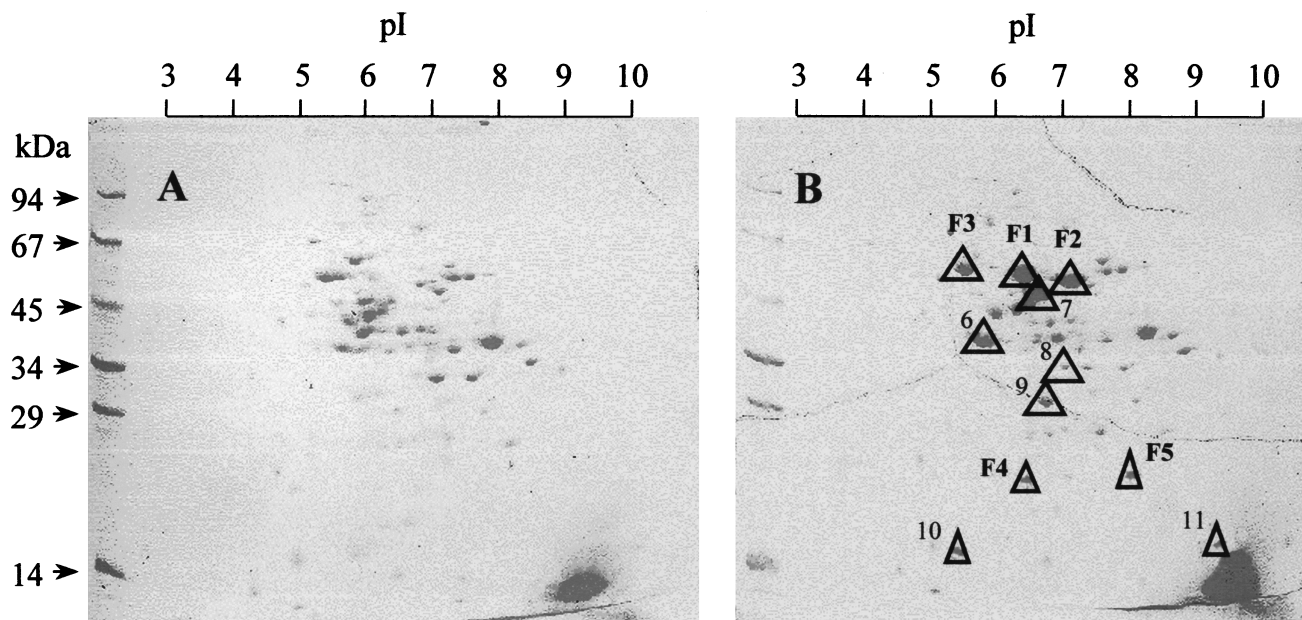


FIG. 2. Two-dimensional gel electrophoresis of soluble proteins (supernatant obtained after centrifugation at $100,000 \times g$) of *T. aromatica* anaerobically grown on 4-hydroxybenzoate (A) and phenol and CO_2 (B). Triangles indicate phenol-induced proteins. F1 to F5 were isolated and their N-terminal amino acid sequences were determined.

Expression of the *T. aromatica* genes. Heterologous expression of the genes was performed as previously described (13) for *E. coli* K38 containing the pGP1-2 plasmid, which contains the gene for the heat-inducible T7 RNA polymerase. Strain K38 was transformed according to a previously described method (18) with the pBluescript vector containing different parts of the phenol-induced gene cluster of *T. aromatica* under the control of a T7 promoter.

Computer analysis. The nucleotide and amino acid sequences were analyzed using the PC/gene software package (Genofit) and the Open Reading Frame Finder (ORF Finder; <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Similar sequences were identified using the BLAST search using the TBLASTN algorithm provided by the National Center for Biotechnology Information.

Nucleotide sequence accession number. All sequence data have been deposited in the EMBL nucleotide sequence database under accession no. AJ272115.

RESULTS

Detection of phenol-induced proteins. Growth on phenol and nitrate obviously requires induction of proteins that are required for anaerobic phenol metabolism. This follows from the observation that 4-hydroxybenzoate-grown cells were not able to metabolize phenol immediately, but only after a prolonged adaptation period of several hours. Furthermore, phenol-grown cells contained several protein bands in SDS-PAGE that were missing in 4-hydroxybenzoate-grown cells (not shown). Therefore, the soluble fraction (supernatant obtained after centrifugation at $100,000 \times g$) and the membrane fraction (pellet obtained after centrifugation at $100,000 \times g$, not shown) of extracts of cells that were grown anaerobically on phenol and 4-hydroxybenzoate, respectively, were compared in more detail by two-dimensional gel electrophoresis (Fig. 2).

The soluble fraction contained up to 11 phenol-induced proteins. Three of them, called F1, F2, and F3, showing apparent molecular masses of about 60, 58, and 67 kDa, respectively, were strongly visible and were stained with similar intensity on SDS-polyacrylamide gels. The pI values were estimated to be 6.5, 7.0, and 5.5, respectively. Two further induced proteins, called F4 and F5, with apparent molecular masses of about 20 kDa and pI values of approximately 6.5 and 8.0, respectively, could be identified. Another two protein spots (no. 10 and 11 in Fig. 2) were only weakly stained and, because of their size (apparently about 14 kDa), were hardly visible. Their pI values

were estimated to be 5.5 and 9.0. Up to four additional phenol-induced proteins were not clearly resolved from neighbor proteins; their apparent molecular masses were in the range of 30 to 55 kDa, and their pI values were between 5.8 and 7.0. The membrane fraction did not contain proteins that were strongly phenol induced.

Partial purification of phenol-induced proteins. The high content of phenol-induced proteins encouraged us to purify phenylphosphate carboxylase and CO_2 isotope exchange activity, despite its oxygen sensitivity. A chromatographic separation of cell extract on a DEAE-Sepharose fast-flow column is shown in Fig. 3A. Both activities coeluted in two fractions, and both contained phenol-induced proteins F1 and F2, although at different ratios. Both activities seemed to require the presence of some amount of the protein F1. Neither activity was measured in a third fraction containing the protein F3. The subfractions of the first two fractions were pooled and tested for both activities. In the first fraction, 35% of the residual activity of the CO_2 isotope exchange reaction and 15% of the phenylphosphate carboxylase activity could be detected, and in the second fraction, 20 and 10% of the activities, respectively, were detected. No combination of the three fractions restored full activity.

The two pooled DEAE fractions containing mainly F1 and F2 were chromatographed isocratically on MonoQ HR5/5, and the pooled DEAE fraction containing mainly F3 was chromatographed on MonoQ HR5/5 under refined conditions by using a linear KCl gradient. This resulted in further purification of the three proteins (Fig. 3B), but activity was completely lost. When the three DEAE fractions were applied to blue Sepharose at pH 7.0, F1 to F3 bound to the column and were eluted with 1 M KCl. At pH 7.5, F2 was specifically eluted with 10 mM phenylphosphate (Fig. 3C).

N-terminal sequencing of five phenol-induced proteins (F1 to F5), peptide sequencing of F2, and synthesis of degenerate primers. Three phenol-induced proteins, F1 to F3, were enriched by chromatographic techniques in the enzymatically inactive form, and two other phenol-induced proteins, F4 and

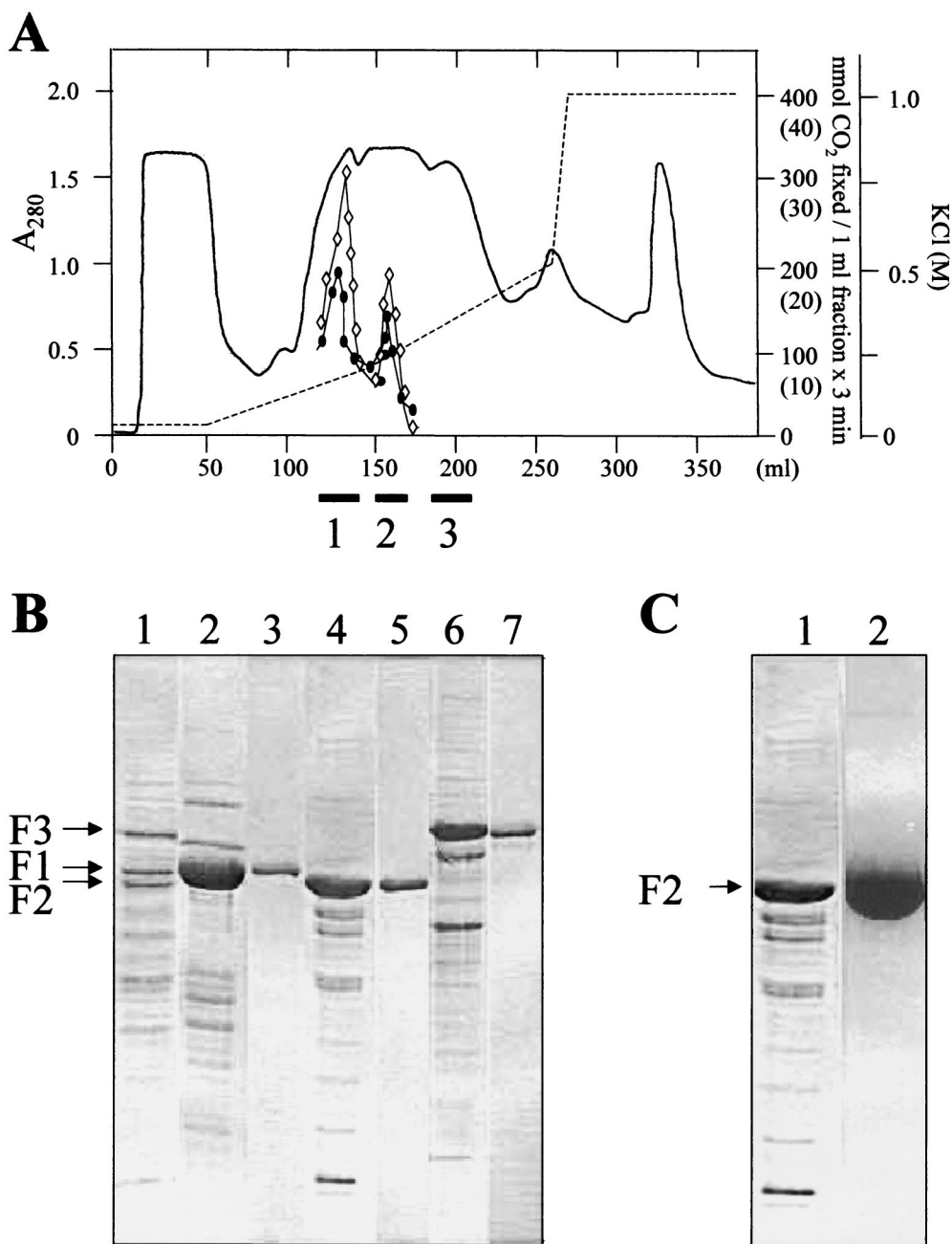


FIG. 3. Partial purification of phenol-induced proteins. (A) DEAE-Sepharose fast-flow chromatography of 15 ml of the soluble fraction (supernatant obtained after centrifugation at $10^5 \times g$) of *T. aromatica* grown on phenol and nitrate. Fractions containing the F1, F2, and F3 proteins (identified by SDS-PAGE) that were pooled are indicated as black bars (1, 2, and 3, respectively) below the x axis. Only 10 to 20% of both the phenylphosphate carboxylating activity and the isotope exchange were detectable in the F1 and F2 pools. In the F3 pool, none of the activities were measurable. Circle, phenylphosphate carboxylase activity (values in parentheses). Rhombus, isotope-exchange activity. (B) SDS-10% PAGE of the fractions obtained by chromatography of the soluble protein fraction on a DEAE-Sepharose fast-flow column. Lane 1, extract of cells grown with phenol and nitrate; lane 2, pooled fractions containing F1; lane 4, pooled fractions containing F2; lane 6, pooled fractions containing F3; lanes 3, 5, and 7, F1 to F3 were further purified by chromatography on MonoQ. The three proteins eluted with 60, 200, and 330 mM KCl, respectively. (C) SDS-10% PAGE of F2 (fractionated by DEAE-Sepharose fast flow) after chromatography on blue Sepharose. Lane 1, DEAE fraction of F2; lane 2, main fraction of F2 eluting with 10 mM phenylphosphate from blue Sepharose.

F5, were obtained from two-dimensional gels. The material was sufficient for N-terminal sequencing and peptide sequencing of F2. Up to 40 amino acids were determined, with only a few ambiguous amino acid residues. Comparison of the experimentally determined sequences with the sequences deduced from the genes (see below) showed very good correspondence, with only very few mismatches, and most uncertain amino acids were confirmed to be correct (Fig. 4). The results of the N-

terminal sequencing were used to design degenerate primers, taking into account the known codon usage of *T. aromatica*. Amino acid sequences that were used as templates are underlined in Fig. 4.

Screening of a λ EMBL3 and a λ ZAP express gene library. Based on the N-terminal amino acid sequences of F2 and of an internal fragment of F2, degenerate oligonucleotides were designed. The primers used for the generation of labeled probes

	N-Terminal Amino Acid Sequence Determined by Edman Degradation	N-Terminal Amino Acid Sequence Deduced from the Genes
F1	gKIS APKNN REFIE AsVKS GDAVR IRQEV DWDNE AGAIV rRA	MGKIS APKNN REFIE ACVKS GDAVR IRQEV DWDNE AGAIV RRA
F2	<u>MDLRY</u> <u>FINQX</u> AEAHE LKRIT TEVDW NLEIS HVsKL XXe (F2H)	<u>MDLRY</u> <u>FINQC</u> AEAHE LKRIT TEVDW NLEIS HVsKL TEE
F3	MKFPV PHDIQ AKTIP GTEGW ERMYP XXXAF VXd	MKFPV PHDIQ AKTIP GTEGW ERMYP YHYQF VTD
F4	MEQAK NIKLV	MEQAK NIKLV
F5	MRIVV GMXGA	MRIVV GMSGA
N-Terminal Amino Acid Sequence of Internal Fragments of F2 after Trypsin Digest Determined by Edman Degradation		
	.FHEGG gg. .MQMLD DK. .QVADA VIASN TGSYg M. .FWSVV DER. .IXTEV DWNLE ISXV. .TATLW TELEQ MR. .YIGTM VSVVL YDPET GR. .GQQAE <u>FLMAX</u> XXXXP VXAGA EIVLE XGI. (F2T43R) .GQQAE FLM..	

FIG. 4. Determined and deduced N-terminal amino acid sequences of phenol-induced proteins F1 to F5 and internal sequences of protein F2. X, amino acids that could not be sequenced; small letters, amino acids that were uncertain. Underlined amino acids were used as templates for primer design (names of the forward and reverse primers are given in parentheses). F1 corresponds to Orf6, F2, to Orf4, F3 to Orf1, F4 to Orf5, and F5 to Orf8.

to screen the libraries are listed in Materials and Methods. A labeled 500-bp PCR product corresponding to the N-terminal sequence of F2 (F2H/F2T43R) was used as a probe for screening a λ EMBL3 gene library. One phage hybridized with the probe. The insert DNA was prepared and subcloned in pBlue-script vectors. About 9 kbp of sequence information of both DNA strands was obtained.

To get more sequence information 5' of the first segment, a new probe was amplified by PCR (fubrei p374 and fubrei 375) and used for gene bank screening. Two positive phages whose DNA was subcloned in pBluescript vectors were obtained. The sequences overlapped with the 5' sequences of the clone obtained by the first screen.

To obtain more sequence information 3' of the sequences of the first screen, a λ ZAPExpress gene library was screened with a 600-bp probe derived from the 3' end of the sequences (λ 15H/ λ 15R). One positive clone was detected, and the phagemid DNA was prepared. The size of the DNA insert was estimated to be at least 8 kbp by agarose gel electrophoresis. The 5' sequences of the phagemid insert overlapped with the 3' sequences of the first screen.

DNA sequencing and identification of the genes coding for putative proteins involved in anaerobic phenol metabolism. Screening of the gene libraries and sequencing of the subcloned DNA resulted in sequence information for 14,272 bp. Eleven open reading frames (ORFs) were detected (Fig. 5). Table 1 summarizes the location and orientation of ORFs 1 to

11 and, the number of amino acids encoded by each ORF, as well as the percent similarity and percent identity of the amino acid sequences of ORFs 1 to 11 with known sequences in the databases.

Ten ORFs are transcribed in the same direction. The first six ORFs (*orf1* to *orf6*) are separated by less than 65 bp and amount to 7,210 bp. This cluster of putative genes is followed by a 658-bp noncoding region whose transcript contains two putative hairpin mRNA secondary structures. Another cluster of four putative genes with fewer than 40 bp of intergenic regions amounts to 2,702 bp. Downstream of *orf10*, 840 bp which did not code for proteins were sequenced. Upstream of *orf1* and transcribed in the opposite direction, another putative gene was found, *orf11*, which was separated by 428 bp from *orf1*.

Comparison of deduced amino acid sequences of ORFs 1 to 11 with the experimentally determined N-terminal and internal amino acid sequences of phenol-induced proteins revealed that the following genes code for known proteins: *orf1* for F3, *orf4* for F2, *orf5* for F4, *orf6* for F1, and *orf8* for F5 (Fig. 4). The predicted molecular masses agree reasonably well with the experimentally determined masses (Table 1). The similarities of the gene products possibly involved in anaerobic phenol metabolism with other proteins in the databases, comparison of deduced and experimentally determined sequences, and some extrapolated parameters of the proteins compared to

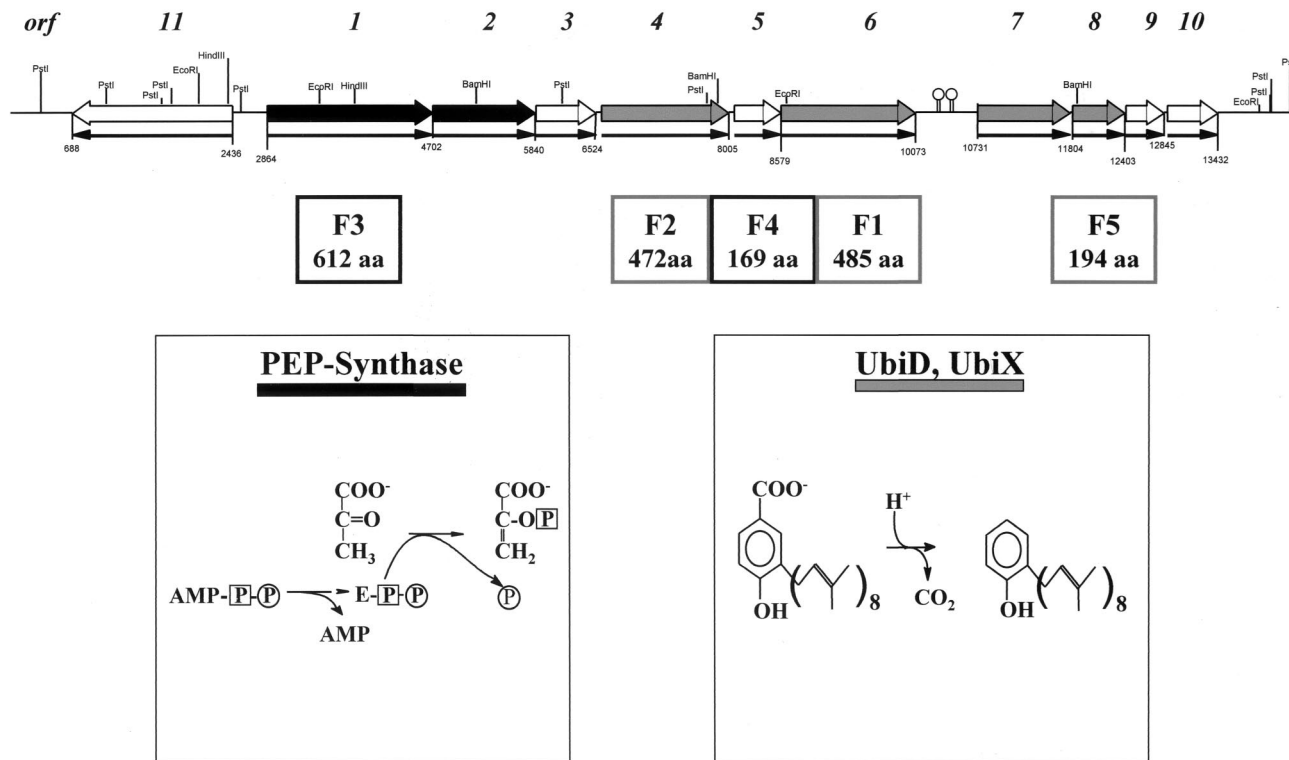


FIG. 5. Organization of the clusters of genes possibly involved in anaerobic phenol metabolism in *T. aromatica*. Phenol-induced proteins F1 to F5 and the corresponding number of amino acids (aa) are shown below the ORFs. The reactions catalyzed by PEP synthase and 3-octaprenyl-4-hydroxybenzoate carboxy lyase (isoenzymes UbiD and UbiX) are shown below; these enzymes show similarity to some of the proteins possibly involved in phenol metabolism. Like colors (black and grey) stand for genes and gene products with similarities. Black arrows indicate the direction of transcription. The coding regions (sizes in base pairs) are indicated, as well as the restriction sites of various enzymes.

those of the phenol-induced protein spots are summarized in Table 1.

Protein F3 (encoded by *orf1*) and the *orf2* gene product show similarity to parts of phosphoenolpyruvate synthase (PEP synthase) of *E. coli*. The reaction catalyzed by PEP synthase is shown in Fig. 5. The C-terminal half of F3 is similar to the central part of PEP synthases and contains a highly conserved histidine (amino acid 569) which is known to become phosphorylated in the PEP synthase reaction (histidine 421 in the central part of PEP synthase of *E. coli*). The *orf2* gene product shows similarity to the N-terminal domain of PEP synthase. In *E. coli*, this domain is probably involved in ATP binding. We propose that ORFs 1 and 2 code for two proteins belonging to the phenylphosphate synthase system, whereby phenol ultimately becomes phosphorylated. This proposal seems plausible, considering the structural similarity between the enol form of pyruvate and phenol, respectively.

ORFs 4, 6, and 7 code for F2, F1, and a not-yet-identified protein, respectively. Sequence alignment shows that each of these gene products is overall similar to the *ubiD* gene product of *E. coli*, 3-octaprenyl-4-hydroxybenzoate carboxy lyase. This enzyme is involved in the biosynthesis of ubiquinone; the reaction catalyzed is shown in Fig. 4. *orf8* codes for F5 and shows high similarity to *ubiX*, a gene which codes for an isoenzyme of 3-octaprenyl-4-hydroxybenzoate carboxy lyase (UbiD). UbiX and F5 were also similar to phenylacrylic acid decarboxylase from *Saccharomyces cerevisiae*. An alignment of a conserved amino acid sequence motif showed that all three proteins have a common E-X-P motif. We propose that the proteins encoded by *orf4* and *orf6* to *orf8* are involved in the carboxylation of

phenylphosphate or similar phenolic compounds; this proposal is based on the similarity of the known reactions of the similar proteins, decarboxylation of 3-octaprenyl-4-hydroxybenzoate or phenylacrylic acid, to the phenylphosphate carboxylase reaction.

The function of four gene products is enigmatic. Orf3 showed similarity to a putative inosine-5'-monophosphate dehydrogenase of *Methanobacterium thermoautotrophicum*. Orf5 (F4) and Orf9 showed similarities to hypothetical and conserved proteins whose functions are unknown. Orf10 has high similarity to the putative Mutator T protein (MutT) of *Methanococcus jannaschii*.

orf11 codes for a protein that shows strong similarity to the DmpR gene regulator of *Pseudomonas putida*, which regulates expression of genes involved in aerobic metabolism of phenolic compounds (41, 57, 58). Therefore, we assume that Orf11 is the regulator of anaerobic phenol metabolism.

Expression of F1 to F5 proteins in *E. coli*. Five of the 11 ORFs were identified as genes coding for F1 to F5. However, there was no proof that the other putative genes were expressed as well. Therefore, several DNA fragments were ligated into pBluescript KS under the control of the heat-inducible T7 polymerase promoter and transformed into *E. coli* K38, and expression was induced. The following DNA fragments were used (compare with Fig. 5): 3.7-kbp *PstI* fragment containing *orf1* (which codes for the F3 protein) and *orf2*; a 2.7-kbp *BamHI* fragment containing *orf3* and *orf4* (which codes for F2); a 4.0-kbp *BamHI* fragment containing *orf5* (which codes for F4), *orf6* (which codes for F1), and *orf7*; and a 5.25-kbp *EcoRI* fragment containing the 3' end of *orf6*, the intergenic

TABLE 1. Properties of the gene products involved in anaerobic phenol metabolism and similarities with other proteins in the databases^a

Gene product	Similar protein ^b	Gene properties ^c		Protein properties ^d				Protein correspondence ^e				Protein with highest similarity ^f	
		Dir	Range	Length (aa)	Mol mass (kDa)		pI	% Identity	% Similarity	E value	References	Protein	Protein
					Deduced	Exp							
Orf11	(7)	←	688–2436	582	63.756	55	6.14	6.5	72.3	IE–20	gnl P D t1010531 (D63814)	DmpR (transcriptional activator)	
Orf1	F3	→	2864–4702	612	70.261	67	5.06	5.5	39.3	4E–10	gjl147146 (M69116)	PEP synthase (central part)	
Orf2	(6)	→	4716–5840	374	40.423	40	5.38	5.5	34.5	1E–63	gjl147146 (M69116)	PEP synthase (N-terminal part)	
Orf3	(9)	→	5853–6524	223	24.244	30	6.19	6.5	30.2	1E–8	gij2621183 (AE000803)	Inosine-5'-monophosphate dehydrogenase	
Orf4	F2	→	6587–8005	472	52.605	58	6.06	7.0	58.95	5E–47	gij549586 sp P26615 UbiD	3-Octaprenyl-4-hydroxybenzoate carboxylase	
Orf5	F4	→	8070–8579	169	18.450	18	5.62	6.5	63.8	2E–25	gij2851406 sp P45396 YrbI	Hypothetical protein	
Orf6	F1	→	8616–10073	485	53.940	60	5.64	6.5	57.1	1E–31	gij549586 sp P26615 UbiD	3-Octaprenyl-4-hydroxybenzoate carboxylase	
Orf7	(8)	→	10731–11804	357	38.198	35	6.68	7.0	47.5	7E–25	gij549586 sp P26615 UbiD	3-Octaprenyl-4-hydroxybenzoate carboxylase	
Orf8	F5	→	11819–12403	194	21.262	20	6.97	8.0	86.8	5E–56	gij2507150 sp P09550 UbiX	3-Octaprenyl-4-hydroxybenzoate carboxylase	
Orf9	(11)	→	12414–12845	143	15.209	17	7.60	9.0	64.8	8E–13	gij2622617 (AE000910)	Hypothetical protein	
Orf10	(10)	→	12884–13432	182	19.936	18	5.76	5.5	62.7	2E–9	gij2129134 pir D64443	Mutator protein MurT	

^a The deduced properties of the gene products of *orf1* to *orf11* (as determined by diverse computer programs) are compared with the properties of phenol-induced proteins detected in two-dimensional gels.

^b Numbers in parentheses refer to the numbers of the spots of corresponding proteins in a two-dimensional gel in Fig. 2.

^c Dir, direction of transcription. The range is of the nucleotides of the corresponding gene.

^d Mol mass, molecular mass; pI, isoelectric point; Exp., experimentally determined (two-dimensional gels); aa, amino acids. The length is the number of amino acids of the corresponding translated gene product.

^e Overall similarity and identity of the amino acid sequence to proteins in the databases are given. % Identity, the percentage of amino acids that are identical or conserved between the two proteins; E value, Expect value. The Expect value estimates the statistical significance of the match, specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

^f Given are references of similar proteins. Database accession numbers are in parentheses.

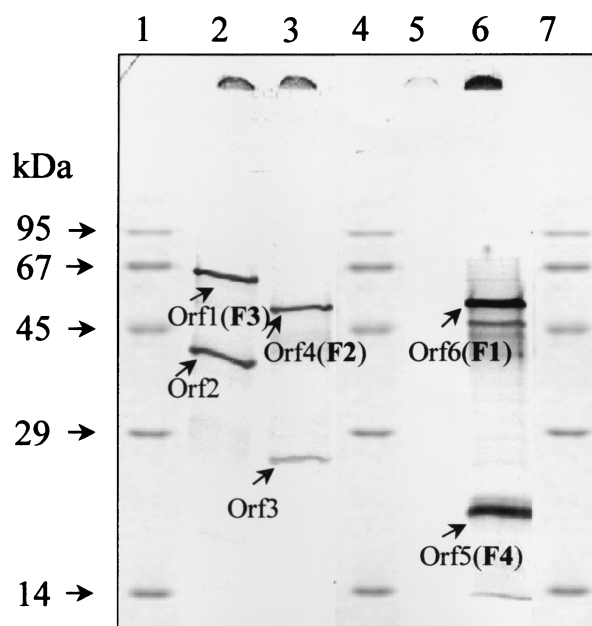


FIG. 6. Expression of ORFs 1 to 6 in *E. coli* (T7 polymerase experiment). A volume of 25 μ l was loaded onto each lane. Lanes 1, 4, and 7, marker proteins; lane 2, 3.7-kbp *Pst*I fragment containing ORF1 and ORF2; lane 3, 2.7-kbp *Bam*HI fragment containing ORF3 and ORF4; lane 5, 5.25-kbp *Eco*RI fragment containing ORF7, ORF8, ORF9, and ORF10; lane 6, 4.0-kbp *Bam*HI fragment containing ORF5, ORF6, and ORF7.

region between *orf6* and *orf7*, and the complete sequences of *orf7*, *orf8* (coding for F5), *orf9*, and *orf10*. The proteins were separated by SDS-PAGE and localized by autoradiography (Fig. 6).

orf1 and *orf2* were obviously transcribed by the T7 polymerase. On the SDS-polyacrylamide gel, the resulting proteins F3 and Orf2 migrated at apparent molecular masses of 67 and 41 kDa, respectively, which agreed reasonably well with the predicted molecular masses. *orf3* and *orf4* were also both transcribed by the T7 polymerase. F2 (encoded by *orf4*) was strongly induced and had an apparent molecular mass of approximately 58 kDa. This was slightly larger than expected according to the nucleotide sequence (52 kDa), but agreed with the apparent molecular mass of F2 prepared from *T. aromatica*. In contrast, expression of *orf3* was not strongly induced and ORF3 was hardly visible on the autoradiogram. However, the apparent molecular mass of 24 kDa agreed with the predicted molecular mass. The experiment with the 4.0-kbp *Bam*HI fragment containing *orf5* to *orf7* showed the expression of only two proteins. Their apparent sizes of approximately 19 and 60 kDa corresponded to those of the phenol-induced proteins F4 and F1, respectively. As in cell extract, F1 ran with a molecular mass larger than that expected from the nucleotide sequence (54 kDa). No expression was observed for *orf7*, which would have resulted in a 39-kDa protein. The putative secondary mRNA structures between *orf6* and *orf7* may have prevented translation of *orf7*.

Using the 5.25-kbp *Eco*RI fragment, none of the ORFs from *orf7* to *orf10* were expressed. This may be due to the fact that the secondary structures downstream of *orf6* may have prevented transcription of *orf7* to *orf10*. Despite several attempts, we did not succeed in subcloning the DNA sequence of *orf7* to *orf10* without the putative secondary structures and thus could not express these genes. However, expression of *orf8* is clearly induced in *T. aromatica* in the presence of phenol, as F5 is one

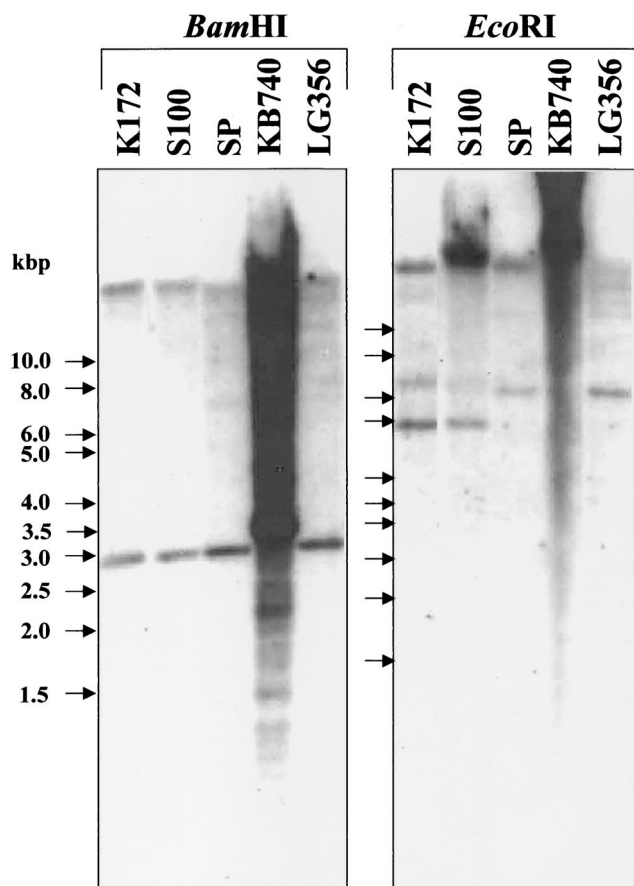


FIG. 7. Southern blot of genomic DNA of *T. aromatica* strains (type strain K172, strains S100, SP, and LG356) and *A. Evansii* type strain KB740. The DNA was restricted with *Bam*HI and *Eco*RI. After separation with agarose gel electrophoresis, DNA fragments were blotted onto a nylon membrane and hybridized with a 32 P-labeled probe for the 5'-terminal part of the gene of F2. Arrows indicate the positions of the marker DNA, whose sizes are given in kilobase pairs (kbp).

of the phenol-induced proteins. This suggests that the second gene cluster consisting of *orf7* to *orf10* indeed codes for structural phenol-induced proteins.

Genomic Southern hybridization of chromosomal DNA of *T. aromatica* and other bacterial strains. Genetic work with *T. aromatica* is troublesome because of poor growth on agar plates. To obtain data on a possible correlation of the phenol-induced genes and their function in anaerobic phenol metabolism, a 32 P-labeled 750-bp PCR product (F2H/F2t43R) that corresponded to the 5' part of the gene coding for F2 (*orf4*) was used as a probe for Southern hybridization. The probe should only hybridize with the DNA of those strains that were closely related to *T. aromatica* and able to metabolize phenol. The 16S rRNA of *Thauera* sp. strain S100 is 100% identical and that of strain SP is more than 98.9% identical to that of *T. aromatica* type strain K172 (E. Stackebrandt and G. Fuchs, unpublished results). The phenol-induced genes should not be present in strains that could not have been shown to grow on phenol, like *Thauera* sp. strain LG356 (which has 100% 16S rRNA sequence identity with strain SP) and *Azoarcus Evansii* type strain KB740.

The result of the genomic Southern experiment was surprising (Fig. 7). DNA of all organisms hybridized with the labeled probe. Moreover, the sizes of the hybridizing fragments seemed to be very similar in most of the organisms. In

all organisms, the probe hybridized with the undigested DNA and/or fragments larger than 15 kbp. The experimentally determined sizes of hybridizing fragments of the digested chromosomal DNA of *T. aromatica* were consistent with the expected sizes. Only after *Eco*RI digestion did the probe hybridize with two fragments instead of one, as expected. The strongly hybridizing fragment of about 5 kbp corresponded to the expected one, and the 7-kbp fragment hybridized only weakly, which may be due to unspecific binding of the probe. *T. aromatica* type strain K172 and strain S100 showed the same sizes and patterns of hybridizing fragments; strain SP differed slightly in its hybridization pattern. Hybridization of strain SP DNA was expected, because this organism is also able to grow on phenol. Slight differences in the hybridization patterns may be explained with slight differences in the genome sequences of the three organisms.

Unexpectedly, chromosomal DNA of strain LG356 also hybridized. Moreover, the hybridization pattern of strains LG356 and SP were quite different, although these organisms have 100% identical 16S rRNA sequences. Even *A. Evansii* DNA hybridized; however, the hybridization pattern was totally different from those of the *Thauera* strains, which may be due to major differences in the genome sequences of *Thauera* and *Azoarcus* species. The results of the genomic Southern experiment showed that there is no simple correlation between the presence of the F2 gene and reported growth on phenol.

DISCUSSION

Genes and gene organization. Eleven ORFs were cloned and sequenced, and up to 11 phenol-induced proteins were identified by two-dimensional gel electrophoresis. Table 1 summarizes the deduced sizes and isoelectric points of these gene products. Five of the genes coded for the identified phenol-induced proteins F1 to F5. Comparison of the experimentally determined and deduced values showed that the molecular masses agreed reasonably well with the prediction.

However, the deduced pI values (as determined by diverse computer programs) did not fit exactly to those estimated experimentally. In general, the deduced pI values were 0.5 to 1.0 U lower than the experimentally determined values. This discrepancy may be due to inaccurate determination of the pI values, which were extrapolated assuming an immobilized linear pH gradient of 3 to 10. Six phenol-induced protein spots could not be directly attributed to the corresponding genes by purification or extraction mainly due to the poor separation of the proteins. Although the estimated molecular masses and pI values fit reasonably well with those predicted from ORFs 2, 3, 7, and 9 to 11, the identification is very preliminary and may be incorrect in some cases. For example, protein spot no. 7 may essentially fit to the gene product of *orf11*, which encodes the putative regulator protein of phenol metabolism, but it is unlikely that a regulator protein is induced in such amounts.

Ten of the 11 genes are transcribed in the same direction and are organized in two gene clusters. To underline the correlation between the genes and the induced proteins, the genes were cloned under the control of the T7 polymerase promoter and were expressed in *E. coli*. All genes of the first gene cluster (*orf1* to *orf6*) were transcribed and expressed. Probably due to the possible RNA hairpin secondary structures between the two clusters, we did not observe expression of the second cluster. However, the fact that the induced protein F5 (which is encoded by *orf8* of the second gene cluster) is expressed in the presence of phenol in *T. aromatica* leaves no doubt that the second gene cluster is expressed as well.

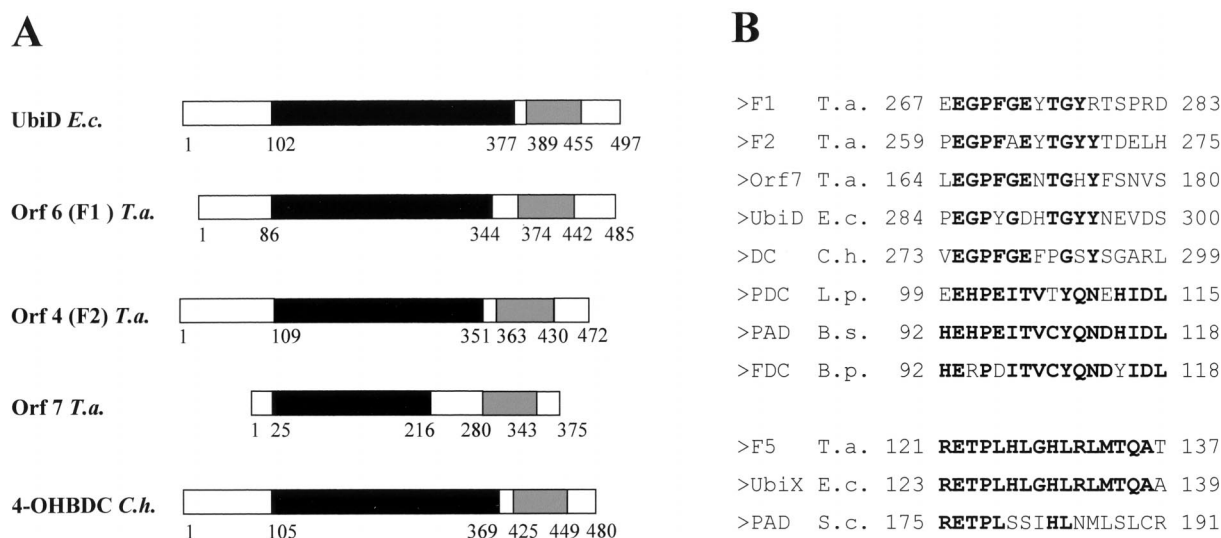


FIG. 8. Alignment of the conserved regions of F1, F2, and Orf7 possibly involved in phenol metabolism showing similarity to 3-octaprenyl-4-hydroxybenzoate carboxy lyase of *E. coli* and to 4-hydroxybenzoate decarboxylase of *C. hydroxybenzoicum* (A) and regions of conserved amino acid sequences in several decarboxylases (B). (A) Boxes with like colors (black and grey) represent similar domains. UbiD, 3-octaprenyl-4-hydroxybenzoate carboxy lyase; 4-OHBDC, 4-hydroxybenzoate decarboxylase; F1, F2, and Orf7, phenol-induced proteins of *T. aromatica*. (B) PDC, *p*-coumaric acid decarboxylase; PAD, phenylacrylic acid decarboxylase; FDC, ferulate decarboxylase. Bold letters represent amino acids that are identical in more than 50% of the sequences. The numbers refer to the first and last positions of the motif in the corresponding sequence. *E.c.*, *E. coli*; *T.a.*, *T. aromatica*; *C.h.*, *C. hydroxybenzoicum*; *S.c.*, *Saccharomyces cerevisiae*; *L.p.*, *Lactobacillus plantarum*; *B.s.*, *Bacillus subtilis*; *B.p.*, *Bacillus pumilus*.

Possible functional assignment of the genes. The two essential steps in phenol metabolism are the phosphorylation of phenol to phenylphosphate and the subsequent carboxylation of phenylphosphate to 4-hydroxybenzoate. Two gene products showed similarity to PEP synthase and four gene products showed similarity to 3-octaprenyl-4-hydroxybenzoate carboxy lyase, respectively, both of *E. coli*. Because of the similar chemistry involved in the respective reactions, we suggest that these genes are coding for phenol-phosphorylating and phenylphosphate-carboxylating enzymes, respectively.

Orf1 and Orf2, homologues of PEP synthase. Two ORFs, *orf1* (coding for F3) and *orf2*, code for proteins of 70.2 and 40.4 kDa with similarity to the central and the N-terminal parts, respectively, of PEP synthase. Thus, the gene products appear to belong to the family of the structurally and functionally related PEP-utilizing enzymes, which comprises three groups of proteins (19, 20): (i) PEP synthase (or pyruvate:water dikinase [EC 2.7.9.2]), (ii) pyruvate:orthophosphate dikinase (EC 2.7.9.1), and (iii) PEP-protein phosphotransferase (EC 2.7.3.9), which is enzyme I of the PEP-dependent phosphotransferase system. Two sequence signatures are shared by all these proteins and by no other proteins in the current protein databases. The first sequence signature (PROSITE, accession no. PS00370) contains an active-site histidine residue that mediates phosphoryl transfer. The second sequence signature (PROSITE, accession no. PS00742) is a conserved region, the exact role of which is unknown. The functional similarity of the enzymes lies in the transfer of a phosphoryl group via a phospho-histidine intermediate and in the utilization of PEP or pyruvate as a substrate; the corresponding active sites are located on independently folded domains. The *E. coli* PEP synthase (175 kDa) is a dissociable dimer of identical subunits (87.4 kDa), and histidine residue 442 has been identified as the phosphorylation site (6, 42). In the thermophilic archaeobacterium *Staphylothermus marinus*, PEP synthase is a homomultimeric enzyme complex whose subunits have a similar structure as the subunits of the homodimeric *E. coli* enzyme (20).

T. aromatica Orf1 (protein F3) shows 54% similarity to the

central part of PEP synthase of *E. coli* and carries the conserved sequence motif of PEP-utilizing enzymes (PROSITE, accession no. PS00370) that contains the active-site histidine residue. The PEP synthase of *B. subtilis* contains the (putative) active-site histidine at the C-terminal part of the protein, as does F3. The N-terminal part of F3 shows no similarity to any region of the known PEP synthase sequences or to other sequences in the databases. Orf2 showed 60% similarity to the N-terminal part of PEP synthase of *E. coli*. We believe that the gene products of *orf1* and *orf2* together form an active enzyme complex responsible for phosphorylation of phenol. The phosphoryl donor for this reaction remains unknown, although a number of potential cosubstrates have been tested (33).

Orf4, -6, -7, and -8, homologues of 3-octaprenyl-4-hydroxybenzoate carboxy lyase. The gene products of *orf4* (F2), *orf6* (F1), and *orf7* showed 47 to 63.8% similarity to UbiD (55.2 kDa) (Table 1 and Fig. 8), and the product of *orf8* (F5) showed 86.8% similarity to UbiX (20.7 kDa). UbiD catalyzes the third reaction in ubiquinone biosynthesis, namely, the decarboxylation of 3-octaprenyl-4-hydroxybenzoate to 3-octaprenylphenol (Fig. 5). The gene *ubiX* encodes an isoenzyme of UbiD that catalyzes the same reaction. So far, only a few biochemical data are available on both enzymes. The holoenzyme UbiD of *E. coli* has a molecular mass of 340 kDa, and its activity is dependent on the presence of Mn^{2+} and an unidentified, heat-stable factor with a mass of <10 kDa (36). Moreover, the activity was increased by adding membrane preparations or phospholipids, indicating that the enzyme normally functions in association with the membrane.

We assume that some or all of those four genes code for enzymes involved in carboxylation of phenylphosphate to 4-hydroxybenzoate because decarboxylation of 3-octaprenyl-4-hydroxybenzoate to 3-octaprenylphenol can be compared to the reverse reaction of phenylphosphate carboxylase. Interestingly, the four genes of *T. aromatica* are distributed on two different clusters of genes. F1 and F2 are encoded in one cluster together with the genes possibly encoding the phenol-phosphorylating enzymes (F3 and Orf2), whereas Orf7 and F5 are

	75		138
MutT (75-149)	L [!] Y [!] L [!] H	P [!] A [!] V [!] A [!] V [!] D [!] G [!] I [!] E	K [!] D [!] N [!] K [!] I [!] L [!] L [!] I [!] K [!] R
Orf10 (33-107)	F [!] H [!] H [!] K	P [!] A [!] P [!] V [!] V [!] L [!] A [!] V [!] I [!] E	H [!] A [!] G [!] Q [!] L [!] V [!] L [!] I [!] R [!] R
Consensus	l [!] h [!] h [!]	p [!] a [!] p [!] a [!] v [!] d [!] a [!] l [!] e [!]	h [!] a [!] n [!] q [!] i [!] l [!] l [!] i [!] r [!] r
	139	149	
MutT (75-149)	K [!] S [!] L [!] L [!] G [!] V [!] Y [!] S [!] S [!] P	D	
Orf10 (33-107)	D [!] E [!] L [!] L [!] G [!] V [!] Y [!] S [!] Q [!] A	D	
Consensus	d [!] e [!] l [!] i [!] G [!] V [!] Y [!] s [!] q [!] a	D	

FIG. 9. Alignment of highly conserved regions of a protein of *T. aromatica* encoded by *orf10* possibly involved in anaerobic phenol metabolism showing similarity to putative 8-oxo-dGTPase/ADP-ribose pyrophosphatase (MutT) of *M. jannaschii*. In parentheses are given the amino acid regions of the domains. !, either I or V; %, either F or Y; #, either N, D, Q, or E.

encoded in a second gene cluster where no phosphorylating enzymes seem to be encoded (Fig. 5). Both clusters are expressed in the presence of the substrate phenol, as gene products of both were identified as strongly phenol-induced proteins.

Like the *E. coli* enzyme UbiD, the phenylphosphate carboxylase and isotope exchange activities were both dependent on Mn^{2+} . Phenylphosphate carboxylase of *T. aromatica* could be purified by gel filtration chromatography with little loss of activity. This indicated that, unlike 3-octaprenyl-4-hydroxybenzoate decarboxylase, no low-molecular-weight factor is required for enzyme activity.

There are recent reports that enzymes involved in anaerobic degradation of vanillic acid (4-hydroxy-3-methoxybenzoic acid) (17), 3,4-dihydroxybenzoic acid (28a) and 4-hydroxybenzoic acid (30) show similarity to the UbiD/UbiX system of *E. coli*, and this similarity is extended to the phenol system in *T. aromatica* (Fig. 8). The *vdcBCD* gene cluster in *Streptomyces* sp. strain D7 codes for proteins specifically involved in vanillic acid decarboxylation to 2-methoxyphenol (guaiacol). VdcC is similar to UbiD in *E. coli* and to Orf4, -6, and -7 in *T. aromatica*. VdcB is similar to UbiX and phenylacrylic acid decarboxylase of *S. cerevisiae* and to Orf8 of *T. aromatica*. VdcD shows similarity to an unknown protein of *Sphingomonas* and YcdD (function not known) of *Bacillus subtilis*; in *T. aromatica*, no gene product similar to VdcD was found. Wiegel and co-workers identified the gene coding for the (reversible) 4-hydroxybenzoate decarboxylase (54.4 kDa) in *Clostridium hydroxybenzoicum* (30). This enzyme was similar to the three UbiD-like gene products of *T. aromatica* and to UbiD from *E. coli* (53.4 kDa) (17). Two other ORFs, *orf2* and *orf3*, downstream of the gene for the 4-hydroxybenzoate decarboxylase were similar to the *vdcDB* genes of *Streptomyces* sp.; however, *orf3* seems not to be complete. A sequence motif with highly conserved amino acids was also found in three decarboxylases from *Lactobacillus plantarum* (15), *B. subtilis* (16), and *Bacillus pumilus* (66). In all listed protein sequences, an E-X-P motif is conserved and may therefore play a role in substrate binding or catalysis of (hydroxy-) aryl acid decarboxylation or carboxylation of the corresponding phenolic compounds.

Obviously, in the *Clostridium*, *Streptomyces*, and *Thauera* species, the genes coding for UbiD-like decarboxylases are arranged in one or two clusters together with *ubiX* homologues. In *E. coli*, however, *ubiD* and *ubiX* are widely separated on the chromosome. There remain open questions about *T. aromatica*: why there are four genes with similarity to (reversible) decarboxylases organized in two clusters and why both clusters are transcribed in the presence of phenol. Perhaps phenol acts as a gratuitous inducer of the second gene cluster that may play a role in the metabolism of other phenolic compounds (for a classical example of gratuitous induction, see reference 5).

Orf10, homologue of a mutator T protein. The gene product of *orf10* showed approximately 60% similarity to the mutator protein MutT of *M. jannaschii* (Table 1 and Fig. 9). Mutator

proteins are enzymes involved in DNA metabolism which are widespread in prokaryotes and eukaryotes. The 15 members of the MutT family of proteins are believed to act in nucleoside phosphate metabolic pathways. 8-Oxo-7,8-dGTP (8-oxo-dGTP) is formed in the nucleotide pool of a cell during normal cellular metabolism and causes AT-to-GC transversion mutations when it is incorporated into DNA (7, 22, 53). The MutT protein of *E. coli* and related mammalian enzymes specifically hydrolyze 8-oxo-dGTP to 8-oxo-dGMP and PP_i , thereby preventing the occurrence of transversion mutation (38, 43). Members of this class of enzymes require two divalent cations per active site for activity, one coordinated by the enzyme and the other by the enzyme-bound nucleoside triphosphate (39). The substrate of proteins of the MutT family varies from 8-oxo-dGTP and diphosphoinositol polyphosphate (49) to adenosine(5')triphospho(5')adenosine, ADP-ribose, and NADH (43). One can only speculate which role Orf10 plays in the anaerobic metabolism of phenol in *T. aromatica*, e.g., in phenol activation.

Orf11, a putative regulator protein. One ORF located 5' of the two gene clusters and transcribed in the opposite direction codes for a protein with 72% similarity to the regulator protein DmpR of *P. putida* CF600, which regulates aerobic (dimethyl) phenol metabolism (Fig. 10). DmpR is a member of the NtrC family of transcriptional activators and activates transcription of the *dmp* operon, which contains all 15 structural genes needed for the degradation of (dimethyl)phenol, in response to aromatic effector compounds (58). These effectors are phenol, *o*-cresol, *m*-cresol, *p*-cresol, and 3,4-dimethylphenol, and also ethylphenol, catechol, and 3- and 4-methylcatechol. MopR, the regulator of phenol degradation in *Acinetobacter calcoaceticus* NCIB8250, also belongs to the NtrC family (51). Like all regulators of the NtrC family, Orf11 contains several conserved protein domains (40). The highly conserved central (C) domain probably binds and hydrolyzes ATP and promotes open-complex formation with RNA polymerase. The C-terminal (D) domain is expected to bind to the corresponding target DNA sequence ("enhancer"). The highly variable sensory domain is joined to the C domain by short interdomain linker B. NtrC-like regulators sense their respective signals by direct binding of the effector to the A domain (56). Effector binding to the A domain then leads to release of the C domain (21, 56), thus permitting the binding of ATP followed by an ATP-driven cycle of multimerization and demultimerization of the regulator at the enhancer sites (45). This supports open-complex formation of σ^{54} -containing RNA polymerase with the promoter (65). We propose that Orf11 controls the expression of the first or both gene clusters by a similar mechanism and acts as a transcriptional activator, when phenol or a similar compound plus ATP is present.

In *P. putida* strains P35X and CF600, the difference in the spectrum of aromatic growth substrates seems to be due to the different specificity of effector binding to the regulator protein rather than due to the different substrate specificity of the

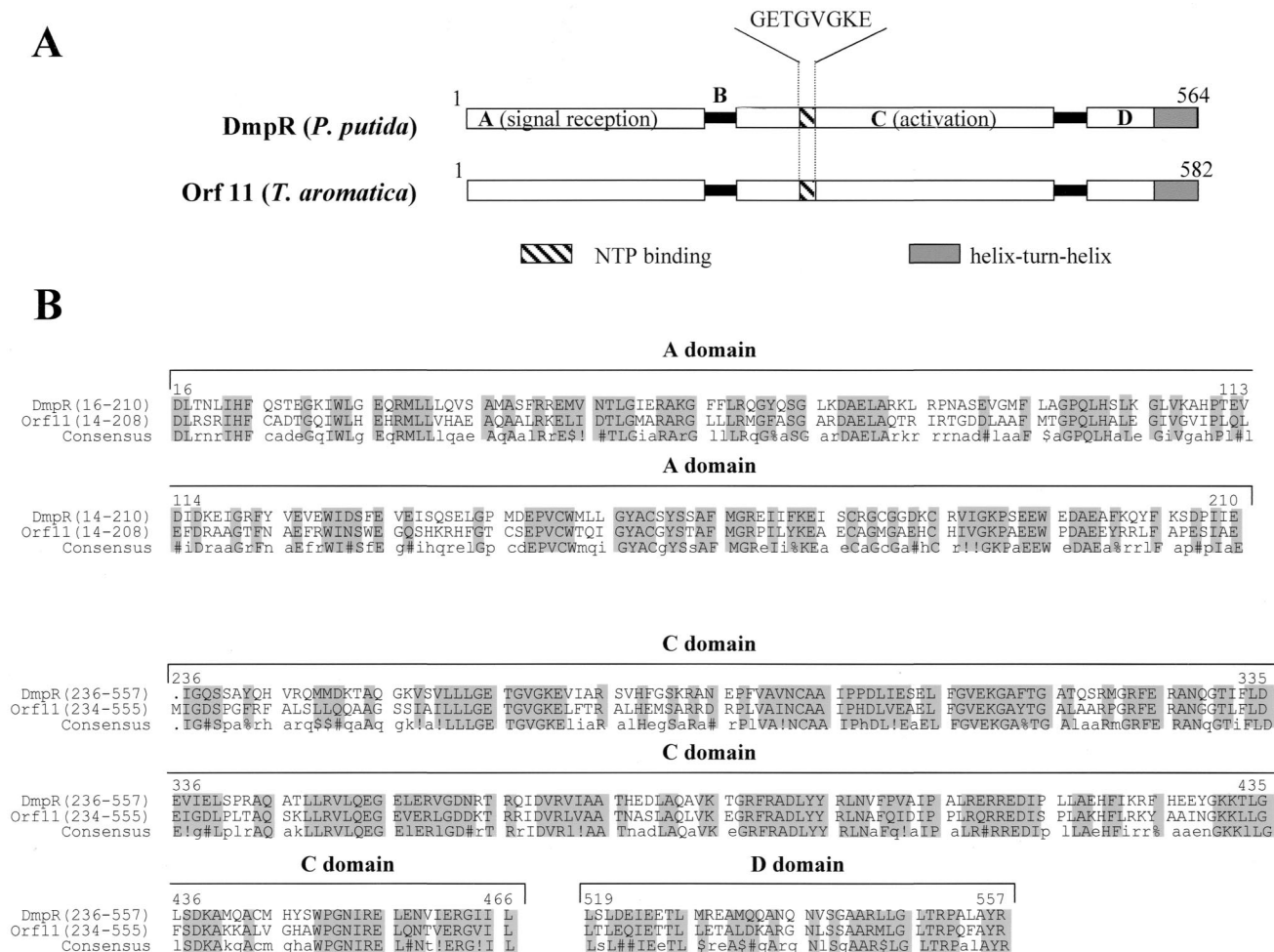


FIG. 10. Orf11 of *T. aromatica* showing similarity to a regulator protein in aerobic (dimethyl)phenol metabolism (DmpR) of *P. putida*: comparison of the domain structures of DmpR of *P. putida* CF600 and Orf11 of *T. aromatica* (A) and alignment of the conserved amino acid regions (B). Structures were adapted from reference 35. The domains A, C, and D and the hinge region B of DmpR are indicated. In parentheses are given the amino acid regions of the conserved regions. !, either I or V; \$, either L or M; %, either F or Y; #, either N, D, Q, or E.

catabolic enzymes (58). However, this hypothesis has yet to be proven. All DmpR-like effector proteins (>20 known) mediate their action through a single binding site within the A domain (44). Depending on the effector, this results in differential effects on ATPase activity of the C domain and its productive coupling to the transcriptional apparatus.

Promoter region. Transcriptional activators of the NtrC-like family usually regulate transcription from σ^{54} -dependent promoters, as was shown in *P. putida* for the positive regulator DmpR (57). The structural *dmp* genes of the aerobic phenol degradation pathway are clustered in a single operon that lies downstream of a *nif/ntr*-like promoter sequence. Upon inspection of the first gene cluster in *T. aromatica*, a similar *nif/ntr*-like promoter sequence was indeed found 5' of *orf1* (Fig. 11). Primer extension analysis has to be performed to show whether transcription really starts at the expected site. So far, nothing is known about promoter sequences in *T. aromatica*, so the -24 and -12 consensus sequences are speculative. Upstream of the second gene cluster, no *nif/ntr*-like promoter sequences were found. All σ^{54} -dependent promoters analyzed so far are regulated by positive transcriptional activators that usually bind to specific DNA sequences ("enhancer sequences," mostly inverted repeats) located relatively far upstream of the promoter (100 to 200 bp) (31).

Regulation of transcription. We suggest that phenol acts as an effector of a transcriptional activator protein, as there is an *ntr*-like regulator gene present upstream of the two gene clusters and a *nif/ntr*-like promoter sequence upstream of the first cluster of genes. In addition to the regulation by phenol, there may be additional catabolite repression, but no experimental data are available. As *T. aromatica* could not be shown to metabolize phenol in the presence of oxygen, it has to be shown whether O₂ represses the induction of transcription of the genes involved in phenol metabolism.

Presence of genes possibly involved in anaerobic phenol metabolism in different strains of *T. aromatica* and various *Azoarcus* species. So far, it has not been possible to unequivocally correlate genes coding for phenol-induced proteins and their function. Southern hybridization of a labeled probe of *orf4* (coding for F2) with chromosomal DNA of different strains seemed to indicate that all tested *Thauera* and *Azoarcus* strains have a gene similar to *orf4*, whether or not they have been shown to metabolize phenol. One plausible assumption is that all strains are able to utilize phenol, but so far the appropriate growth conditions have not been applied. Alternatively, rearrangement and deletion may be the reason why some *Thauera* and *Azoarcus* strains are able to metabolize phenol and some are not.

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