

Phenylphosphate Synthase: a New Phosphotransferase Catalyzing the First Step in Anaerobic Phenol Metabolism in *Thauera aromatica*

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The anaerobic metabolism of phenol in the beta-proteobacterium *Thauera aromatica* proceeds via *para*-carboxylation of phenol (biological Kolbe-Schmitt carboxylation). In the first step, phenol is converted to phenylphosphate which is then carboxylated to 4-hydroxybenzoate in the second step. Phenylphosphate formation is catalyzed by the novel enzyme phenylphosphate synthase, which was studied. Phenylphosphate synthase consists of three proteins whose genes are located adjacent to each other on the phenol operon and were overproduced in *Escherichia coli*. The promoter region and operon structure of the phenol gene cluster were investigated. Protein 1 (70 kDa) resembles the central part of classical phosphoenolpyruvate synthase which contains a conserved histidine residue. It catalyzes the exchange of free [¹⁴C]phenol and the phenol moiety of phenylphosphate but not the phosphorylation of phenol. Phosphorylation of phenol requires protein 1, MgATP, and another protein, protein 2 (40 kDa), which resembles the N-terminal part of phosphoenolpyruvate synthase. Proteins 1 and 2 catalyze the following reaction: phenol + MgATP + H₂O → phenylphosphate + MgAMP + orthophosphate. The phosphoryl group in phenylphosphate is derived from the β-phosphate group of ATP. The free energy of ATP hydrolysis obviously favors the trapping of phenol (*K_m*, 0.04 mM), even at a low ambient substrate concentration. The reaction is stimulated severalfold by another protein, protein 3 (24 kDa), which contains two cystathionine-β-synthase domains of unknown function but does not show significant overall similarity to known proteins. The molecular and catalytic features of phenylphosphate synthase resemble those of phosphoenolpyruvate synthase, albeit with interesting modifications.

Phenol is a natural substrate which is formed from a variety of natural compounds. Phenol arises from tyrosine by tyrosine phenol lyase, but phenol also arises during the degradation of many secondary phenolic plant constituents, notably in the course of the degradation of lignin and phenylpropanoid compounds. Besides phenol, there are many other phenolic compounds, both natural and synthetic ones. Their mineralization proceeds via completely different pathways, depending on whether oxygen is available or not. For instance, groundwater and landfills are free of oxygen. Therefore, anaerobic metabolism of phenolic compounds is of general interest, from both scientific and applied aspects.

The initial steps in aerobic phenol metabolism are catalyzed by oxygenases. Phenol is oxidized to catechol (1,2-dihydroxybenzene) by phenol monooxygenases followed by oxygenolytic ring cleavage catalyzed by catechol dioxygenase. Hence, aerobic metabolism of phenol requires molecular oxygen for both ring hydroxylation and ring cleavage. In contrast to aerobic metabolism, anaerobic metabolism cannot rely on oxygen- and oxygenase-dependent steps. Therefore, anaerobic metabolism of phenol and related phenolic compounds promises unprecedented biochemistry. Anaerobic growth of pure cultures on phenol has been shown for sulfate-reducing (5), denitrifying (47, 50, 51), and iron-reducing (35) bacteria. The list of bacteria growing anaerobically with phenolic compounds is steadily growing (see references in reference 43). In all cases stud-

ied, anaerobic growth on phenol requires the presence of CO₂ (50); CO₂ is required as a cosubstrate for phenol carboxylation which results in the formation of 4-hydroxybenzoate. Phenol carboxylation has been known in chemistry for more than 100 years and is referred to as Kolbe-Schmitt carboxylation.

Anaerobic phenol metabolism by pure cultures has been studied in some detail only in the denitrifying beta-proteobacterium *Thauera aromatica* (2, 3, 16, 24, 29–31, 43, 50, 51). It involves two initial steps (Fig. 1). In the first step, phenol is converted to phenylphosphate. This reaction is catalyzed by an enzyme, E₁, tentatively named phenylphosphate synthase (30). Phenylphosphate is the substrate of a second enzyme, E₂, or phenylphosphate carboxylase, which catalyzes the carboxylation of phenylphosphate to 4-hydroxybenzoate (43). The corresponding enzymes are encoded by a large gene cluster which is phenol induced (Fig. 2A) (16). The phenol-induced gene products found in phenol-grown (induced) cells are missing in 4-hydroxybenzoate-grown (noninduced) cells which served as a control.

Phenylphosphate synthase E₁ (equation 1) was postulated to consist of two proteins, proteins 1 and 2, based on a sequence comparison of two phenol-induced genes (16). Putative protein 1 resembles the central part and putative protein 2 resembles the N-terminal part of classical phosphoenolpyruvate (PEP) synthase (EC 2.7.9.2) of, e.g., *Escherichia coli*. Phenol-induced cells, rather than noninduced cells, catalyzed an exchange of free [¹⁴C]phenol and the phenol moiety of phenylphosphate (30) (a kind of esterase reaction [equation 2]). This suggests that enzyme E₁ is phosphorylated by phenylphosphate in the course of this exchange reaction (equations 3 and 4) and that,

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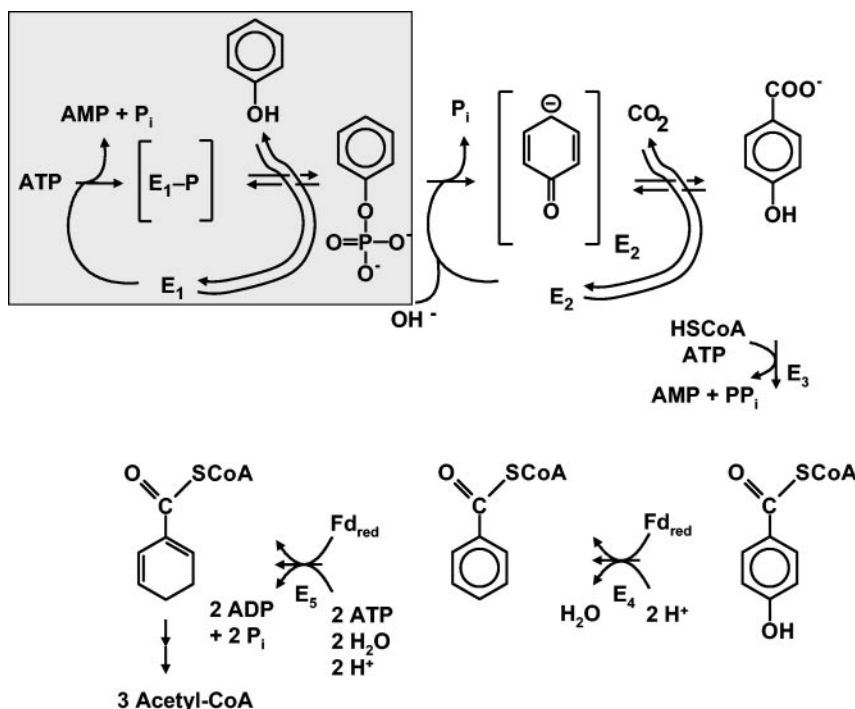
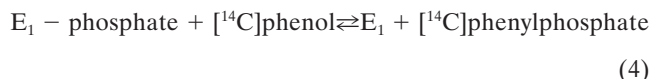
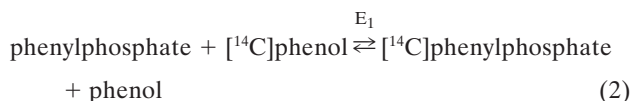


FIG. 1. Proposed pathway of anaerobic metabolism of phenol in the denitrifying bacterium *T. aromatica*. E₁, phenylphosphate synthase (reaction shown in the boxed area [studied in this work]) (EC 2.7.9.x); E₂, phenylphosphate carboxylase (EC 4.1.1.x, studied in previous work) (43). The enzymes catalyzing the subsequent conversion of 4-hydroxybenzoate to three molecules of acetyl-CoA and one molecule of CO₂ have been studied previously (8, 10, 11, 13, 15, 22, 23). The proposed enzyme-bound intermediates are shown in brackets.

in the course of phenol phosphorylation, the enzyme is phosphorylated by another phosphoryl donor in an essentially irreversible step (equation 5). The phosphorylated enzyme E₁ is supposed to subsequently transform phenol to phenylphosphate in a reversible reaction (equation 6). These features are consistent with a ping-pong mechanism. The whole reaction (equation 1) is understood as the sum of equations 5 and 6. The phosphoryl donor X~P of enzyme E₁ was unknown.



It was the aim of this work to purify and study the first enzyme (E₁) of the pathway that transforms phenol into phenylphosphate. Notably, despite many attempts, the phosphorylation of phenol could not be brought about in cell extracts, and the phosphoryl donor was therefore unknown. Furthermore, it was to be shown whether the enzyme catalyzing the exchange of free

[¹⁴C]phenol and the phenol moiety of phenylphosphate (exchange reaction) and the phenol moiety of phenylphosphate (net phosphorylation) are identical and whether the exchange of [¹⁴C]phenol is catalyzed by a partial fraction of the native enzyme.

MATERIALS AND METHODS

Gases, chemicals, biochemicals, materials for chromatography, and radioisotopes. [¹⁴C]phenol (73 mCi mmol⁻¹) was obtained from American Radio-labeled Chemicals/Biotrend (Cologne, Germany) and Hartmann Analytic (Braunschweig, Germany); [³²P]ATP (6,000 Ci mmol⁻¹) was obtained from Amersham Pharmacia Biotech (Freiburg, Germany); Aminex-HPX-87H high-performance liquid chromatography (HPLC) columns were obtained from Bio-Rad (Munich, Germany); Nucleosil 100-10SB HPLC columns were obtained from Machery-Nagel (Düren, Germany); LiChroCART 125-4 HPLC columns filled with LiChrospher 100 PR-18 (C₁₈) and silica gel 60 F₂₅₄ thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany); amylose affinity columns (fast-performance liquid chromatography [FPLC]) were obtained from New England Biolabs (Frankfurt, Germany); and DEAE-Sephacrose (FPLC) was obtained from Amersham Pharmacia Biotech. Chemicals were reagent grade and purchased from Fluka (Neu-Ulm, Germany), Merck, Serva (Heidelberg, Germany), and Sigma-Aldrich (Deisenhofen, Germany). Scintillation cocktail and acrylamide stock solution were purchased from Roth (Karlsruhe, Germany). Nitrogen, helium, and an N₂-H₂ gas mixture (95:5, vol/vol) were purchased from Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany). Restriction enzymes were obtained from MBI Fermentas (St. Leon-Rot, Germany).

Growth conditions of *T. aromatica*, cell harvesting, and storage. *T. aromatica* type strain K172 (DSMZ 6984) (2, 50) was cultured anaerobically in a fed-batch mode (21) at 30°C in a modified mineral salt medium (13, 50) which initially contained 0.5 mM phenol, 10 mM bicarbonate, and 2 mM nitrate. As a control, cells grown on 4-hydroxybenzoate (5 mM) and nitrate (15 mM) were used. The medium (pH 7.8) contained 32 mM K₂HPO₄, 4.3 mM NaH₂PO₄, 10 mM NH₄Cl, 0.8 mM MgCl₂, 0.1 mM CaCl₂, and carbon and electron sources as indicated. Vitamins (B₁₂, 0.1 μg ml⁻¹; B₆, 0.3 μg ml⁻¹; pantothenate, 0.1 μg ml⁻¹; B₁, 0.2 μg ml⁻¹; nicotinic acid, 0.2 μg ml⁻¹; 4-aminobenzoate, 0.08 μg ml⁻¹; biotin, 0.02

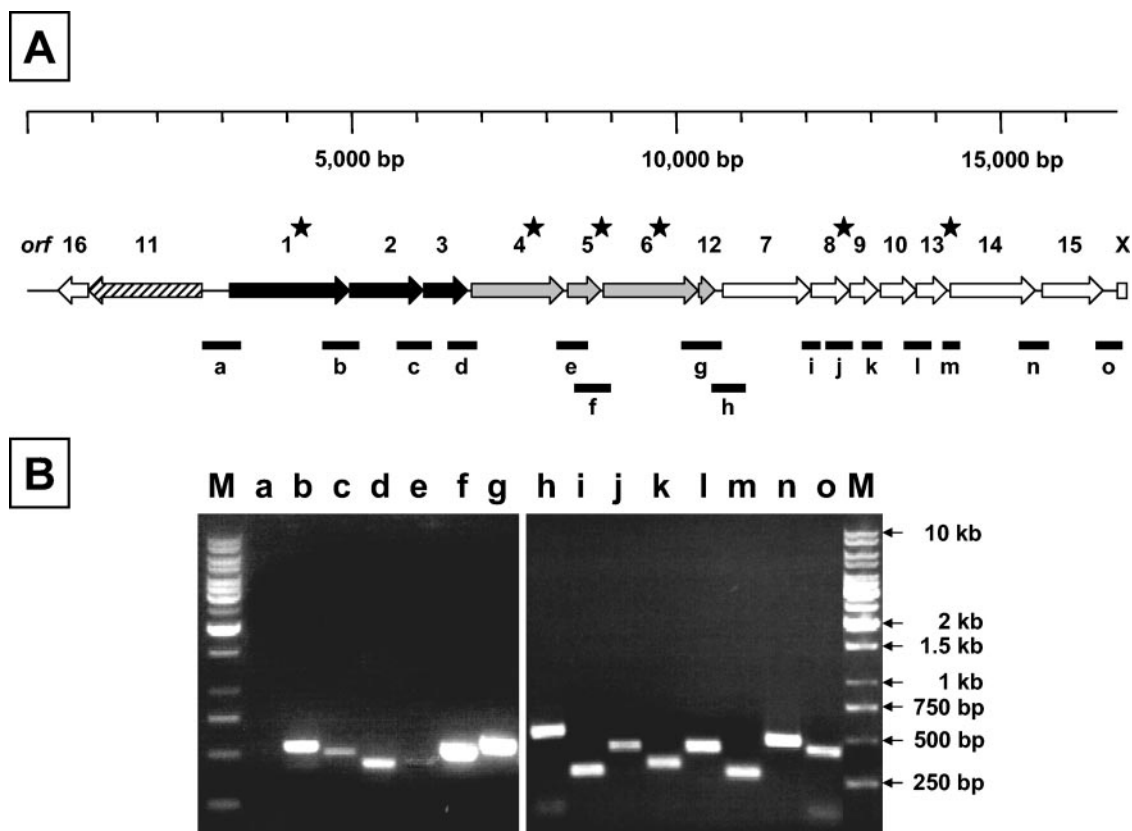


FIG. 2. A. Organization of the phenol gene cluster of *T. aromatica* (GenBank accession number AJ272115). The numbers refer to the ORF numbers of *T. aromatica*. Arrows indicate the direction of transcription. Black, genes encoding subunits of phenylphosphate synthase; grey, genes encoding subunits of phenylphosphate carboxylase. Genes coding for phenol-induced proteins identified in two-dimensional gel electrophoresis are indicated by stars (16, 43). ORF11 is a putative σ^{54} -dependent regulator. ORFs 1 to 3 are subunits of phenylphosphate synthase. ORFs 4 to 6 and 12 are subunits of phenylphosphate carboxylase. ORFs 7 and 8 are putative ubiD/ubiX type (de)carboxylases (16, 43). ORFs 9, 10, 13, X, and 16 are hypothetical. ORF14 is similar to aromatic acid:CoA ligases. ORF15 is similar to proteins involved in the *meta* pathway of aerobic phenol metabolism in various species of bacteria. Black bars below the ORFs (b to o) represent the locations of fragments amplified in RT-PCR experiments. B. Results of RT-PCR experiments. a: negative control; fragment a could not be amplified on the RNA level; M: molecular mass standard; e: weakness of signal is due to suboptimal primer binding and is also observed in PCR experiments with chromosomal DNA. For details, see Materials and Methods.

$\mu\text{g ml}^{-1}$) (40) and trace elements (ZnCl₂, 0.5 μM ; MnCl₂, 0.5 μM ; CoCl₂, 0.8 μM ; CuCl₂, 0.04 μM ; NiCl₂, 0.1 μM ; Na₂MoO₄, 0.15 μM ; H₃BO₃, 0.01 μM ; FeCl₂, 15 μM ; nitrilotriacetate, 750 μM) were added from 1,000-fold stock solutions. Phenol and nitrate were fed from a 0.5 M phenol–1.8 M KNO₃ stock solution, and 4-hydroxybenzoate was fed from a 0.5 M sodium 4-hydroxybenzoate–1.8 M KNO₃ stock solution. Growth was measured by determining the optical density at 578 nm (OD₅₇₈). An OD₅₇₈ of 1.0 (1-cm light path) 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.

Plasmids, primers, and *E. coli* strains and their growth conditions. The plasmid pETBlue-1 (Amp^r *lacZ* P_{T7}) was from Novagen (Bad Soden, Germany); pMal-c2X (Amp^r *lacI*^q *lacZ* R_{lac} *malE*) was from New England BioLabs. The oligonucleotide primers used were obtained from G. Igloi (University of Freiburg, Freiburg, Germany) or from MWG Biotech (Ebersberg, Germany) and are summarized in Table 1. *E. coli* SURE {e14⁻ (McrA⁻) Δ (*mc**rCB*-*h**sdSMR*-*mrr*)171 *endA1* *supE44* *thi-1* *gyrA96* *relA1* *lac* *recB* *recJ* *sbcC* *umuC*::Tn5 (Kan^r) *uvrC* [F⁻ *proAB* *lacI*^q Δ M15 Tn10 (Tet^r)]} was obtained from Stratagene (Amsterdam, The Netherlands), and *E. coli* TUNER(DE3)pLacI cells [F⁻ *ompT* *h**sdS*_B (τ_{B} m_B) *gal* *dcm* *lacY1* (DE3) pLacI(Cm^r)] were obtained from Novagen. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (42) and, if necessary, with 0.1 mg of ampicillin ml⁻¹.

Preparation of cell extract and protein determination. Cells were broken by two different methods (1). One part of cells (fresh weight) was suspended in 1 volume of a solution containing 15% (vol/vol) glycerol, 40 mM mercaptoethanol, and 0.5 mg DNase I per ml, followed by French press treatment at 137 MPa at 4°C (2). One part of cells (fresh weight) was suspended in 1.5 volumes of a

solution containing 15% (vol/vol) glycerol, 1 mM EDTA, 40 mM mercaptoethanol, and 0.5 mg of DNase I per ml, followed by incubation with 90 μg of lysozyme per g of cells for 10 min at 20°C. This method was used for *T. aromatica* cells exclusively.

Cell extracts of *T. aromatica* and of *E. coli* containing an open reading frame 1 (ORF1) protein were centrifuged at 100,000 $\times g$ for 30 min at 4°C. Twenty percent (vol/vol) polyethyleneglycol (PEG 600) was added to the supernatant of *T. aromatica* cell extract, and ultracentrifugation was repeated. The resulting polyethyleneglycol-containing supernatant was used in the assays. *E. coli* cell extracts containing ORF2 and ORF3 proteins were used for the preparation of inclusion bodies. The approximate protein concentrations, determined by the method of Bradford (14), were 80 mg ml⁻¹ (method 1) and 10 mg ml⁻¹ (method 2).

Enzyme assays and assays of individual ORFs. The radioactive assays followed the incorporation of radioactivity from [U-¹⁴C]phenol into phenylphosphate in a 0.1-ml assay mixture at 30°C; the reaction was started by adding cell extract or protein preparation. After 10 min of incubation, the reaction was stopped by adding 15 μl of 2 M H₂SO₄ followed by centrifugation. The supernatant was analyzed by thin-layer chromatography (10- to 20- μl samples) and radioautography or by HPLC (20 or 50 μl samples) and radiodetection by solid-state scintillation counting. Routinely, 0.2 mg of ORF1 and/or 0.2 mg of ORF2 was used. When ORF3 was tested, 0.025 to 0.2 mg of ORF3 was added.

(i) [¹⁴C]phenol exchange reaction. The exchange of [¹⁴C]phenol and the phenol moiety of phenylphosphate was routinely tested by following the phenylphosphate-dependent formation of labeled products from [U-¹⁴C]phenol. The routinely used assay mixture contained 100 mM Tris-HCl (pH 8.4), 20 mM

TABLE 1. Oligonucleotide primers used in this work^a

Primer no.	Primer	Sequence	Characteristics and use
1	ORF1pMalfor	5'- <i>G</i> AAGATCTATGAA-GTTTCCTGTTCCG-3'	5' primer for DNA fragment containing <i>orf1</i> used for pMal-c2X vector
2	ORF1polyTfor	5'-ATGAAGTTTCCTGTTCCGCAC-3'	5' primer for DNA fragment containing <i>orf1</i> used for pETBlue-1 vector
3	ORF1SalIrev	5'-CCATGTCG <i>A</i> CCACTCCCTCAATCC-3'	3' primer for DNA fragment containing <i>orf1</i> used for pETBlue-1 and pMal-c2X vectors
4	ORF2Bg/IIfor	5'-GAGGGA <i>A</i> GATCTGACATGGGAAG-3'	5' primer for DNA fragment containing <i>orf2</i> used for pMal-c2X vector
5	ORF2polyTfor	5'-ATGGGAAGTATCGTTCCAC-3'	5' primer for DNA fragment containing <i>orf2</i> used for pETBlue-1 vector
6	PEPSSalIrev	5'-GATCGGATTGGT <i>C</i> GACATCCA-3'	3' primer for DNA fragment containing <i>orf2</i> used for pETBlue-1 and pMal-c2X vectors
7	ORF3polyTfor	5'-ATGATCGTACGCAACTGGATG-3'	5' primer for DNA fragment containing <i>orf3</i> used for pETBlue-1 vector
8	ORF3polyTrev	5'-GACCTCGGTGGTATTCTCTT-3'	3' primer for DNA fragment containing <i>orf3</i> used for pETBlue-1 vector
9	5' <i>orf1</i> for	5'-CACCAGGGTTCGATTGA-3'	RT-PCR, negative control
10	orf1Cy5rev	5'-CTCCGGGTAATGCAATCCGTCG-3'	RT-PCR, negative control and primer extension experiment
11	orf1for	5'-GCTCTGGGGGGTCCACCA-3'	RT-PCR, amplification of intergenic region between ORFs 1 and 2
12	orf2rev	5'-CGCACTTGCCGCCACCA-3'	RT-PCR, amplification of intergenic region between ORFs 1 and 2
13	orf2for	5'-GACAACCTTCGTCGTCACAAG-3'	RT-PCR, amplification of intergenic region between ORFs 2 and 3
14	orf3rev	5'-GTGGATATTGGCTTCGGAAA-3'	RT-PCR, amplification of intergenic region between ORFs 2 and 3
15	orf3for	5'-TGTTCTCGCTGGCGGCGCAT-3'	RT-PCR, amplification of intergenic region between ORFs 3 and 4
16	orf4rev	5'-AAGAGAATCACCACCGAGGTG-3'	RT-PCR, amplification of intergenic region between ORFs 3 and 4
17	orf4for	5'-CCTACGAGTGGGAAGCAGAGC-3'	RT-PCR, amplification of intergenic region between ORFs 4 and 5
18	orf5rev	5'-TGTTTCATCTCCTCGAGCA-3'	RT-PCR, amplification of intergenic region between ORFs 4 and 5
19	orf5for	5'-CGGCATGGGCGTATCGTGTGG-3'	RT-PCR, amplification of intergenic region between ORFs 5 and 6
20	orf6rev	5'-CCCAGTCCACTCCTGTCTGAT-3'	RT-PCR, amplification of intergenic region between ORFs 5 and 6
21	orf6for	5'-GGAGCGCGGCATCCACGTGTTTC-3'	RT-PCR, amplification of intergenic region between ORFs 6 and 12
22	orf12rev	5'-GCCGCGGGCGGACAAAAGGC-3'	RT-PCR, amplification of intergenic region between ORFs 6 and 12
23	orf12for	5'-CCGCATCATCGAGACCGTCCA-3'	RT-PCR, amplification of intergenic region between ORFs 7 and 12
24	orf7rev	5'-AAGGAAGTGTATCCACGCC-3'	RT-PCR, amplification of intergenic region between ORFs 7 and 12
25	orf7for	5'-GGCACATCGAAAGTCGGGT-3'	RT-PCR; amplification of intergenic region between ORFs 7 and 8
26	orf8rev	5'-TGGTGATGTCGGATTTCGG-3'	RT-PCR, amplification of intergenic region between ORFs 7 and 8
27	orf8for	5'-GGCATGATCATCGCGCCCTGT T-3'	RT-PCR, amplification of intergenic region between ORFs 8 and 9
28	orf9rev	5'-GATGCCACCGATCGCCCTTCC-3'	RT-PCR, amplification of intergenic region between ORFs 8 and 9
29	orf9for	5'-GGGATACAGCATCCGCAT-3'	RT-PCR, amplification of intergenic region between ORFs 9 and 10
30	orf10rev	5'-GCCATGTGAATCTCCTTGCTGACG-3'	RT-PCR, amplification of intergenic region between ORFs 9 and 10
31	orf10for	5'-GCATACCGATCGAACACTGG-3'	RT-PCR, amplification of intergenic region between ORFs 10 and 13
32	orf13rev	5'-CGTTCCCTTGACGAGGAAGG-3'	RT-PCR, amplification of intergenic region between ORFs 10 and 13
33	orf13for	5'-GGCGCTGACGAAGATGGA-3'	RT-PCR, amplification of intergenic region between ORFs 13 and 14
34	orf14rev	5'-TCCACTTGTGGTGTAGAAGC-3'	RT-PCR, amplification of intergenic region between ORFs 13 and 14
35	orf14for	5'-GAGTTCGGCATCAAGTACG-3'	RT-PCR, amplification of intergenic region between ORFs 14 and 15
36	orf15rev	5'-CTCCGTTCCGATACTGGTTCG-3'	RT-PCR, amplification of intergenic region between ORFs 14 and 15
37	orf15for	5'-CATTGCGCAATGGCAGCACG-3'	RT-PCR, amplification of intergenic region between ORFs 15 and X
38	orfXrev	5'-CTTGAGCCGCTCCTTGCTCG-3'	RT-PCR, amplification of intergenic region between <i>orfs</i> 15 and X

^a The mutated nucleotides are marked in italic type, and the restriction sites are marked in boldface type. For gene order, see Fig. 2A.

MgCl₂, 1 mM MnCl₂, 40 mM mercaptoethanol, 0.125 mM [¹⁴C]phenol (1.8 kBq), 1 mM phenylphosphate, and cell extract or protein sample (in 0.1 ml).

(ii) **Net phenol phosphorylation reaction.** The formation of phenylphosphate was routinely tested by monitoring the MgATP-dependent formation of labeled products from [¹⁴C]phenol. The routinely used assay mixture contained 100 mM Tris-HCl (pH 8.6), 20 mM MgCl₂, 1 mM MnCl₂, 10 mM ATP, 40 mM mercaptoethanol, 0.125 mM [¹⁴C]phenol (1.8 kBq), and cell extract or protein sample (in 0.1 ml).

(iii) **Test for other possible phosphoryl donors.** The following compounds (5 mM) were tested in the net phenol phosphorylation assay for their ability to transfer a phosphoryl group to [¹⁴C]phenol: ATP, GTP, ITP, UTP, CTP, phosphoenolpyruvate, acetylphosphate, carbamoylphosphate, creatine phosphate, ATP plus P_i, and ADP plus PP_i.

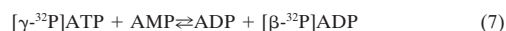
(iv) **Analysis of adenine nucleotides.** To identify the products formed from ATP during net phenol phosphorylation, a modified net phosphorylation assay mixture (100 μl) containing 2 mM unlabeled phenol, 2 mM MgATP, 20 μl (each) of ORF1 and ORF2 protein solution (0.2 mg of each protein), and 40 μl of ORF3 protein solution (0.2 mg) was used. The assays were incubated for 15 h at 30°C, and then the reaction was stopped by adding 15 μl of 2 M H₂SO₄. After centrifugation, a sample of the supernatant (100 μl) was analyzed for adenine nucleotides by HPLC (see below). To investigate whether the subunits of phenylphosphate synthase have ATPase activity in the absence of the substrate phenol, the same assay was used with the exception that phenol was omitted and the subunits were tested individually or in combination.

(v) **Study of enzyme requirements and kinetics.** The oxygen sensitivity of phenylphosphate synthase was checked. Therefore, the preparation of cell extracts and assays was performed under aerobic and strict anaerobic conditions. The pH dependence of the reactions was tested by varying the pH values in the

range of 7 to 10 for net phosphorylation by using 100 mM Tris-HCl and glycine-KOH. The exchange reaction was tested in the pH range of 4.5 to 10 by using 100 mM histidine-KOH, TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-KOH, Tris-HCl, and glycine-KOH. The Mn²⁺ dependence was tested by altering the concentration of Mn²⁺ ions in the assay between 0 and 9 mM. For the determination of the apparent K_m values for the substrates of net phenol phosphorylation, the (co)substrate concentrations were varied at saturating concentrations of the other (co)substrates (10 mM ATP, 0.4 mM phenol, 20 mM MgCl₂). Assays were stopped after 5 min of incubation.

Experiments with [³²P]ATP and determination of products of ATP hydrolysis.

(i) **Preparation of β-³²P-labeled ATP.** β-³²P-labeled ATP was enzymatically synthesized from γ-³²P-labeled ATP. In the first step, a myokinase reaction (5 U of myokinase) (equation 7) was performed at 35°C for 15 min with a 270-μl reaction mixture containing 20 mM MgCl₂, 10 mM AMP, 2 mM [γ-³²P]ATP (100 μCi), and 150 mM K-morpholinepropanesulfonic acid (K-MOPS) buffer (pH 7.4).



The assay was stopped by adding 27 μl of 10% perchloric acid following centrifugation. Next, 5.5 μl of a 5 M potassium carbonate solution was added, followed by centrifugation to remove precipitated potassium perchlorate. The supernatant (300 μl) was loaded onto a Q-Sepharose fast-flow column (0.5 ml) previously equilibrated with buffer A (50 mM K-phosphate, pH 6.7). After washing with 1 bed volume of buffer A, AMP was eluted with 3 bed volumes of buffer A containing 30 mM KCl, ADP was eluted with 3 bed volumes of buffer A containing 80 mM KCl, and ATP together with some remaining ADP was eluted with 3 bed volumes of buffer A containing 500 mM KCl. The purity of the ADP-containing fraction (1.5 ml) was proved by HPLC analysis. In a second step,

[β - 32 P]ADP was completely converted in 15 min at 35°C into [β - 32 P]ATP by pyruvate kinase (8 U) (equation 8) in an assay mixture containing 5 mM MgCl₂ and 1 mM phosphoenolpyruvate.



The assay was stopped by adding 45 μ l of 30% (wt/vol) perchloric acid following centrifugation, and neutralization was done with 23 μ l of a 5 M potassium carbonate solution following centrifugation. The radioactivity content of the supernatant was determined by liquid scintillation counting (1.1 kBq μ l⁻¹). This solution was used directly in the assays after adding appropriate amounts of unlabeled ATP.

(ii) Detection of labeled phenylphosphate. A modified net phenol phosphorylation assay (0.2-ml assay mixture) was used in which 2 mM labeled ATP ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\beta\text{-}^{32}\text{P}]\text{ATP}$, respectively [5 kBq each]) and 1 mM unlabeled phenol were added. The reaction was started by the addition of 40 μ l (each) of ORF1 and ORF2 protein solution (0.4 mg of each protein) or a combination of both. After 10 min of incubation at 30°C, the reaction was stopped by adding 30 μ l of 2 M H₂SO₄ followed by centrifugation. Aliquots of the supernatant (20 μ l, 500 Bq) were analyzed by TLC and radioautography.

HPLC, TLC, and detection methods. Both HPLC and TLC were used to analyze the products of the reactions in which [^{14}C]phenol or [^{32}P]ATP was used.

(i) HPLC. Detection was done by using a photodiode array detector (260 nm) and/or a solid scintillation radioactivity detector. For the separation of ^{14}C -labeled products, an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad) was used with solvent 10 mM H₂SO₄ (isocratic) and a flow rate of 0.6 ml min⁻¹ for a 50- μ l sample; the retention time for phenol was 67 min, and the retention time for phenylphosphate was 8.5 min. For the separation of nucleotides, a Mono-Q column (1 ml; Pharmacia) with a flow rate of 1 ml min⁻¹ and a sample size of 100 μ l was used; solvents included buffer A (50 mM K-phosphate, pH 6.7) and buffer A plus 500 mM KCl. Separation was achieved by using a linear gradient over 25 min from 0 to 500 mM KCl. Retention times were as follows: AMP, 3 min; phenylphosphate, 5 min; ADP, 6 min; ATP, 8 min.

(ii) TLC. For the separation of ^{14}C - and ^{32}P -labeled products, Silica gel 60 F₂₅₄ (20 \times 20 cm) plates with a solvent of ethanol-dichloromethane-water (80:10:10, vol/vol/vol) was used; the sample size was 10 to 20 μ l (180 Bq of ^{14}C or 500 Bq of ^{32}P); development was done for 3 to 3.5 h at room temperature, followed by UV detection (254 nm; unlabeled standards), and radioactivity detection was done by phosphorimaging (Fujix BAS-IP MP 2040S, Fuji, Tokyo, Japan). Radioactivity was quantified after a 24-h incubation by scanning the radioautogram with a resolution of 100 μ m (Molecular Imager FX; Bio-Rad), and radioactivity evaluation was done by using the program Quantity One (Bio-Rad). The method was calibrated by using samples whose radioactivity content was determined by liquid scintillation counting.

Molecular biological techniques. DNA was isolated and purified according to the method described by Murray and Thomson (38). Plasmid DNA was isolated according to the method described by Birnboim and Doly (9). Standard procedures were used for concentration and buffer change of plasmid DNA and DNA fragments (42). Total RNA used in primer extension experiments was isolated via the hot-phenol method (1). Total RNA used for reverse transcription (RT)-PCR was isolated by using an RNeasy Total RNA kit (QIAGEN, Hilden, Germany). DNA and RNA concentrations were determined photometrically (260 nm).

(i) Cloning of gene fragments. Primers were derived to amplify *orf1*, *orf2*, and *orf3* via PCR (Table 1). PCR (41) was performed under the following conditions: 100 ng of target DNA; 200 nM each primer; 200 μ M each dATP, dCTP, dTTP, and dGTP; 50 mM KCl; 1.5 mM MgCl₂; 10 mM Tris-HCl (pH 9.0), and 1 U of *Taq* DNA polymerase (Amersham Biosciences). The assay mixture (100 μ l) was overlaid with 75 μ l of paraffin oil. PCR parameters were as follows: 45 s at 95°C and 45 s at 50°C and 30 cycles of 2 min (*orf1* and *orf2* fragment) and 1 min (*orf3* fragment and colony PCR) at 72°C. Fragments containing *orf1*, *orf2*, or *orf3* were cloned into the pETBlue-1 expression vector (Novagene, Darmstadt, Germany); fragments harboring *orf1* or *orf2* were also cloned into the pMal-c2X expression vector. Success of cloning was proved by colony PCR after transformation.

(ii) Overexpression of cloned genes. Chemical transformation of bacterial cells was done according to standard protocols (18). The pETBlue-1 vector constructs were transformed and expressed in *E. coli* TUNER(DE3)pLacI cells, and the pMal-c2X vector constructs were transformed in *E. coli* SURE cells (in Luria-Bertani medium at 37°C under aerobic conditions). Growth was monitored by measuring the optical density at 578 nm. At an OD₅₇₈ of 0.5, cultures were induced by adding 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside). Incubation continued for 3 h with shaking at 37°C. The formation of overproduced protein was monitored by analyzing samples of whole IPTG-induced cells at

different time points by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

(iii) RT-PCR. Contaminating DNA in total RNA preparations was removed with RNase-free DNase (5 U/ μ g, 30 min, 37°C; MBI Fermentas). Complete removal of DNA was tested by PCR (see Fig. 2B, lane a, and primer pair 5'*orf1for-orf1Cy5rev* in Table 1; expected fragment length, 555 bp). A cDNA library was obtained by an RT-PCR using random hexamer priming mix (MBI Fermentas). The cDNA was used in subsequent PCRs with specific primers. To confirm the expression of the gene cluster as an operon, primers were derived from the coding regions and used in PCR to amplify fragments including the intergenic regions between two ORFs (b, *orf1for-orf2rev* [530 bp]; c, *orf2for-orf3rev* [497 bp]; d, *orf3for-orf4rev* [450 bp]; e, *orf4for-orf5rev* [444 bp]; f, *orf5for-orf6rev* [526 bp]; g, *orf6for-orf12rev* [582 bp]; h, *orf12for-orf7rev* [491 bp]; i, *orf7for-orf8rev*; [242 bp]; j, *orf8for-orf9rev* [380 bp]; k, *orf9for-orf10rev* [277 bp]; l, *orf10for-orf13rev* [388 bp]; m, *orf13for-orf14rev* [235 bp]; n, *orf14for-orf15rev* [427 bp]; o, *orf15for-orfXrev* [379 bp] [Table 1A and Fig. 2B]). For a molecular mass standard, a GeneRuler 1-kb DNA ladder (MBI Fermentas) was used.

(iv) Mapping of the 5' end of mRNA. The transcription start site of the mRNA encoded by the phenol gene cluster was mapped via primer extension (12, 34). A Cy5-labeled primer was derived from the 5' end of *orf1* (*orf1rev*). For a control, RNA of cells grown anaerobically on 4-hydroxybenzoate was used. Extension products were analyzed by use of an ALFexpress sequencer by J. Alt-Mörbe (Labor für DNA-Analytik, Freiburg, Germany).

Electrophoretic techniques. DNA agarose gel electrophoresis (42), SDS-PAGE (10 and 12.5%) (32), and visualization of protein bands by staining with Coomassie blue R 250 was done according to standard methods.

Purification of overproduced ORF1 protein. [^{14}C]phenol exchange into phenylphosphate, followed by TLC separation of products and quantification of [^{14}C]phenylphosphate spots, was used as an assay to monitor ORF1 activity throughout purification. All steps were performed at 4°C.

(i) DEAE-Sepharose chromatography. A DEAE-Sepharose column (volume, 16 ml; diameter, 16 mm; Amersham Pharmacia Biotech) equilibrated with buffer B (20 mM Tris-Cl [pH 7.5], 20% [vol/vol] glycerol, 20 mM mercaptoethanol) was used at a flow rate of 2 ml min⁻¹. The sample (20-ml French press cell extract, 400 μ g of protein diluted with 20 ml of buffer B) was applied, and the column was washed with 3 bed volumes of buffer B, followed by a two-step elution with buffer B containing 75 and 150 mM ammonium sulfate, respectively. Fractions of 10 ml were collected and analyzed for enzyme activity. ORF1 eluted at a 150 mM salt concentration. All fractions containing predominantly ORF1 (~40 ml, 50 mg of protein) were combined and applied to the Butyl-Sepharose column.

(ii) Butyl-Sepharose chromatography. Ammonium sulfate was added to the combined fractions to a final concentration of 1 M. After a short centrifugation (5 min, 5,000 \times g), the supernatant was loaded onto a Butyl-Sepharose column (volume, 28 ml; diameter, 26 mm; Amersham Pharmacia Biotech) previously equilibrated with buffer B plus 1 M ammonium sulfate and run at a flow rate of 2 ml min⁻¹. After washing with 3 bed volumes of buffer B plus 1 M ammonium sulfate, the column was eluted in two steps with buffer B containing 150 and 0 mM ammonium sulfate, respectively. ORF1 protein eluted in the 0 mM salt fraction. The application of a linear salt gradient did not improve purification. Fractions of 10 ml were collected and analyzed. The fractions containing mainly ORF1 (30 ml, 20 mg of protein) were combined and applied to the Mono-Q column.

(iii) Mono-Q chromatography. Pooled fractions of the previous chromatography techniques were loaded onto a Mono-Q FPLC column (1 ml; Amersham Pharmacia Biotech) equilibrated with buffer B plus 50 mM NaCl. After washing with 3 bed volumes, the column was developed with a linear gradient (100 ml) of 0 to 500 mM NaCl in buffer B. Fractions of 5 ml were collected and analyzed. ORF1 eluted at a 230 to 280 mM salt concentration. Fractions containing ORF1 (15 ml) were combined and concentrated via an Amicon concentrator cell (10-kDa exclusion size membrane) to a protein concentration of 10 mg ml⁻¹.

Purification of MalE-ORF1 protein. An Amylose column (volume, 12 ml; diameter, 16 mm; Amersham Pharmacia Biotech) equilibrated with buffer C (20 mM Tris-Cl [pH 7.4], 200 mM NaCl, 1 mM EDTA) was used at a flow rate of 1 ml min⁻¹. The sample (20-ml French press cell extract, 120 to 160 mg of protein diluted with 20 ml of buffer C) was applied, and the column was washed with 12 bed volumes of buffer C followed by elution with buffer C containing 10 mM maltose. Active fractions were combined and concentrated via an Amicon concentrator cell to a protein concentration of 10 mg ml⁻¹.

Refolding of the proteins ORF2 and ORF3. **(i) Preparation of inclusion bodies.** Overproduced ORF2, ORF3, and MalE-ORF2 were almost exclusively present in inclusion bodies. Therefore, inclusion bodies formed in the pET system were prepared from extracts after French press treatment of the cells.

TABLE 2. Purification of overproduced ORF1 from 10 g of cells (fresh cell mass) of *E. coli* TUNER (pET vector system)^a

Purification step	Total activity (nmol min ⁻¹)	Total protein (mg)	Sp ac (nmol min ⁻¹ mg ⁻¹)	Yield (%)	Enrichment (fold)
Soluble extract	24	400	0.06	100	1
DEAE-Sepharose	27.5	50	0.55	114	9.2
Butyl-Sepharose	16.1	18.7	0.86	67	14.3
Mono-Q	10.5	9.5	1.1	43	18.3

^a The exchange of radioactivity from [¹⁴C]phenol into phenylphosphate was measured.

Extraction and refolding of the proteins ORF2 and ORF3 followed in part the protocols described by Coligan et al. (19). The cell extract was centrifuged at 5,000 × g for 10 min (4°C), the supernatant was discarded, and the pellet (inclusion bodies) was washed (resuspended or centrifuged) in two successive steps. The first washing step (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 1% [vol/vol] Triton X-100) is thought to remove lipids and membrane fragments, while the second washing step (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 0.5 M urea) should remove proteins adsorbed on the inclusion body surface.

(ii) **Refolding of ORF2 and ORF3.** The resulting pellet was suspended in a 1.5- to 2-fold volume of buffered urea solution (100 mM Tris-Cl [pH 8.5], 8 M urea, 50 mM mercaptoethanol). After 1 h of stirring on ice and 30 min of centrifugation (40,000 × g), the supernatant was separated from the resulting pellet. The pellet was discarded, and the supernatant was added dropwise into cold refolding solution (15% [vol/vol] glycerol, 50 mM Tris-Cl [pH 8.5], 10 mM mercaptoethanol) while stirring on ice. The final protein concentration in the refolding buffer was 0.1 mg ml⁻¹. This protein solution was subsequently concentrated via an Amicon concentrator cell (10-kDa exclusion size) to 10 mg ml⁻¹ for ORF2 and 5 mg ml⁻¹ for ORF3, as determined by the method of Lowry et al. (36). Successful refolding was monitored by reconstitution of net phosphorylation activity after complementation with purified ORF1 or ORF2 and ORF3. A standard assay, as described above, was used. Refolding of the insoluble proteins ORF2 and ORF3 rendered them soluble but possibly mostly still inactive. Significant amounts of both proteins were precipitating during the first minutes of the enzyme assay. It is likely that these proteins did not possess their native conformations.

UV-VIS spectroscopy. UV-visible (VIS) spectra of solutions of proteins ORF1, ORF2, and ORF3 were recorded between 250 and 600 nm against buffer. The protein was dissolved in 25 mM Tris-HCl (pH 8.5) buffer containing 15% (vol/vol) glycerol at a concentration of approximately 0.5 mg ml⁻¹.

RESULTS

[¹⁴C]phenol exchange reaction and phosphorylation of phenol by extracts of *T. aromatica* cells. Extracts of cells grown under denitrifying conditions with phenol (induced cells) and 4-hydroxybenzoate (noninduced cells) were studied for the [¹⁴C]phenol exchange reaction and the nucleoside triphosphate-dependent phosphorylation of phenol, two reactions which are considered pertinent to phenol metabolism. Induced cells catalyzed the exchange of free [¹⁴C]phenol and the phenol moiety of phenylphosphate at a specific rate of 0.11 nmol min⁻¹ mg⁻¹ of protein. This phenylphosphate-dependent formation of [¹⁴C]phenylphosphate in the presence of [U-¹⁴C]phenol was not observed in noninduced cells. Performing the assays under anaerobic conditions did not enhance the overall enzyme activity. The reaction was stimulated by Mn²⁺ (1 mM), and the activity in the absence of added Mn²⁺ was 14%. In contrast to previous results (30), the optimal pH for the [¹⁴C]phenol exchange reaction in cell extract was 8.5; the rate at pH 7.5 was 10% (data not shown). The different pH response may be due to buffer effects on both the reaction rate and TLC separation of phenylphosphate. The reaction was linearly time dependent in the range of 0 to 8 min and linearly

protein dependent in the range of 0 to 8 mg of protein ml⁻¹. Induced cells did not catalyze the exchange of free [³²P]phosphate and the phosphoryl group of phenylphosphate (16).

Extracts of induced cells also catalyzed the MgATP-dependent formation of [¹⁴C]phenylphosphate from [U-¹⁴C]phenol at a specific rate of 1.5 nmol min⁻¹ mg⁻¹ of protein. Noninduced cells were inactive. Activity was reliably detected in cell extracts only if cells were lysed by lysozyme treatment followed by 20% (vol/vol) PEG 600 precipitation; the activity was recovered in the supernatant. This reaction was slightly stimulated (20%) by 1 mM Mn²⁺. No [¹⁴C]phenol phosphorylation was observed with GTP, CTP, ITP, phosphoenolpyruvate, acetylphosphate, carbamoylphosphate, or creatine phosphate. Inorganic phosphate or pyrophosphate did not stimulate the ATP-dependent reaction. The reaction had a narrow pH optimum at pH 9.0; below pH 7, no activity was detectable. The reaction slowly leveled off and was linearly dependent on time only in the first few minutes. Protein dependence was nonlinear and almost quadratic, suggesting that the net phosphorylation reaction was catalyzed by a multicomponent system, in contrast to the [¹⁴C]phenol exchange reaction. Half-maximal rates were obtained with 40 ± 10 μM phenol. The apparent *K_m* value for ATP could not be determined because of interfering activities in the cell extract.

Overexpression of two putative phenylphosphate synthase genes in *E. coli*. Two genes (*orf1* and *orf2*) out of a cluster of up to 16 genes (Fig. 2A) are considered to play a role in phenol phosphorylation (16, 43). However, overexpression experiments with a recombinant pMal-c2 vector encoding both ORFs resulted only in the overproduction of ORF1; therefore, the two ORFs were cloned and expressed separately. Attempts to purify protein ORF2 from *T. aromatica* cell extract were unsuccessful. The ORF2 assay was based on the complementa-

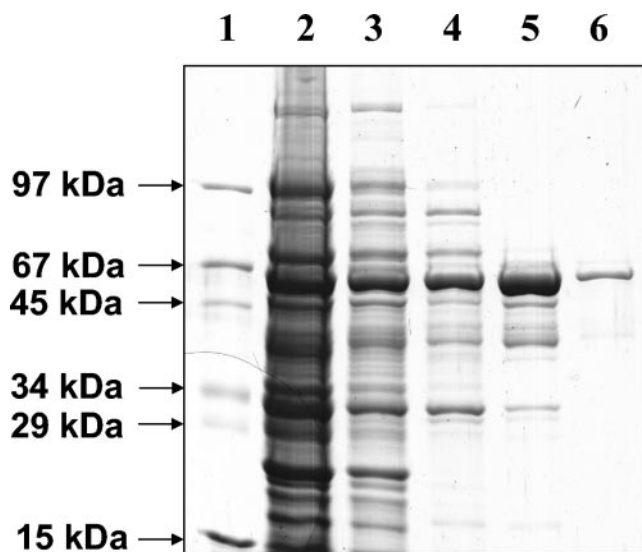


FIG. 3. Purification of ORF1 protein overproduced in *E. coli* TUNER. SDS-PAGE (10%) of various steps of the purification is shown. Lanes: 1, molecular mass standard; 2, total cell protein of induced *E. coli* cells (30 μg of protein); 3, soluble fraction of cell extract (induced cells) (20 μg of protein); 4, DEAE-Sepharose fraction (6 μg of protein); 5, Butyl-Sepharose fraction (5 μg of protein); 6, Mono-Q fraction (2 μg of protein).

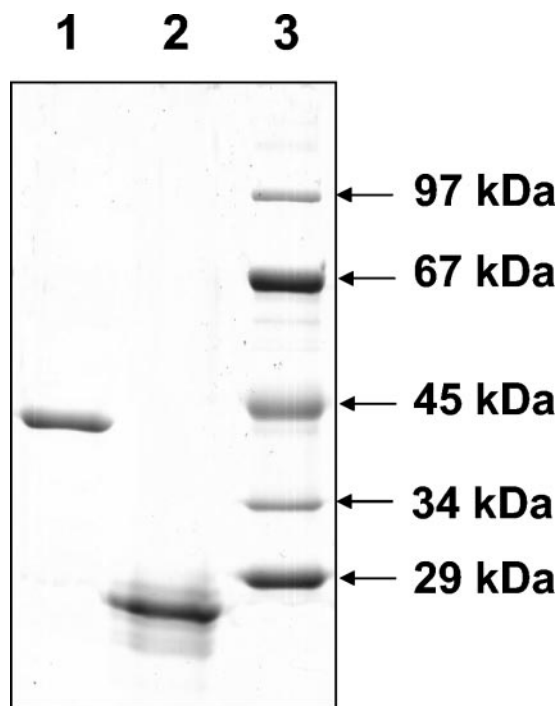


FIG. 4. Purification of overproduced proteins ORF2 and ORF3. SDS-PAGE (12.5%) of ORF2 and ORF3 after refolding is shown. Lanes: 1, ORF2 (5 μ g of protein); 2, ORF3 (5 μ g of protein); 3, molecular mass standard.

tion of phenylphosphate synthase activity when ORF1 was present (see below). Therefore, both ORFs were cloned and overexpressed in two different vector systems in *E. coli*. While a considerable amount of protein ORF1 was soluble (30 to 50% of overproduced protein), protein ORF2 consistently

formed inclusion bodies when the pMal or the pETBlue vector system was used. ORF1 could be purified by several chromatographic steps, while ORF2 had to be refolded from the inclusion bodies formed when the pETBlue-1 vector was used.

Purification of phenylphosphate synthase proteins ORF1 and ORF2. ORF1 without a tag was purified from 400 mg of cell extract protein, yielding 10 mg of ORF1 which was more than 80% pure (SDS-PAGE) (Table 2 and Fig. 3). In the case of the N-terminal fusion protein MalE-ORF1, an amylose affinity column was used, and the purified fusion protein was not proteolytically processed. From 120 mg of cell extract protein, 8 mg of MalE-ORF1 which was 70% pure was obtained (SDS-PAGE) (data not shown). Inclusion bodies of ORF2 formed in the pET system were prepared from cell extract. From the urea extract of 350 mg of protein of inclusion bodies, 180 mg of soluble ORF2 protein which was more than 90% pure was obtained (SDS-PAGE) (Fig. 4).

[U- 14 C]phenol exchange into phenylphosphate catalyzed by protein ORF1. Overproduced and purified wild-type ORF1 protein and the fusion protein MalE-ORF1 were tested for enzyme activities related to phenol phosphorylation. Two reactions were studied, the exchange of free [U- 14 C]phenol and the phenol moiety of phenylphosphate (exchange reaction) and the ATP-dependent formation of [14 C]phenylphosphate from [U- 14 C]phenol (net phenol phosphorylation). Both ORF1 preparations (with and without a MalE tag) catalyzed the exchange reaction with a specific activity of 1.1 nmol min $^{-1}$ mg $^{-1}$ of protein; however, they were unable to catalyze the ATP-dependent net phenol phosphorylation. The reaction was dependent on Mn $^{2+}$ (1 mM). No exchange activity was detectable with 1 mM Ca $^{2+}$, Mg $^{2+}$, Zn $^{2+}$, or Co $^{2+}$. The optimum pH of the exchange reaction was around 8.7, with half-maximal rates at pHs 8.2 and 9.4.

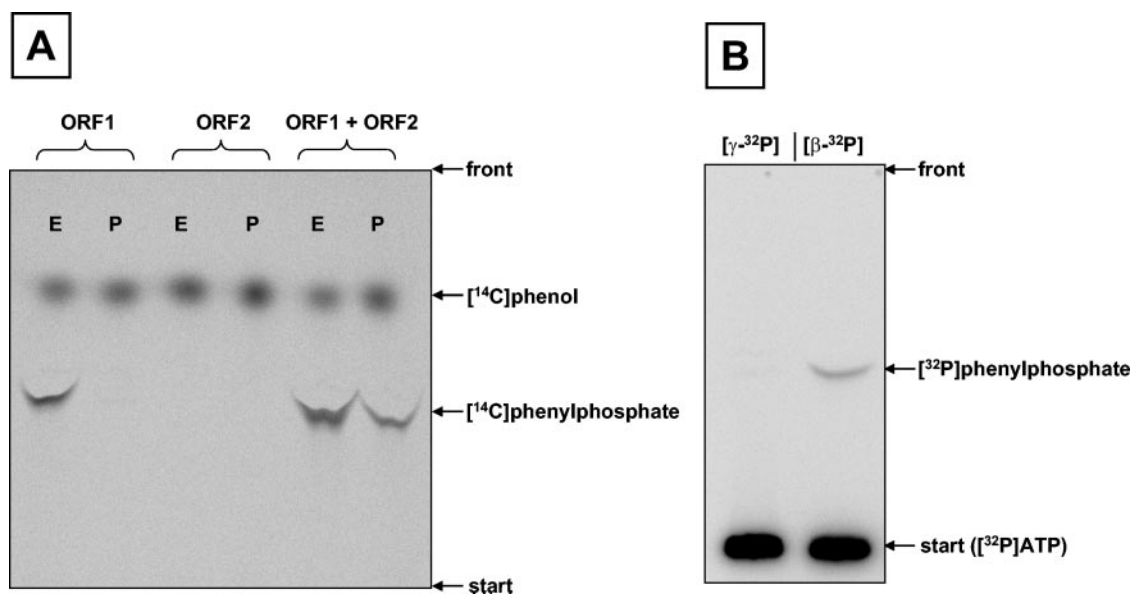


FIG. 5. Characterization of phenylphosphate synthase. A. Study of ATP-dependent net phosphorylation of [14 C]phenol to phenylphosphate (P) and of [14 C]phenol exchange into phenylphosphate (E). From left to right: either ORF1 (0.2 mg) alone, ORF2 alone (0.2 mg), or ORF1 and ORF2 combined (0.2 mg each) were tested in the two assays (P and E). B. Study of the incorporation of 32 P into phenylphosphate from either γ - 32 P-labeled ATP ([γ - 32 P]) or β - 32 P-labeled ATP ([β - 32 P]). Autoradiograms of TLC plates are shown. For experimental details, see Materials and Methods.

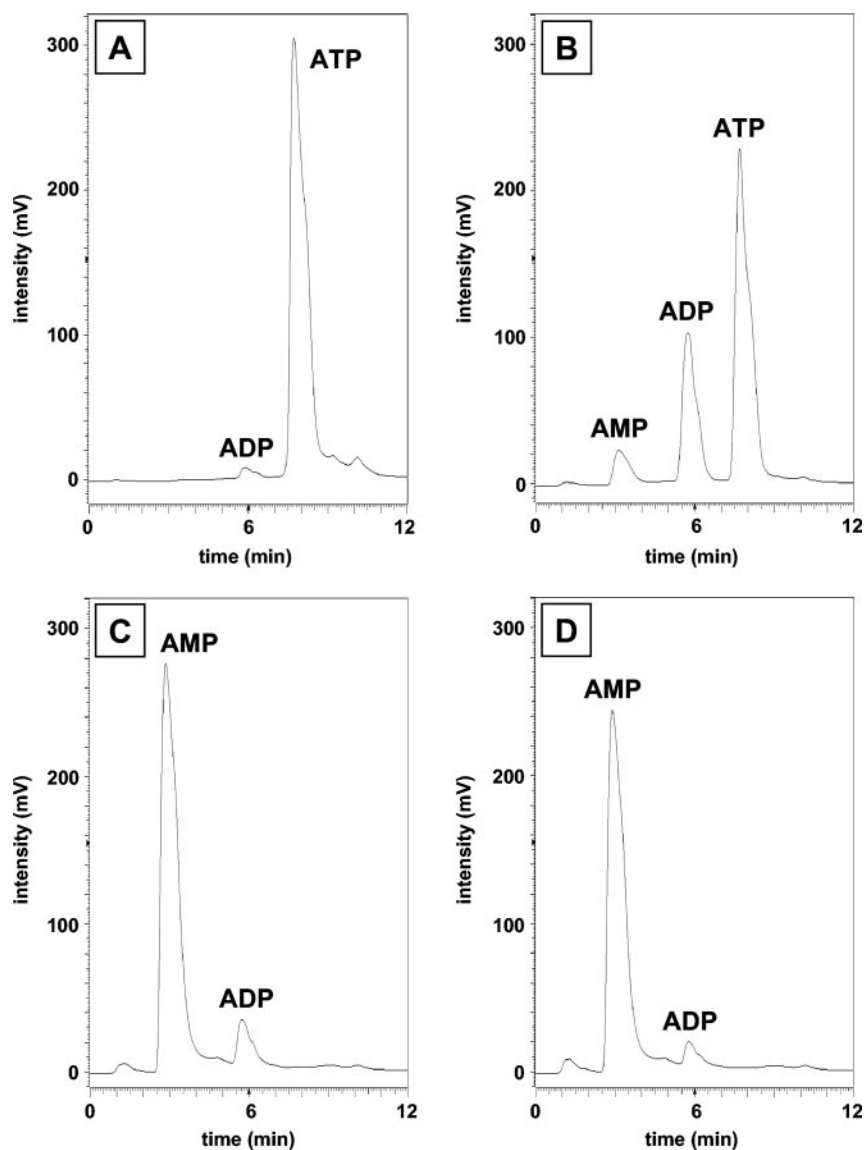


FIG. 6. HPLC analysis of products derived from 2 mM MgATP which were formed by subunits of phenylphosphate synthase in the absence of phenol. A. Control in which ATP was incubated for 15 h in the assay mixture in the absence of protein. B. Assay containing purified ORF2 (0.2 mg). C. Assay containing purified ORF1 (0.2 mg) plus ORF2 (0.2 mg). D. Assay containing purified ORF1 (0.2 mg) plus ORF2 (0.2 mg) plus ORF3 (0.2 mg). Elution times: AMP, 3 min; ADP, 6 min; ATP, 8 min.

Reconstitution of phenylphosphate synthase activity by combining proteins ORF1 and ORF2. ORF2 alone showed neither exchange nor net phenol phosphorylation activity (Fig. 5A). A combination of both ORF1 (without tag) and ORF2 exhibited both exchange activity and net phosphorylation activity ($1.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of protein) (Fig. 5A). In contrast, the combination of the MalE-ORF1 fusion protein and ORF2 did not reconstitute net phosphorylation activity (data not shown). Mn^{2+} (1 mM) stimulated the rate of the net phosphorylation reaction fourfold.

Determination of products of ATP hydrolysis. The products of ATP hydrolysis formed by the reconstituted phenylphosphate synthase (ORF1 and ORF2) in the presence of 2 mM phenol were determined. AMP was the only product formed,

besides phenylphosphate and trace amounts of ADP (data not shown).

ORF2 shows sequence similarity to the N-terminal part of phosphoenolpyruvate synthase which is supposed to contain the ATP binding site (25). Moreover, ORF2 was needed to reconstitute net phenol phosphorylation activity. This finding implies that ORF2 is involved in the binding and cleavage of ATP. When ORF2 alone was incubated in the absence of phenol, ATP was very slowly hydrolyzed, resulting mostly in the formation of ADP, but small amounts of AMP were also formed (Fig. 6B). This result suggests that ORF2 has low ATPase activity. Note that a small amount of ADP was also formed in the absence of enzyme (Fig. 6A). The combined subunits ORF1 and ORF2 catalyzed the formation of AMP

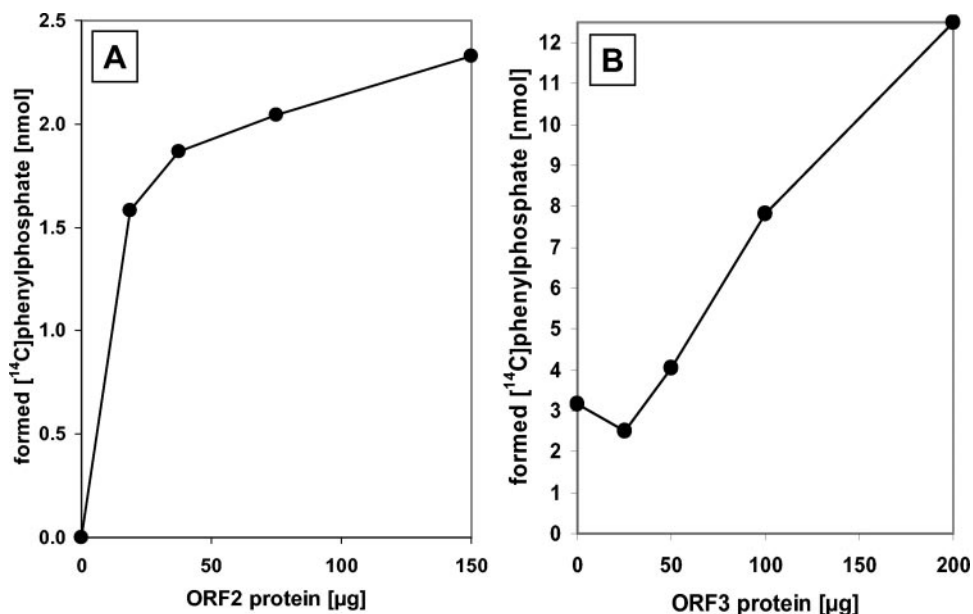


FIG. 7. Dependence of phenylphosphate synthase activity on (A) increasing amounts of ORF2 and (B) increasing amounts of ORF3. A. A constant amount of 840 μg of ORF1 was present in a 200-μl assay mixture (5-min incubation). B. Constant amounts of 100 μg of ORF1 and 200 μg of ORF2 were present in a 100-μl assay mixture (10-min incubation).

and trace amounts of ADP from ATP under the same conditions (Fig. 6C). The addition of ORF3 to ORF1 and ORF2 gave the same result (Fig. 6D), indicating that the reaction catalyzed by phenylphosphate synthase was as follows: phenol + MgATP + H₂O → phenylphosphate + MgAMP + inorganic phosphate.

Further catalytic properties of phenylphosphate synthase.

Purified phenylphosphate synthase consisting of ORF1 and ORF2 was characterized with respect to cosubstrate specificity and kinetic properties. Besides ATP, UTP (50% activity) was the only nucleoside triphosphate which could be used as a cosubstrate for phenol phosphorylation. The divalent metal ions Mg²⁺ and Mn²⁺ were required as cofactors. Mg²⁺ was strictly required, as expected for an ATP-consuming enzyme. Without Mn²⁺, the rate of net phosphorylation dropped to 25% of the rate with Mn²⁺. The addition of monovalent cations such as K⁺ (50 mM) or NH₄⁺ (30 mM) did not stimulate enzyme activity. Mercaptoethanol (40 mM) stimulated phenylphosphate formation (70% increase) and resulted in better reproducibility of the results. Apparent *K_m* values were 0.04 mM for phenol and 2 mM for ATP. The reaction was linearly time dependent in the range of 0 to 5 min maximally. The dependence of the rate of net phosphorylation on the amount of ORF2 is shown in Fig. 7A; a constant amount of ORF1 (4.2 mg/ml) was added. The net phenol phosphorylation reaction had a pH optimum of around 8.5, with half-maximal rates at pHs 7.5 and 9.2. UV-VIS spectra of the proteins ORF1, ORF2, and ORF3 showed only protein absorption maxima at around 280 nm. ORF1 showed a shoulder at 290 nm, which is most likely due to its relatively high (3.8%) tryptophan content.

Demonstration that the β-phosphoryl group of ATP is incorporated into phenylphosphate. Whether the β- or the γ-phosphoryl group of ATP was transferred to phenol was studied. When [γ -³²P]ATP was used in the assay, no radioactive

phenylphosphate was formed. In contrast, labeled phenylphosphate was formed when β-³²P-labeled ATP was used (Fig. 5B). This result showed that the β-phosphate of ATP is transferred to phenol in the course of the reaction and that the γ-phosphate must have been released as phosphate.

Stimulation of net phenol phosphorylation by ORF3 protein.

The previous experiments indicated that proteins encoded by *orf1* and *orf2* are basically sufficient to constitute net phosphorylation activity of phenylphosphate synthase. Nevertheless, the observed rates of this activity were very low. This result might indicate that one or both of the overexpressed proteins were largely inactive and/or that a component was missing. A plausible candidate for a missing component was the product of gene *orf3* which is adjacent to *orf2*. Therefore, *orf3* was cloned and overexpressed in *E. coli*, and whether it had an effect on phenol phosphorylation rate was tested. The procedure for cloning, overexpression, and refolding from inclusion bodies followed the protocol given for ORF2, and purification resulted in 70% pure ORF3 (Fig. 4). When increasing amounts of purified ORF3 were added to assays containing ORF1 and ORF2, an increase of up to fourfold in ATP-dependent phenylphosphate synthase activity was observed (Fig. 7B). This effect was not due to an increase of the protein concentration since bovine serum albumin did not show such a stimulatory effect. ORF3 had no effect on the rate of the [^{14}C]phenol exchange reaction.

Possible promoter region and primer extension analysis.

orf1 is the first gene of the phenol gene cluster (Fig. 2A). A transcription start site upstream of *orf1* was identified via a primer extension experiment. A labeled primer was derived from the 5' region of *orf1* and used in a reverse transcription assay with total RNA isolated from cells grown anaerobically on phenol and 4-hydroxybenzoate. The cDNA products were analyzed on a sequencing gel. With RNA isolated from phe-

nol-grown cells, a specific cDNA product corresponding to a transcription start at a guanosine 65 bp upstream of the ATG translation start codon of *orf1* was observed (Fig. 8). In the area 12 to 24 bp 5' of the transcription start, a σ^{54} -RNA polymerase-specific promoter sequence was found (GTGGCA CACCCTTGCA) (6) (Fig. 8). No cDNA product was observed with total RNA isolated from 4-hydroxybenzoate-grown cells, indicating that the transcription of the phenol gene cluster is phenol induced.

Expression of the gene cluster as an operon. RT-PCR was carried out to identify which of the genes encoded by the phenol gene cluster are coordinately expressed. A cDNA library of total RNA isolated from cells grown anoxically on phenol was prepared and used in PCR. Primers were derived from the coding regions of various genes and used to amplify PCR fragments covering the intergenic regions between two respective ORFs. These experiments demonstrated that ORFs 1 to 12, whose products are directly involved in phenol carboxylation, are transcribed as one mRNA product (Fig. 2B). Interestingly, ORFs 7 to 10 and 13 to 15, some of which encode phenol-induced proteins, are also located on the transcript. Furthermore, the operon seems not to be complete with *orf15*. The 5' part of an additional ORF (*orfX*) was found downstream of *orf15*. RT-PCR experiments indicate that this ORF is also cotranscribed. Hence, at least 15 genes of the cluster probably form an operon and are encoded on one transcript.

DISCUSSION

General properties of phenylphosphate synthase. We have described an enzyme that catalyzes the following reaction: phenol + MgATP + H₂O → phenylphosphate + MgAMP + orthophosphate. Two phosphate groups are transferred from the donor ATP to two different acceptors, phenol and water. The new enzyme, therefore, has the EC number 2.7.9.x and belongs to phosphotransferases with paired acceptors. The recommended name is phenylphosphate synthase or phenol:water dikinase. The systematic name is ATP:phenol:water phosphotransferase. The reaction catalyzed by phenylphosphate synthase is similar to that of phosphoenolpyruvate synthase (pyruvate: water dikinase) (EC 2.7.9.2), and so are the primary structures of the two 70- and 40-kDa subunits of the enzyme (see below). In contrast to phosphoenolpyruvate synthase, phenylphosphate synthase is stimulated by another small 23-kDa protein which does not have any counterpart in the literature. Enzyme activity is stimulated by Mn²⁺ and is not strongly affected by molecular oxygen, if at all.

Molecular composition and catalytic properties of phenylphosphate synthase. Phenylphosphate synthase activity clearly depends on ORF1 and ORF2 and is stimulated severalfold by ORF3. A strict dependence on ORF3 was not observed. Unfortunately, the overproduction of ORF2 and ORF3 resulted in the formation of inclusion bodies, and refolding of the insoluble proteins rendered them soluble but possibly mostly still inactive. Large portions of both proteins were precipitating during the first minutes of the enzyme assay. This result indicates that these proteins did not possess their native conformations. Therefore, the turnover rate of the reconstituted enzyme was much lower than expected. Also, no reliable information about the native subunit composition of the enzyme could be

A *Thauera aromatica*

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                ← ORF11
2641  CCGGAGATCA CCGCGTCTA TGCAGTTCAT ATCGTGAAGC TTGGCCATCA
2691  CCCTTCCCTCC TGAAGTGGTC CTTTTACGCG CAGCCACCAC GGGTGCTATT
      ←-----→
2741  GACGTGCGTC AAACGGCCCG GCGCGCGACT GCGCAGCGCC GGAAACGAAG
2791  AGAAGCCCTT GCGTTTCATCT AATGGTCAAT CCTGACGCCG GCCGGAAGGA
2841  GAACTGATCA TTTGATGAAT CGCATCCAAT GGCCGCTTTT TCCAATTACC
2891  CGGCACAAAC GCCCCGCCAG AAATTTATTT TTTGCAACTG CATGAAATGC
2941  TCGAAAGGCC TGCACAACGG GCAAACAGCG CTCGCCGCGT ATGCGCCCGA
2991  AGGCTGAATT GCTGCTCTCG CGCAATTAAT CGTGGCACAC CCTTTGCATT
      -24          -12
3041  GGATGCCTGG GAGGCGTCGT CCAACAAATC CGTTCGCAAC GATCGACAAC
      ORF1 →
3091  GGAAATAGCA AAGGAGGGG ATCAGATGAA GTTTCCTGTT CCGCAGACA
  
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B *Geobacter metallireducens*

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      ← "ORF11" (gi:23053807)
1    GTCATTTTCC CACCTCACCG CAAATTTGGCA CCAGAATCAC TTTTGAACC
51   GTCATTTTCAT AACGATATTT TATTATCATT GCCTAATTTG ATAGATTGTC
101  AGATGAACCA GTTTCGTACC ATACAAAACG GTCGTTATAC ATTTTACATG
151  TACATGACGA ATATGTCACG GCGAAATCCA CCACACTGCC CGCCTCTCAC
201  CCAAGCCAGG GGCAACAACG TACCGCCAAA TCTCCGTTCC ATCGCATTAT
      →
251  GCACCGTTAC CGCGGGGCCA TGGCAAACGG TATCCCTGCC ACGAGTTGAC
301  AAAATGTGCC AATTCCCGGC CGAGTAAACG AGAATAGTTA CAAATGGTCC
351  GACTGCCGAA AACAGGGGTC TGATCACCAC TGACGATTCA GCCTGGGTTT
401  CTTGCAGGCA ATTTGCGAAG CGCGCAAATC GCTCATTAGC CAGATAAATC
451  AGCCTGATTC GCTTAACACG TTCGATAGAC CTGTTGCGCC CCCACTCATT
501  ACAAACACT GTTTGGCACA GAGCATGCTC ATACCCCTCC CAAATGTCA
      -24          -12
551  ACTCACCACA AACCAGAACC TTCGTCGACG GCAACGAGGC CTCGCATCAC
      "ORF1" (gi:23053807) →
601  TAACCATGGA GCATCAACGT ACATTCGGGA GACACGCGAT GAAATTTCCA
  
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FIG. 8. Promoter structure of the phenol gene clusters of (A) *T. aromatica* and (B) *G. metallireducens*. The intergenic sequence between *orf1* and *orf11* (regulator gene) in *T. aromatica* and in *G. metallireducens* is shown. The ATG start codons are marked in boldface type. Numbers in front of the lines indicate the nucleotide positions within the phenol gene clusters (*T. aromatica* [GenBank accession number AJ272115] and *G. metallireducens*, intergenic region between putative ORFs [Joint Genome Institute accession numbers gi|23053807 and gi|23053806]). Shine-Dalgarno sequences are underlined. -24/-12 σ^{54} promoters are shown in grey. Arrows: inverted repeats; box: *T. aromatica* transcription start; dashed box: *G. metallireducens* proposed transcription start (bp + 1). The proposed ATG start codon for *orf1* of *Geobacter* was corrected (see the text).

obtained. Interestingly, even in concentrated cell extracts, the observed phenylphosphate synthase activity was extremely low and could be reliably measured only when cell extract was prepared by lysozyme treatment of cells, the extract was precipitated by polyethyleneglycol, and the protein content in the assay mixture was high. This finding suggests that the native enzyme easily decomposes into its subunits and becomes inactive. A similar behavior is known for some other enzymes; for instance, acetyl-coenzyme A (CoA) carboxylase is notorious for being completely inactive in cell extracts of most bacteria, and low enzyme activity can be achieved only by recombining the three separately purified subunits (20). The natural sub-

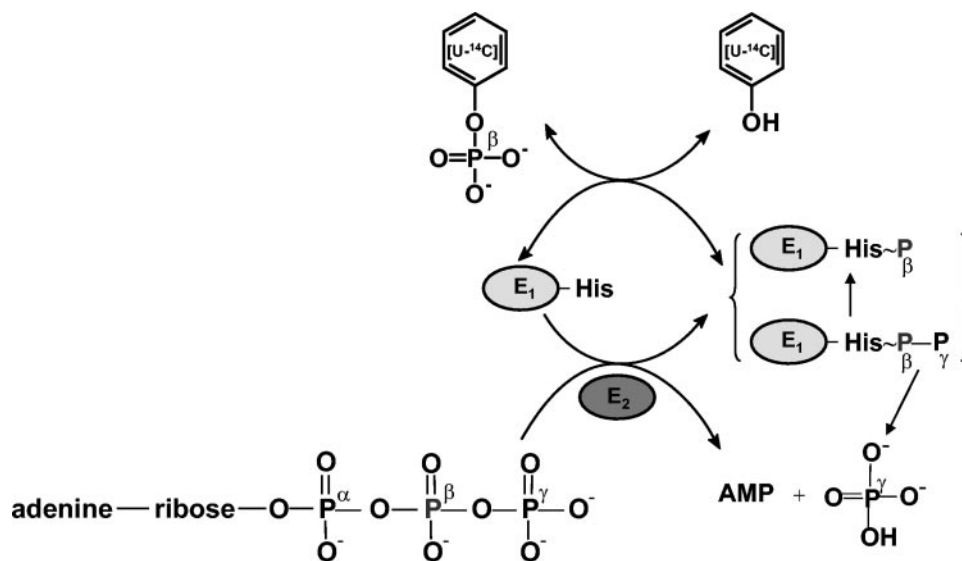


FIG. 9. Hypothetical catalytic mechanism of the complete phenylphosphate synthase reaction catalyzed by the combined action of ORF1 and ORF2 and of the partial reaction, the exchange of free $[^{14}\text{C}]$ phenol, and the phenol moiety of phenylphosphate (exchange reaction) catalyzed by ORF1 alone. Note that the role of ORF3 is not explained by this scheme.

strates of the enzyme are phenol (apparent K_m , 0.04 mM) and MgATP (apparent K_m , 2 mM). The low apparent K_m value for phenol obviously allows the bacterium to grow at rather low ambient concentrations of phenol.

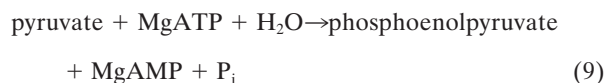
Function of ORF1, ORF2, and ORF3 of phenylphosphate synthase. The conclusion that ORF1 alone is sufficient to catalyze the $[^{14}\text{C}]$ phenol exchange reaction was derived from experiments with ORF1 (without and with fusion protein) overproduced in *E. coli*. ATP-dependent net phosphorylation of phenol required the addition of ORF2, indicating that the active holoenzyme can be reconstituted from its subunits and therefore may not form a stable enzyme complex. The N-terminal 42-kDa MalE protein fusion to ORF1 seemed to disturb ORF1 and ORF2 interaction since this combination was inactive in ATP-dependent phenol phosphorylation, whereas the fusion protein was active in the $[^{14}\text{C}]$ phenol exchange reaction.

The dependence of phenol phosphorylation on the amount of ORF2 added to a constant amount of ORF1 resulted in a saturation curve (Fig. 7A) rather than in a sharp titration curve, again indicating that the two subunits may not form a stable complex. For unknown reasons, the rate of phenylphosphate formation rapidly decreased with time. This rate decrease cannot be explained by rapidly reaching an equilibrium between $[^{14}\text{C}]$ phenol and $[^{14}\text{C}]$ phenylphosphate.

The role of ORF3 is at issue. Adding protein ORF3 to the standard net phosphorylation assay improves the overall rate of phenylphosphate formation. ORF3 contains a pair of cystathionine- β -synthase domains (7). These domains of so-far-unknown function are proposed to participate in the binding of the adenosyl moiety or may play a role as a structural or regulatory component (27, 44). A similar function could be proposed for ORF3.

Proposed catalytic mechanism and similarity of phenylphosphate synthase to PEP synthase. Phenylphosphate synthase transfers the β -phosphoryl rather than the γ group from ATP (donor) to the acceptor phenol. This behavior has been

reported for PEP synthase, which catalyzes a similar reaction (equation 9).



The catalytic features of phenylphosphate synthase are consistent with a ping-pong mechanism, as in PEP synthase (39). ORF2 contains an ATP binding domain, suggesting that this protein catalyzes the transfer of the pyrophosphoryl group of ATP to the conserved target histidine residue (His569) in ORF1; this residue corresponds to the conserved histidine 421 in *E. coli* PEP synthase which is thought to be reversibly transformed into a pyrophosphorylated histidine- β -phosphate- γ -phosphate. The terminal γ -phosphate group is proposed to be irreversibly hydrolysed off (transferred to water) from ORF1, giving rise to free γ -phosphate, whereas the histidine- β -phosphate is reversibly transferred to phenol (note that phenol formally corresponds to the enol form of pyruvate). The phosphoryl group transfer from phosphorylated ORF1 to phenol giving rise to phenylphosphate and nonphosphorylated ORF1 must be reversible, resulting in the observed exchange reaction of $[^{14}\text{C}]$ phenol and the phenol moiety of phenylphosphate. This means that the group transfer potential of phosphohistidine of ORF1 and that of phenylphosphate should not be too dissimilar. The role of Mn^{2+} in this catalytic cycle is at issue. Maybe Mn^{2+} is required to stabilize the enzyme-phosphate intermediate or the interaction between ORF1 and phenylphosphate, since the exchange reaction was mainly affected by Mn^{2+} . Some kinases require another divalent cation for full activity in addition to Mg^{2+} , which coordinates with the nucleotide. Examples are pyruvate kinase and phosphoenolpyruvate carboxykinase (4, 33, 49). The second divalent metal cation has a separate distinct binding site (37). Hence, the proposed mechanism for phenylphosphate synthase shown in Fig. 9 is completely analogous to that of PEP synthase. This

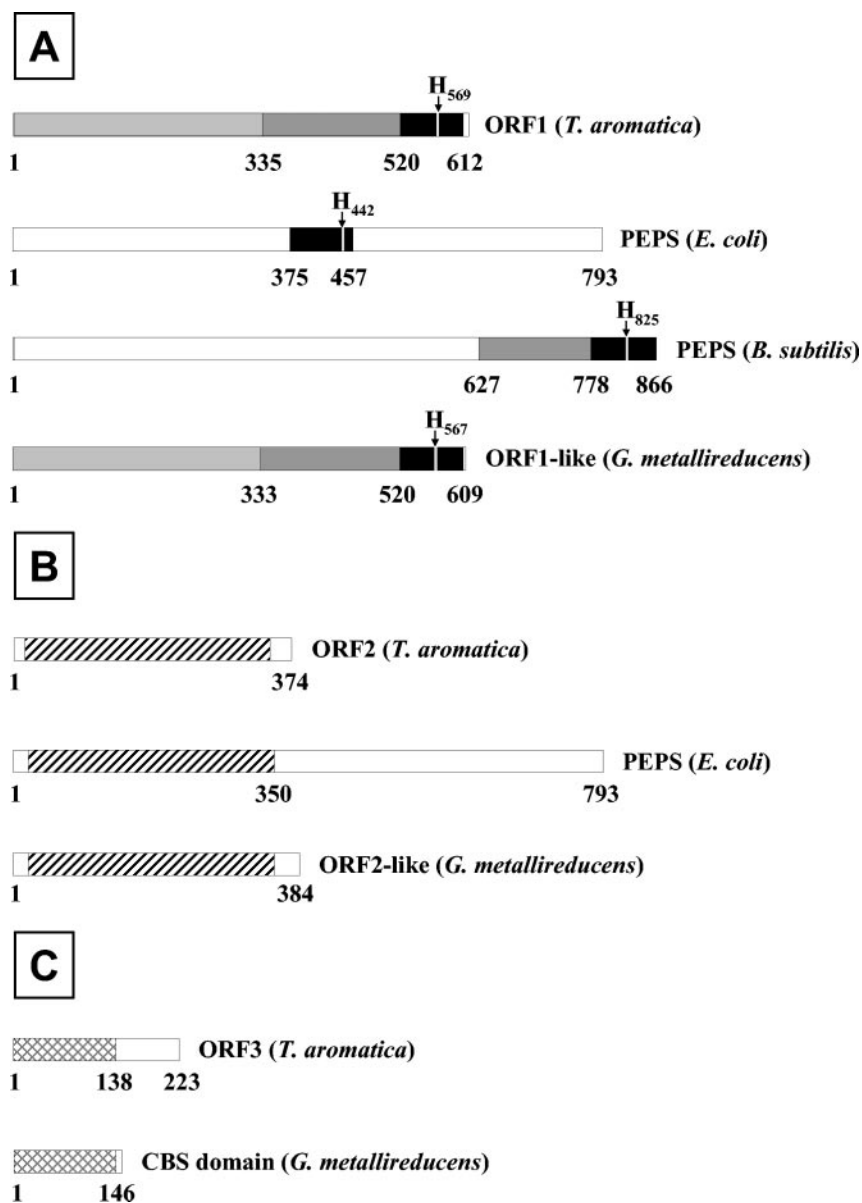


FIG. 10. Comparison of the proteins ORF1 (A), ORF2 (B), and ORF3 (C) with proteins in databases. The numbers refer to the positions of the amino acids. A. Highly similar sequences are marked in black, and sequences with weak or no similarities are in white. Dark grey indicates an extended conserved region in the proteins of *T. aromatica*, *B. subtilis*, and *G. metallireducens*, and light grey indicates an additional sequence similarity between the proteins of *T. aromatica* and *G. metallireducens*. H marks a conserved histidine residue in all four proteins. Striping (B) and cross-hatching (C) indicate sequence similarities. For details, see Discussion. CBS, cystathionine- β -synthase; PEPS, PEP synthase.

scheme does not account for the role of ORF3, however, and therefore must be incomplete.

Similar entries in databases. *Geobacter metallireducens* is the only one of the bacteria which reportedly grows with phenol under anaerobic conditions whose genome has been partially sequenced (NCBI accession no. gi|48851373|ref|NZ_AAAS00000000.12). This genome contains orthologues of ORFs 1, 2, and 3 located near each other (discussed in reference 43). This suggests that phenol metabolism in this bacterium is similar to that in *T. aromatica*. The following similar proteins were reported in the database (Fig. 10).

(i) **ORF1.** ORF1 (612 amino acids [aa], 70.26 kDa) has the highest similarity (percentage of identical or similar

amino acids) with a hypothetical, ORF1-like protein of similar size from *G. metallireducens* (NCBI accession no. gi|48845085|ref|ZP_00299374.1) (78% identity and 88% similarity). The N-terminal part of approximately 500 amino acids does not show strong similarity with other known proteins. However, the approximately 100-amino-acid C-terminal part of the protein has high similarity with the central part of PEP synthase from *E. coli* (accession number gi|16129658|ref|NP_416217) (37% identity and 55% similarity). Interestingly, an extended C-terminal region of ORF1 (approximately 240 aa) has a counterpart in the C-terminal region of the PEP synthase of *Bacillus subtilis* (accession number gi|16078943|ref|NP_389764) (28% identity and 47% sim-

ilarity). The highly conserved 100-amino-acid C-terminal part of ORF1, which corresponds to the central or C-terminal part of most PEP synthases, contains a conserved histidine which was shown for PEP synthase of *E. coli* and is considered to represent the phosphorylation site for phenylphosphate synthase.

(ii) **ORF2.** ORF2 (374 aa, 40.42 kDa) has the highest similarity with a hypothetical protein of similar size from *G. metallireducens* (NCBI accession no. gi|488450861|ref|ZP_00299375.1) (70% identity and 83% similarity) and with the N-terminal part (approximately 350 aa) of PEP synthase of *E. coli* (44% identity and 61% similarity) and other (putative) PEP synthases from different bacteria (e.g., *B. subtilis* and *Staphylothermus marinus*).

(iii) **ORF3.** ORF3 (223 aa, 24.24 kDa) has the highest similarity with a hypothetical (NCBI Microbial Genomes Annotation Project) protein of similar size from *G. metallireducens* (NCBI accession no. gi|48845089|ref|ZP_00299378.1) (60% identity and 77% similarity). Both proteins contain a pair of cystathionine- β -synthase domains (pfam00571) (7) with so-far-unknown function.

Possible regulator of the gene cluster. A gene for a putative regulator protein, ORF11, is located directly upstream of *orf1* but is transcribed in the opposite direction (16, 43). ORF11 shows high similarity to σ^{54} -dependent transcription activators of gene clusters involved in the aerobic degradation of phenolic [e.g., (dimethyl)phenol] compounds (16). Examples are PoxR of *Ralstonia* sp. (Joint Genome Institute accession number gi|3445531) (54% identity and 70% similarity) and DmpR of *Pseudomonas* sp. (accession number gi|483552) (47% identity and 68% similarity) (26, 45, 46). Orf11 also has high similarity with three putative σ^{54} -dependent activators of *G. metallireducens* (accession numbers gi|23053791, gi|23053793, and gi|23053807) (66% identity and 79% similarity). One of these regulator genes is located as *orf11* in *T. aromatica* (Fig. 2A) (43). They all contain an XylR domain (pfam06505) in the N-terminal part; the binding of the respective effector molecule is expected to lead to their activation (reviewed in references 17 and 48).

Promoter structure. σ^{54} -RNA polymerase specifically binds to promoters with the consensus sequence YTGGCACGN₄T TGCW in the area 12 to 24 bp 5' of the transcription start (6). A putative $-12/-24$ promoter consensus sequence was found 5' of the transcription start of *T. aromatica orf1* (16) (Fig. 8A). A similar putative σ^{54} -dependent promoter sequence was also found in the *Geobacter* gene cluster but was overlapping with the proposed ATG start codon for the *orf1* orthologue (Fig. 8B), which is rather unlikely. Also, no Shine-Dalgarno consensus sequence is found in the proposed -8 -bp region, and the ORF1 orthologue would be N-terminally prolonged by 38 amino acids compared with the *T. aromatica* ORF1 sequence. We therefore propose a corrected ATG start codon for *Geobacter* ORF gi|23053806 (Fig. 8B), which shows a Shine-Dalgarno consensus sequence, and the derived protein has full sequence similarity to the *Thauera* ORF1 protein. The *Geobacter* $-12/-24$ σ^{54} promoter sequence is located about 120 bp upstream of the corrected start of *orf* gi|23053806.

The σ^{54} -dependent activator proteins usually bind to enhancer sequences that are mostly inverted repeats located 100 to 200 bp upstream of the promoter (28). Inverted repeats representing possible binding sites for the regulator protein are

found about 260 bp upstream of the *Thauera* $-12/-24$ promoter consensus sequence and in the region between -220 and -370 bp upstream of the *Geobacter* orthologue (Fig. 8), respectively. Contact between regulator protein and RNA polymerase is mediated by the formation of a DNA loop. In some cases, DNA is bent via the binding of the integration host factor to AT-rich DNA sequences located between the promoter and enhancer regions. This feature also applies to the 5' region of the phenol gene cluster of *Thauera* and to the orthologue of *Geobacter* (accession number gi|23053806) (Fig. 8). We therefore conclude that the phenol gene cluster of *T. aromatica* and the putative phenol gene cluster of *G. metallireducens* are under the transcriptional control of a σ^{54} -dependent regulator, most probably encoded by *orf11* and an *orf11* orthologue of *G. metallireducens*, respectively.

Postulation of operon structure. ORF11 was proposed to control the expression of the phenol gene cluster, acting as a transcriptional activator when binding to phenol or a similar compound (16). It was previously shown (43) that only the 5' part of the phenol gene cluster of *T. aromatica* (ORFs 1 to 6 and 12) is needed for anaerobic degradation of phenol to 4-hydroxybenzoate, and we proposed that the 3' part of the cluster (ORFs 7 to 10 and 13 to 15) may play a role in the degradation of a phenol derivative. Nevertheless, both parts of the gene cluster seemed to be coordinately expressed, as indicated by identification of phenol-induced proteins and RT-PCR experiments. It was shown for DmpR (45, 46) that this regulator protein was activated not only by phenol but also by some (methyl) derivatives. Therefore, it is possible that not only phenol but also similar phenolic compounds may activate ORF11 and lead to the transcription of the gene cluster; the degradation of those compounds may need the proteins encoded by the 3' part of the cluster.

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