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Characterization of Three Distinct Extradiol Dioxygenases Involved in Mineralization of Dibenzofuran by *Terrabacter* sp. Strain DPO360

ANDREAS SCHMID,[1]† BERND ROTHE,[1] JOSEF ALTENBUCHNER,[2] WOLFGANG LUDWIG,[3] AND KARL-HEINRICH ENGESSER[1]‡*

Institut für Mikrobiologie[1] and Institut für industrielle Genetik,[2] Universität Stuttgart, 70569 Stuttgart, and Lehrstuhl für Mikrobiologie, Technische Universität München, 80290 Munich,[3] Germany

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The dibenzofuran-degrading bacterial strain DPO360 represents a new species of the genus *Terrabacter* together with the previously described dibenzofuran-mineralizing bacterial strain DPO1361 (K.-H. Engesser, V. Strubel, K. Christoglou, P. Fischer, and H. G. Rast, FEMS Microbiol. Lett. 65:205–210, 1989; V. Strubel, Ph.D. thesis, University of Stuttgart, Stuttgart, Germany, 1991; V. Strubel, H. G. Rast, W. Fietz, H.-J. Knackmuss, and K.-H. Engesser, FEMS Microbiol. Lett. 58:233–238, 1989). Two 2,3-dihydroxybiphenyl-1,2-dioxygenases (BphC1 and BphC2) and one catechol-2,3-dioxygenase (C23O) were shown to be expressed in *Terrabacter* sp. strain DPO360 growing with dibenzofuran as a sole source of carbon and energy. These enzymes exhibited strong sensitivity to oxygen. They were purified to apparent homogeneity as homodimers (BphC1 and BphC2) and as a homotetrameric catechol-2,3-dioxygenase (C23O). According to their specificity constants k_{cat}/K_m, both BphC1 and BphC2 were shown to be responsible for the cleavage of 2,2′,3-trihydroxybiphenyl, the first metabolite in dibenzofuran mineralization along the angular dioxygenation pathway. With this substrate, BphC2 exhibited a considerably higher k_{cat}/K_m value (183 μM/min) than BphC1 (29 μM/min). Catechol-2,3-dioxygenase was recognized to be not involved in the ring cleavage of 2,2′,3-trihydroxybiphenyl (k_{cat}/K_m, 1 μM/min). Analysis of deduced amino acid sequence data of *bphC1* revealed 36% sequence identity to *nahC* from *Pseudomonas putida* PpG7 (S. Harayama and M. Rekik, J. Biol. Chem. 264:15328–15333, 1989) and about 40% sequence identity to various *bphC* genes from different *Pseudomonas* and *Rhodococcus* strains. In addition, another 2,3-dihydroxybiphenyl-1,2-dioxygenase gene (*bphC3*) was cloned from the genome of *Terrabacter* sp. strain DPO360. Expression of this gene, however, could not be detected in *Terrabacter* sp. strain DPO360 after growth with dibenzofuran.

Due to the environmental significance of its chlorinated derivatives, the metabolism of dibenzofuran has been studied intensively in the last few years (10, 14, 16–18, 48, 58, 59, 65). Accordingly, bacterial mineralization of dibenzofuran is initiated by dibenzofuran 4,4a-dioxygenase, catalyzing the angular dioxygenation of dibenzofuran. This gives rise to the chemically labile hemiacetal 4,4a-dihydro-4,4a-dihydroxydibenzofuran, which, after spontaneous rearomatization, yields 2,2′,3-trihydroxybiphenyl (16, 59). A multicomponent dioxygenase of this angular dioxygenase type was purified from *Sphingomonas* sp. strain RW1 (9) as a four-component class IIA dioxygenase system. Ring cleavage of 2,2′,3-trihydroxybiphenyl was shown to be catalyzed by extradiol dioxygenases yielding 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid (16, 59), which is subsequently hydrolyzed to 2-oxopent-4-enoate and salicylate (8, 16, 59). From *Sphingomonas* sp. strain RW1, a 2,2′,3-trihydroxybiphenyl-cleaving extradiol dioxygenase which was suggested to be involved in dibenzofuran mineralization was cloned and characterized (24). In this paper, three distinct extradiol dioxygenases from *Terrabacter* sp. strain DPO360 are shown to be involved in total mineralization of dibenzofuran and characterized according to their function in the degradative pathway.

* Corresponding author. Mailing address: Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft (ISWA), Abteilung Biologische Abluftreinigung, Universität Stuttgart, Bandtäle 2, D-70569 Stuttgart, Germany. Phone: 49 711 6853734. Fax: 49 711 6853729. E-mail: karl-h.engesser@iswa.uni-stuttgart.de.

† Present address: Institut für Biotechnologie, ETH-Hönggerberg, HPT, CH-8093 Zürich, Switzerland.

‡ Present address: ISWA, Abteilung Biologische Abluftreinigung, Universität Stuttgart, 70569 Stuttgart, Germany.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Terrabacter* sp. strain DPO360 was isolated from tar-contaminated soil in Germany (57), and *Terrabacter* sp. strain DPO1361 was isolated from Rhine water (58). Both gram-positive isolates were catalase positive and cytochrome oxidase negative. Biochemical characterization of these strains using the API-CH50 and API-20NE test kits (Biomerieux) gave identical results, apart from positive reactions for esculin and 5-ketogluconate with *Terrabacter* sp. strain DPO1361, reactions which were negative for *Terrabacter* sp. strain DPO360. Both strains were cultivated as described earlier (58) with dibenzofuran as a source of carbon. Yeast extract (0.005% [wt/vol]) was added to the minimal medium to supply vitamins. L broth (42) was used as a complex medium for *Terrabacter* sp. and *Escherichia coli* strains. *E. coli* JM101 [*supE thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) [47] was used as a recipient for library construction and screening, and *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) [69] was used in all other cloning experiments. The cloning vectors pUC20 and pUC21 (identical with pUC20, except for an inverted polylinker) were obtained from Boehringer Mannheim Biochemicals. Plasmids constructed in the present study are shown in Fig. 3.

Chemicals. Chemicals were of the highest purity commercially available (Merck, Darmstadt, Germany; Sigma-Aldrich, Steinheim, Germany). 3-Phenylcatechol was obtained from Wako Chemicals (Neuss, Germany). 3-Chlorocatechol was obtained by chlorination of catechol by the method of Willstätter and Müller (66). Enzymes and reagents used for DNA manipulations were purchased from Gibco BRL and Boehringer Mannheim Biochemicals and were used as suggested by the suppliers.

HPLC. High-pressure liquid chromatography (HPLC) analysis was carried out with an HPLC system (Merck), equipped with a diode array detector, an RP-8 column (125 by 4.6 mm [internal diameter]; Grom, Herrenberg, Germany), water-methanol (55% methanol, isocratic) as the mobile phase with a final pH of 2.1, and a flow rate of 1 ml/min.

Preparation of crude cell extracts and enzyme assays. Cells were harvested during the late exponential growth phase and resuspended into phosphate buffer (50 mM Na_2HPO_4-KH_2PO_4 [pH 7.5]) for determination of enzyme activity or in

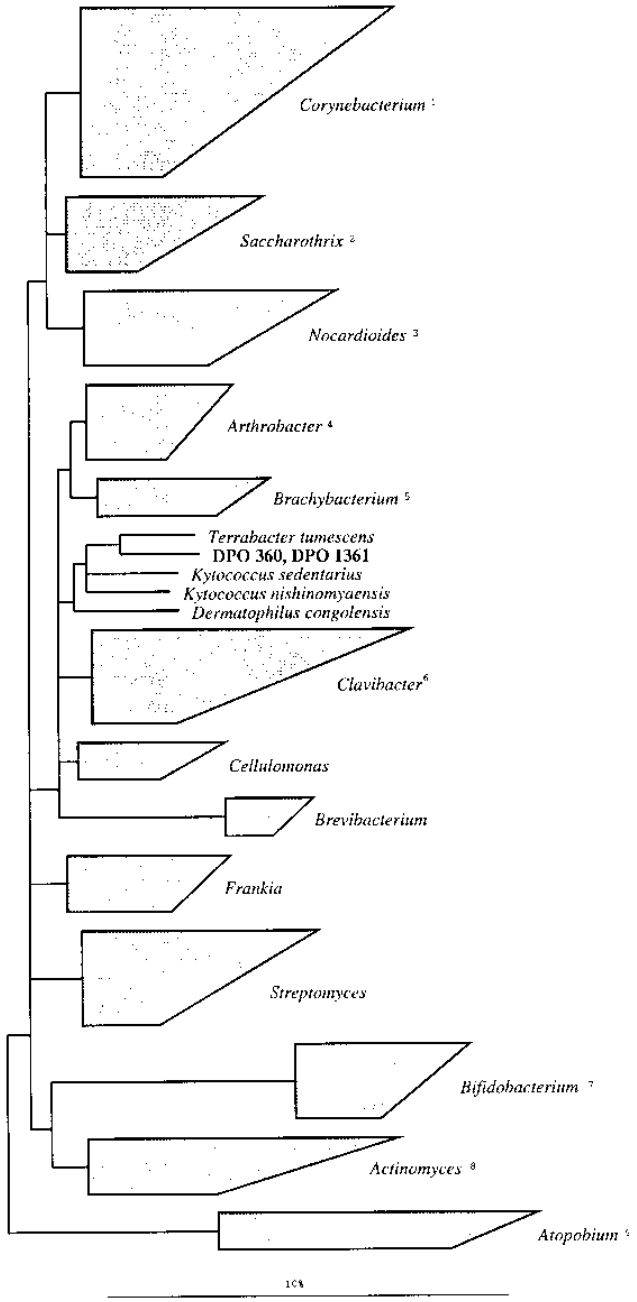


FIG. 1. 16S rRNA-based tree reflecting the phylogenetic position of *Terrabacter* sp. strain DPO360 and *Terrabacter* sp. strain DPO1361. The tree was constructed by performing distance matrix analyses of all available at least 90% complete (in comparison with 16S rRNA from *E. coli*) 16S rRNA primary structures. Only sequence positions which share the same nucleotide in at least 50% of the whole data set were included for the calculations. The tree topology was evaluated according to the results of maximum parsimony and maximum likelihood analyses. Multifurcations indicate that a significant relative branching order could not be found by applying different treeing methods and data sets. The rectangles indicate phylogenetic groups. Horizontal edges reflect maximum and minimum estimated phylogenetic distances among the members of the particular groups; the vertical lines roughly indicate the number of representatives. The bar indicates 10% estimated sequence divergence. Abbreviations: 1, *Corynebacterium*, *Dietzia*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Tsukamurella*; 2, *Actinokineospora*, *Actinosynnema*, *Amycolata*, *Amycolatopsis*, *Kutznera*, *Lentzea*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Thermocrispum*; 3, *Aeromicrobium*, *Micronulatus*, *Nocardioides*, *Propionibacterium*, *Propioniferax*; 4, *Arthrobacter*, *Renibacterium*, *Micrococcus*; 5, *Brachybacterium*, *Dermabacter*; 6, *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium*, *Microbacterium*, *Rathayibacter*; 7, *Bifidobacterium*, *Gardnerella*; 8, *Actinomyces*, *Mobiluncus*; 9, *Atopobium*, *Coriobacterium*.

50 mM Tris-HCl buffer (pH 7.5) for protein chromatography. Cells were disrupted with a French press (Aminco, Silver Spring, Md.). Prior to disruption, lysozyme (16 mg [wet weight] per g) was added to *Terrabacter* sp. strain DPO360, and the cells were incubated at 37°C for 30 min. Cell debris was removed by centrifugation (35 min, 100,000 × *g*). Protein was determined by the Bradford procedure (7) (Bio-Rad, Munich, Germany).

Photometric determination of extradiol dioxygenase activity for catechol and 3-phenyl-catechol was performed at 25°C as described elsewhere (59). Extradiol dioxygenase activity for 2,2′,3-trihydroxybiphenyl was determined polarographically with a Clark-type electrode with a thermostated (25°C) 2-ml reaction vessel (YSI, Yellow Springs, Ohio). 2-Hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid (HOPDA) hydrolase activity was determined in phosphate buffer as a decrease in absorbance at 434 nm (51) and was calculated on the basis of an extinction coefficient of 22 cm^2 μmol^{-1} (19). HOPDA was obtained from 2,3-dihydroxybiphenyl by using purified BphC1 of *Terrabacter* sp. strain DPO360.

Enzyme purification. Purification of extradiol dioxygenase enzymes was performed at ambient temperature by using a fast-performance liquid chromatography (FPLC) system, which consisted of a GP250 controller, two P500 pumps, a UV-1 monitor, a REC-482 recorder, and a FR100 autosampler (all from Pharmacia, Uppsala, Sweden). Extradiol dioxygenase activity in aerobic crude extracts of *Terrabacter* sp. strain DPO360 was stable over several days, whereas preparations of extradiol dioxygenases enriched by aerobic FPLC showed a rapid decrease in activity (half-life of purified BphC1 in 50 mM Tris-HCl buffer [pH 7.5] containing Fe^{2+} [1 mM] and dithiothreitol [1 mM] was 1 h). Therefore, all purification procedures, except preparation of crude extracts, were carried out in an anaerobic chamber (Coy, Ann Arbor, Mich.) (N_2-H_2 [95/5]; palladium catalyst) containing the FPLC unit and a small centrifuge for protein concentration (Centricon tubes; Amicon, Beverly, Mass.). For stabilization, enzyme fractions were adjusted to 1 mM Fe^{2+} and 1 mM dithiothreitol and stored under N_2-H_2 (95/5). They could be stored at 4°C for 1 week without significant loss of activity. The efficiency of single chromatography steps was monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and extradiol dioxygenase activities were characterized by comparing activity ratios of 3-phenylcatechol and catechol.

(i) Anion-exchange chromatography. Crude extract was loaded onto a Q-Sepharose HR 16/10 column equilibrated with buffer A (50 mM Tris-HCl [pH 7.5]) and operated with a flow rate of 3 ml/min. Enzymes were eluted with a linear NaCl gradient (0 to 0.5 M) over 200 min. Purified from *Terrabacter* sp. strain DPO360, BphC1, BphC2, and C23O eluted at NaCl concentrations of 328, 388, and 363 mM, respectively.

(ii) Hydrophobic interaction chromatography. FPLC fractions containing high activity of the separated dioxygenases were pooled, adjusted to a final $(NH_4)_2SO_4$ concentration of 1 M, and centrifuged to remove precipitated protein. Each pool was loaded onto a phenyl-Superose HR10/10 column equilibrated with buffer A containing $(NH_4)_2SO_4$ (1 M). At a flow rate of 1 ml/min, the enzymes were eluted with a linear $(NH_4)_2SO_4$ gradient (1 to 0 M) in 100 min. BphC1, BphC2, and C23O eluted at concentrations of 284 mM, 0 M, and 0 M, respectively; compared with BphC2, C23O eluted 2 min earlier.

(iii) Gel filtration chromatography. FPLC fractions containing high extradiol dioxygenase activity were combined, concentrated, and loaded onto a HiLoad 16/10 Superdex 200 preparation-grade column equilibrated with buffer A containing 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions containing purified extradiol dioxygenase enzymes were combined and concentrated.

SDS-PAGE. SDS-PAGE was performed with a gel chamber system from Biometra (Göttingen, Germany). The protein bands on the gels were stained with Coomassie brilliant blue G250 (Merck).

Molecular weight determination. The relative molecular weights of the native enzymes were determined by gel filtration on a Hiload 16/60 Superdex 200 preparation-grade column (Pharmacia) calibrated with bovine thyroglobulin (670,000), bovine gamma globulin (158,000), ovalbumin (44,000), horse myoglobulin (17,000), and vitamin B_{12} (1,350). The sizes of the subunits were determined by SDS-PAGE with phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) as reference proteins (Pharmacia).

Enzyme kinetics. The kinetic parameters were calculated with the Enzfitter kinetic analysis computer program (Biosoft, Cambridge, Great Britain).

DNA techniques and nucleotide sequence analysis. All DNA techniques, if not specifically stated, were carried out according to established procedures described elsewhere (53).

(i) DNA sequencing and analysis of 16S rRNA genes. In vitro amplification of 16S rRNA encoding DNA and direct sequencing of amplified DNA were performed as described earlier (56). The new 16S rRNA sequences were added to an alignment of about 6,000 homologous completed and partial primary structures from bacteria (44, 63) by using the alignment tool of the ARB program package (60). Phylogenetic analysis was performed by applying distance matrix, maximum parsimony, and maximum likelihood methods with the corresponding tools of the ARB (60) and PHYLIP (15) program packages as well as the fastDNAml program (44). The composition of the data sets varied with respect to the reference sequences as well as alignment positions included. Positional variabilities of the individual alignment positions were determined with the

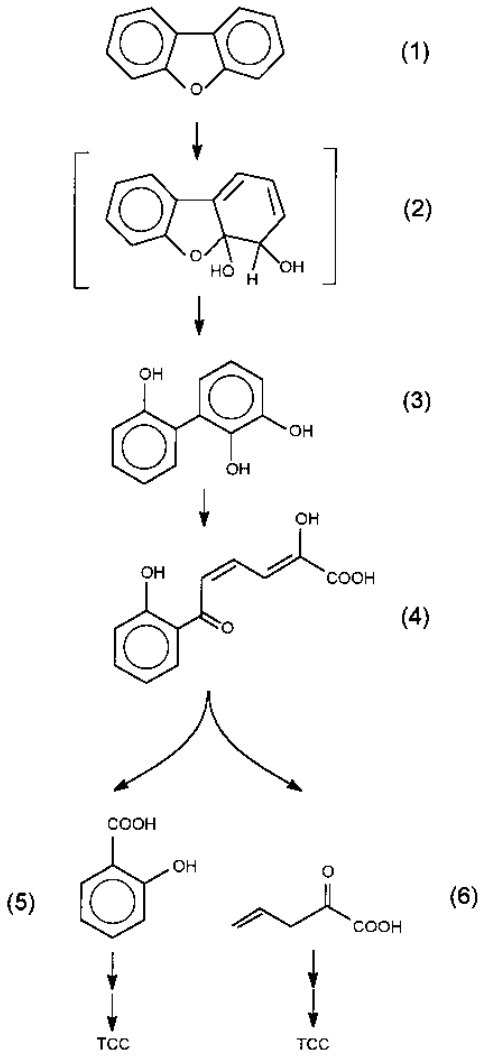


FIG. 2. Pathway proposed for the metabolism of dibenzofuran by *Terrabacter* sp. strain DPO360. 1, dibenzofuran; 2, 4,4a-dihydro-4,4a-dihydroxydibenzofuran; 3, 2,2′,3-trihydroxybiphenyl; 4, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; 5, salicylate; 6, 2-oxo-4-pentenoate. TCC, tricarboxylic acid cycle.

respective tools of the ARB package and used as criteria to remove or include variable positions.

For the determination of the nucleotide sequence, the 2.2-kb *Bgl*II/*Eco*RI fragment (Fig. 5) from *Terrabacter* sp. strain DPO360 containing the *bphC1* gene was inserted into the polylinker sequence of the vector pUC20 (Boehringer, Mannheim, Germany). Smaller fragments were obtained by subcloning or by generating deletions with restriction enzymes cutting in the 2.2-kb *Bgl*II/*Eco*RI fragment and the multiple cloning site (*Sal*I, *Eco*RI, *Eco*RV, *Sac*I, *Xho*I, *Apa*I, *Sma*I, *Hin*dIII). Plasmid DNA was isolated with the QUIAwell 8 Plus kit (Qiagen) and sequenced on an ALF automated fluorescent sequencer with the AutoRead Sequencing kit (Pharmacia) according to the instructions of the manufacturer. The nucleotide sequence was analyzed on a SUN workstation with the programs of the University of Wisconsin Genetics Computer Group (13). Searches in databases were done with the programs BLASTX, BLASTP, and TBLASTN, provided by GenBank (2).

Isolation of total DNA and construction of a DNA library. Total DNA from *Terrabacter* sp. strain DPO360 and *Terrabacter* sp. strain DPO1361 was isolated by modifications of procedures described elsewhere (45). An overnight culture (2 liters of L broth) was harvested and resuspended in 6 ml of TE 5.1 (5 mM Tris-HCl [pH 8.0], 1 mM EDTA). After addition of 20 ml of TE 5.1 containing lysozyme (40 mg) and incubation at 37°C for 15 min, 13 ml of SDS solution (20%) was added, and the suspension was shaken for 10 min. After stabilization of the DNA with 5 ml of a NaCl solution (5 M), about 150 ml of chloroform-isoamylalcohol (24:1) was added. The suspension was gently shaken for 30 min and centrifuged to separate the water phase. After ethanol precipitation, the DNA was resuspended in 45 ml of TE 10.01 (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) and 200 μl of RNase A (10 mg/ml) was added. The solution was fractionated by a CsCl density gradient (1.71 g/ml) for 48 h at 100,000 × *g*. Fractions showing high viscosity were combined, precipitated with ethanol, and resuspended in TE 10.01.

Total DNA of *Terrabacter* sp. strain DPO360 was partially digested with *Sau*3AI and purified in a 0.6% agarose gel, and DNA fragments of 2 to 4 kb in size were electroeluted (Biometra). These fragments were ligated with pUC20 digested with *Bam*HI, and *E. coli* JM101 was transformed (11) with these recombinant plasmids.

DNA hybridization. Southern blotting was performed as described elsewhere (53) with nylon membranes (Biodyne B; Pall, Portsmouth, Great Britain). Labeling, hybridization (67°C), and detection of the 1.2-kb *Sal*I/*Eco*RI fragment carrying *bphC1* were performed with a nonradioactive labeling and detection kit (Boehringer Mannheim Biochemicals).

Nucleotide sequence accession numbers. The nucleotide sequence of *bphC1* reported in this paper has been submitted to GenBank under accession number U57649. The full nucleotide sequence of the 16S rRNA gene was deposited at the EBI databases under accession number Y08853.

RESULTS

Taxonomy of *Terrabacter* sp. strain DPO360. Together with the previously misdescribed "*Brevibacterium*" sp. strain DPO1361 (14, 58), strain DPO360 belongs to a new species of the genus *Terrabacter.*

Full (DPO1361) and partial (DPO360) 16S rRNA sequence analyses were performed for this new group of dibenzofuran-mineralizing bacteria. The sequences were identical. Given that the partial sequence comprises the 5′ half of the molecule, containing the most variable regions, base differences within the 3′ halves are highly unlikely to be assumed. Phylogenetically, the strains are representatives of the gram-positive bacteria with a high DNA G+C content (*Actinomycetes* phylum). *Terrabacter tumescens* is the closest relative among the bacteria analyzed by comparative 16S rRNA sequencing so far. This relationship is reflected by 95.9% overall 16S rRNA sequence similarity. Therefore, strain DPO360 and strain DPO1361 are assigned to the genus *Terrabacter*. The G+C content of *Terrabacter* sp. strain DPO360 was determined by the DSMZ (Braunschweig, Germany) as 71.8% (±0.1 %). The position of the DPO strains among the other important phylogenetic groups of the phylum is shown in the tree of Fig. 1. DNA-DNA hybridization experiments performed by the DSMZ with *Terrabacter* sp. strain DPO360 and *Terrabacter* sp. strain DPO1361 showed 88% sequence similarity, indicating that both strains belong to the same species. DNA-DNA hybridization of *Terrabacter* sp. strain DPO360 and *T. tumescens* DSM20308 gave 31.7% similarity, distinguishing these strains on the species level.

Degradation pathway for dibenzofuran. The proposed pathway for the mineralization of dibenzofuran by *Terrabacter* sp. strain DPO360 (Fig. 2) is identical to the previously described dibenzofuran pathways in *Terrabacter* sp. strain DPO1361 (14, 59), *Sphingomonas* sp. strain RW1 (67), and *Sphingomonas* sp. strain HH69 (16). This could be demonstrated with resting cell experiments using dibenzofuran-grown *Terrabacter* sp. strain DPO360. Resting cells (2 g [dry weight] per liter) were incubated with dibenzofuran (2 mM) and 3-chlorocatechol (2 mM), in order to inhibit extradiol dioxygenase activity (5). In 30 min of incubation, about 1 mM 2,2′,3-trihydroxybiphenyl, the first chemically stable metabolite in dibenzofuran mineralization, accumulated in the medium. Cell extracts of the same dibenzofuran-grown culture showed a turnover of 2,2′,3-trihydroxybiphenyl with a maximum initial activity of 289 nmol per min per mg of protein with simultaneous formation of small amounts of salicylate. In addition, the same cells expressed a maximum salicylate-degrading activity of 74 μM per min per 2 g (dry weight) of cells, as determined by HPLC analysis using whole cells.

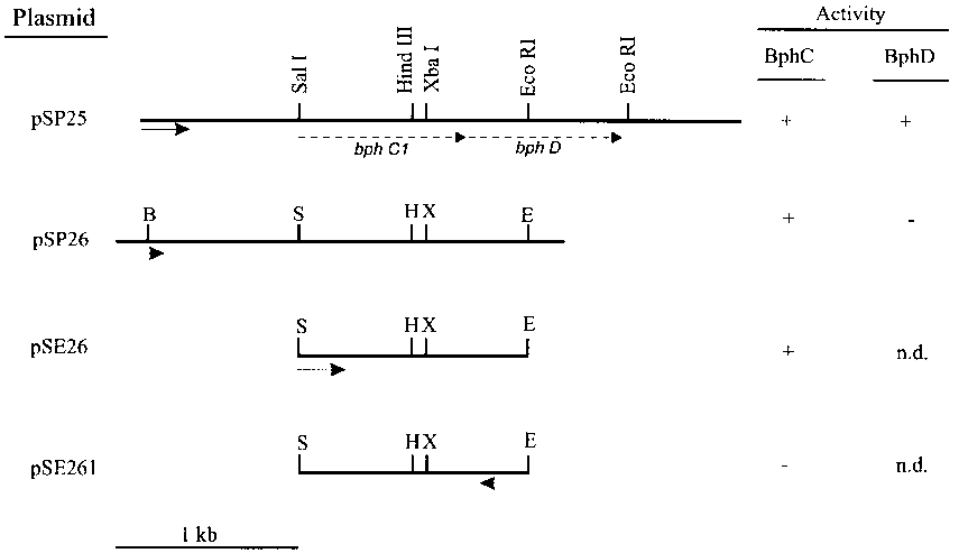


FIG. 3. Restriction map of pSP25 and pSP26 cloned from the genome of *Terrabacter* sp. strain DPO360 (pSE26 and pSE261 are subclones of pSP26). The activity column indicates whether the clone expresses 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC1) or HOPDA hydrolase (BphD) activity. The solid arrows indicate the direction of transcription from the *lac* promoter of the cloning vector (pUC20 for pSP25, pSP26, and pSE261; pUC21 for pSE26). The dashed arrows indicate the direction of transcription as determined by the nucleotide sequence (*bphC1*, full sequence; *bphD*, deduced from the first 306 nucleotides and sequence similarity analysis). n.d., not determined.

Cloning and expression of extradiol dioxygenase genes in *E. coli*. As genetic information of dibenzofuran degradation in gram-positive bacteria was missing, attempts were made to clone essential genes of this pathway.

(i) Upper pathway enzymes. A plasmid library of total DNA of *Terrabacter* sp. strain DPO360 was prepared as described in Materials and Methods. Screening of approximately 6,000 recombinant clones by spraying the colonies with a solution of 2,3-dihydroxybiphenyl (1 mM, in 10% ethanol [22]) yielded 10 colonies turning bright yellow within a few seconds to several minutes. After a transfer of the plasmids with an expression of extradiol dioxygenase activity (6 of 10 after one overnight cultivation) to *E. coli* JM109, the plasmid and expression of extradiol dioxygenase activity appeared to be stable over several subcultivation steps. Restriction mapping of the different cloned DNA fragments showed overlapping areas. This indicated that the different clones carried fragments from the same DNA region. Two representative plasmids (pSP25 and pSP26) are shown in Fig. 3. Subcloning and expression analyses in crude extracts allowed the localization of a 2,3-dihydroxybiphenyl-1,2-dioxygenase gene on a 1.2-kb *Eco*RI/*Sal*I DNA fragment and a neighboring HOPDA hydrolase gene (Fig. 3). Crude extracts of *E. coli* JM109 pSE26 showed relative activities of 100:1 for 2,3-dihydroxybiphenyl and catechol, as determined spectrophotometrically. This characterized the gene as a 2,3-dihydroxybiphenyl-1,2-dioxygenase gene (*bphC1*). Expression of 2,3-dihydroxybiphenyl-1,2-dioxygenase activity could be observed only in pSE26 (derivative of pUC21), not in pSE26-1 (carrying the same fragment in pUC20), indicating the lack of a functional promoter on this DNA fragment and a direction of transcription as shown in Fig. 3. Labeling and hybridization of the 1.2-kb *Sal*I/*Eco*RI fragment with total DNA from *Terrabacter* sp. strain DPO360 confirmed this strain to be the source of the gene. In addition, hybridization of this gene probe with pAW6194 (*Pseudomonas putida* OU83 [34]) and pMlu9 (*Pseudomonas* sp. strain NCIB10643 [52]) showed no signals, indicating a low degree or a lack of similarity of the gene from *Terrabacter* sp. strain DPO360 with either *bphC* gene of the two gram-negative biphenyl degraders *Pseudomonas* sp. strain OU83 and *Pseudomonas* sp. strain NCIB10643.

(ii) Lower pathway enzymes. In order to clone a catechol-2,3-dioxygenase gene involved in salicylate degradation in *Terrabacter* sp. strain DPO360, about 6,000 new recombinants from the library were screened by spraying with a 1 mM cat-

TABLE 1. Purification of extradiol ring cleavage dioxygenases from *Terrabacter* sp. strain DPO3600grown on dibenzofuran

Step	BphC1 (*Terrabacter* sp. strain DPO360)[a]			BphC1 (*E. coli* JM109 pSE26)[a]			BphC2 (*Terrabacter* sp. strain DPO360)[a]			C23O (*Terrabacter* sp. strain DPO360)[a]		
	Protein (mg)	TA[b]	SA[c]	Protein (mg)	TA[b]	SA[c]	Protein (mg)	TA[b]	SA[c]	Protein (mg)	TA[b]	SA[c]
Cell extract	130	337[d]	2.6[d]	45.7	41.1	0.89	130	337[d]	2.6[d]	193	20.21	0.1
Anion exchange	11	251.24	22.84	1.9	19.2	10.32	16.29	30.24	1.87	31.3	27.45	0.88
Hydrophobic interaction	3.25	171.36	52.76	0.28	7.35	26.89	0.77	6.96	9.04	5.6	14.47	2.58
Gel filtration	0.81	82.94	102.1	0.02	2.5	138.89	0.31	3.23	10.4	0.58	4.61	7.95

[a] Organism from which the enzyme was purified.
[b] TA, total activity (micromoles of substrate oxidized per minute). Substrates, 2,3-dihydroxybiphenyl for BphC1 and BphC2 and catechol for C23O.
[c] SA, specific activity (micromoles of substrate oxidized per minute per milligram of protein).
[d] Activity includes that for 2,3-dihydroxybiphenyl of both BphC1 and BphC2.

TABLE 2. Molecular weights of extradiol dioxygenases from *Terrabacter* sp. strain DPO360

Enzyme[a]	Mol wt (native enzyme)[b]	Mol wt (subunits)[c]	Subunit composition
2,3-Dihydroxybiphenyl dioxygenase (BphC1)	80,000	39,000	Homodimer
2,3-Dihydroxybiphenyl dioxygenase (BphC2)	76,000	38,000	Homodimer
Catechol 2,3-dioxygenase (C23O)	140,000	36,500	Homotetramer

[a] BphC1 was purified from *Terrabacter* sp. DPO360, as well as from *E. coli* JM109 pSE26; BphC2 and C23O were purified from *Terrabacter* sp. strain DPO360.
[b] The native molecular weights were determined by gel filtration.
[c] Molecular weights of the subunits were determined by denaturing PAGE.

echol solution. Only one colony turned yellow, indicating catechol-2,3-dioxygenase activity. Restriction analysis of the corresponding 6.1-kb DNA fragment on pSC1 showed a different pattern from that observed for the *bphC1* gene, and no restriction sites for *Xba*I, *Hin*dIII, and *Eco*RI were found on this DNA fragment (data not shown). *E. coli* JM109 pSC1 expressed an extradiol dioxygenase activity with relative activities for 2,3-dihydroxybiphenyl and catechol of 100:40, as determined photometrically in crude extracts. This characterized the enzyme also as a 2,3-dihydroxybiphenyl dioxygenase. No hybridization with the labeled 1.2-kb *Sal*I/*Eco*RI DNA fragment carrying *bphC1* could be observed. Construction of other DNA libraries containing total DNA from *Terrabacter* sp. strain DPO360 using *Mbo*I, *Apa*I, *Mlu*I, *Kpn*I, *Bam*HI, *Pst*I, and *Sst*I; the use of the positive selection vector pJOE 930 (1); and screening of recombinants by spraying with catechol yielded only DNA fragments carrying the *bphC* genes described above. A similar limitation of the strategy of expression screening for extradiol dioxygenase genes was observed by Kim and Zylstra (36).

Purification of ring cleavage dioxygenases. In order to understand which one of the cloned *bphC* genes from *Terrabacter* sp. strain DPO360 is active in the mineralization of dibenzofuran, the extradiol dioxygenases expressed during growth with dibenzofuran were purified. Initial experiments with aerobic purification procedures revealed a high instability of enriched extradiol dioxygenase fractions corresponding to observations of Kojima et al. (39). This suggested the presence of an oxidizable ferrous iron in the active site of the enzymes, as found in most characterized extradiol dioxygenases (6, 20, 23, 49, 50, 55, 68). Therefore, the purification and the handling of enzyme fractions were performed under strictly anaerobic conditions as described above, yielding enzyme fractions stable at 4°C for about 1 week without significant loss of activity (<5%).

Interestingly, three different extradiol dioxygenases were detected in crude extracts of *Terrabacter* sp. strain DPO360. A typical data set of total and specific activities of each enzyme purification is summarized in Table 1. BphC1 was purified from *Terrabacter* sp. strain DPO360 and from *E. coli* JM109 pSE26 with specific activities of 102 and 139 U/mg, respectively. BphC2 was purified from *Terrabacter* sp. strain DPO360 with a specific activity of 10.4 U/mg and an apparent purification factor of 4.

An estimation of the respective proportionate amounts of 2,3-dihydroxybiphenyl-1,2-dioxygenase activity of BphC1, BphC2, and C23O in comparison to the total activity in crude extracts of dibenzofuran-grown *Terrabacter* sp. strain DPO360 was calculated by using the added activities in enzyme fractions after the first purification step (anion-exchange chromatography) containing BphC1, BphC2, or C23O, respectively. Assuming a similar stability of all three enzymes, it could be calculated that about 95% of the total activity for 2,3-dihydroxybiphenyl in crude extracts can be assigned to BphC1, about 4% of the activity can be assigned to BphC2, and 1% at maximum can be assigned to C23O.

C23O was purified to a specific activity of 7.95 U/mg with a purification factor of 79.5. Table 2 gives the estimated sizes of the native enzymes as determined by gel filtration and the subunit structures as determined by denaturing PAGE (Fig. 4). According to these data, BphC1 was purified as a homodimer from *Terrabacter* sp. strain DPO360 and from *E. coli* JM109 pSE26. BphC2 was also purified as a homodimer, and C23O was purified as a homotetrameric enzyme from *Terrabacter* sp. strain DPO360. No formation of higher multimeric aggregates, as claimed for 2,2′,3-trihydroxybiphenyl dioxygenase from *Sphingomonas* sp. strain RW1 (24), could be demonstrated for the extradiol dioxygenases from *Terrabacter* sp. strain DPO360.

The NH_2-terminal amino acid sequence of BphC1 purified from *Terrabacter* sp. strain DPO360 was SKV KEL AYV GYE VSD LAA WEH FGV D. Apart from the missing, probably processed, first amino acid, this sequence was identical to the amino terminus of the cloned and sequenced BphC1 gene (Fig. 5). The NH_2-terminal amino acid sequence of purified BphC2 could not be determined, as the amino terminus was blocked.

The extradiol dioxygenase encoded on the 6.1-kb fragment on pSC1 showed relative activities for 2,3-dihydroxybiphenyl as 100% and for catechol as 40%, determined photometrically in crude extracts of *E. coli* JM109 pSC1. This relation was also found after anion-exchange chromatography of the same crude extract and after a subsequent gel filtration step characterizing

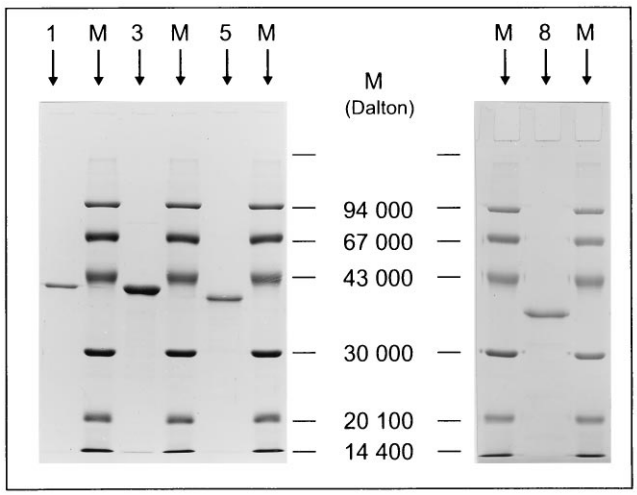
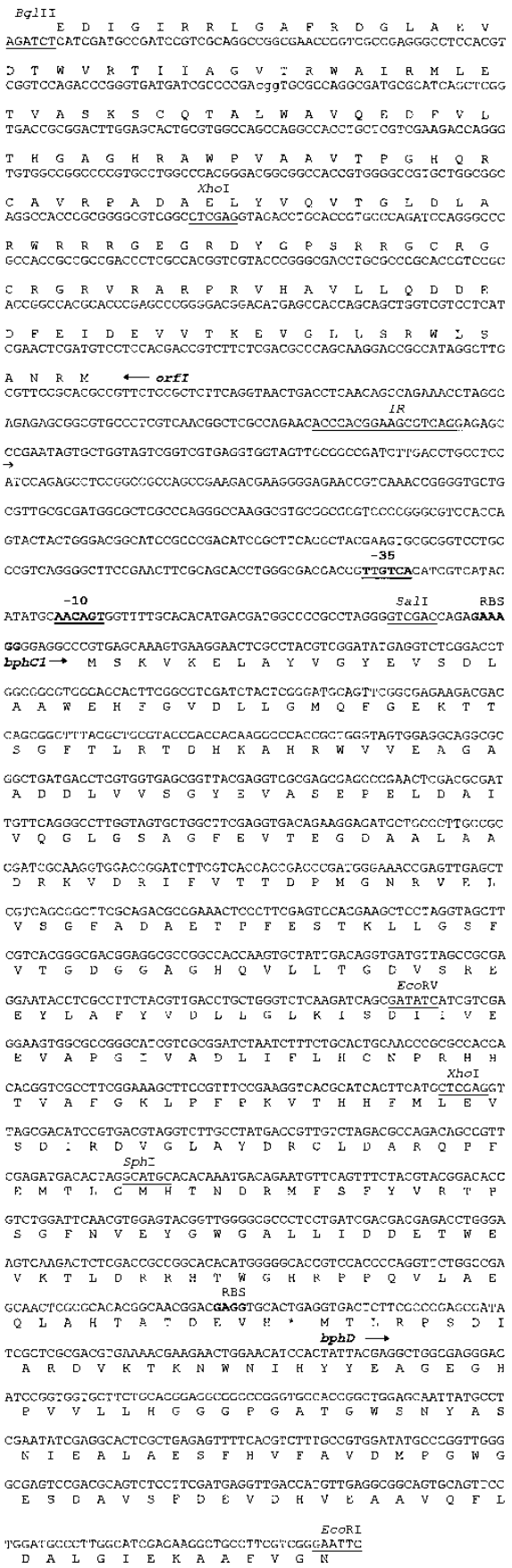


FIG. 4. SDS-PAGE analysis of purified extradiol dioxygenases from *Terrabacter* sp. strain DPO360. 1, BphC1 purified from *E. coli* JM109 pSE26; 3, BphC1 purified from *Terrabacter* sp. strain DPO360; 5, BphC2 purified from *Terrabacter* sp. strain DPO360; 8, C23O purified from *Terrabacter* sp. strain DPO360; M, molecular mass marker.



this enzyme as a third 2,3-dihydroxybiphenyl-1,2-dioxygenase enzyme (BphC3) of *Terrabacter* sp. strain DPO360, different from BphC1 and BphC2. After gel filtration, the molecular weight of native BphC3 was estimated to be in the same range as the molecular weights of BphC1 and BphC2 (data not shown).

Kinetic characterization. BphC1 and BphC2 were shown to be responsible for the ring cleavage of 2,2′,3-trihydroxybiphenyl in *Terrabacter* sp. strain DPO360 growing on dibenzofuran. The kinetic constants determined for the purified extradiol dioxygenases are summarized in Table 3. BphC1, BphC2, and C23O all showed a high affinity for 2,3-dihydroxybiphenyl and 2,2′,3-trihydroxybiphenyl with K_m values in the lower micromolar range. The K_m value for catechol was 0.6 μM for C23O, whereas BphC1 and BphC2 showed K_m values for catechol of 8.9 and 0.9 mM, respectively. All three enzymes being compared, BphC1 shows the highest k_{cat} value for 2,2′,3-trihydroxybiphenyl with 2,640.3 1/min. Interestingly, this situation is not reflected by the specificity constants k_{cat}/K_m. In this respect, BphC2 has the highest value with 183 1/min × 1/μM compared to BphC1 (29 1/min × 1/μM) and C23O (1 1/min × 1/μM). Looking at relative k_{cat} values of the different enzymes, BphC2 is the only enzyme showing the highest value for 2,2′,3-trihydroxybiphenyl (1,242 1/min, 100%) with respect to 2,3-dihydroxybiphenyl (83%) and catechol (6.5%). As expected, C23O shows the highest k_{cat} and k_{cat}/K_m values of all three enzymes for catechol with 771.2 1/min and 1,285 1/min × 1/μM, respectively.

Nucleotide sequence for *bphC1*. BphC1, expressed in *E. coli* JM109 pSE26, has the same relative activities for 2,3-dihydroxybiphenyl and catechol as the enzyme purified from *Terrabacter* sp. strain DPO360. BphC3, encoded on pSC1, has a significantly higher relative activity for catechol compared to 2,3-dihydroxybiphenyl and was not involved in dibenzofuran metabolism (see above). Therefore, the nucleotide sequence of *bphC1*, encoded on pSE26, and its flanking DNA regions was determined for a more detailed characterization (Fig. 5). The deduced NH_2-terminal amino acid sequence of the extradiol dioxygenase encoded on pSE26 is identical to the amino acid sequence determined for BphC1 purified from *Terrabacter* sp. strain DPO360. As BphC1 purified from *Terrabacter* sp. strain DPO360 and the extradiol dioxygenase purified from *E. coli* JM109 pSE26 also show identical mobility in SDS-polyacrylamide gels (Fig. 4), and after gel filtration of the native enzymes, we assume that *bphC1* on pSE26 actually is the gene for BphC1. Located downstream of *bphC1*, there is the beginning of an open reading frame (*bphD*) showing high similarity to HOPDA hydrolase genes of biphenyl-degrading bacteria as determined with the blastX software (2). Indeed, HOPDA-hydrolase activity could be detected in crude cell extracts of *E. coli* JM109 pSP25 (Fig. 3). Putative ribosome binding sites are marked in Fig. 5 for *bphC1* and *bphD*. Also, putative promoter sequences (−10 and −35 regions) are marked for *bphC1*. Upstream of *bphC1* there is an open reading frame (*orf1*) with 26.4% identity to ISAE1, an insertion element of *Alcaligenes eutrophus* H1 (41), including a 33-nucleotide inverted repeat (75% identity to ISAE1 repeat 2). At present, it is not clear

FIG. 5. Nucleotide sequence of a *Bgl*II/*Eco*RI fragment containing *bphC1*, coding for 2,3-dihydroxybiphenyl-1,2-dioxygenase C1; the amino-terminal part of *bphD*, coding for HOPDA-hydrolase; and *orf1*, showing significant similarity to ISAE1, an insertion element from *A. eutrophus* H1. IR, DNA region showing 75% sequence identity to repeat 2 from ISAE1. An asterisk indicates a stop codon. Putative ribosome binding sites (RBS) and a putative promoter region (−10 and −35) are marked.

TABLE 3. Kinetic constants of extradiol dioxygenases from *Terrabacter* sp. strain DPO360 growing on dibenzofuran[a]

Enzyme and substrate	$K_{m,\,app}$ (μM)	V_{max} (U/mg)	k_{cat} (1/min)	k_{cat}/K_m (1/min × μM^{-1})
BphC1				
PC	5.4 (1.6)	108.4 (8)	4,227.6	783
HPC	91.2 (8.8)	67.7 (2.32)	2,640.3	29
BC	8,932 (1,370)	13.04 (1.5)	508.6	0.06
BphC2				
PC	1 (0.2)	27.16 (1.1)	1,032.1	1,032
HPC	6.8 (1.8)	32.7 (1)	1,242.6	183
BC	896 (164)	2.11 (0.2)	80.2	0.09
C23O				
PC	2.3 (0.2)	3.6 (0.08)	131.4	57
HPC	40 (10)	1.16 (0.15)	42.34	1.1
BC	0.6 (0.06)	21.1 (0.45)	771.2	1,285

[a] BphC1, 2,3-dihydroxybiphenyl-1,2-dioxygenase C1, subunit molecular weight, 39,000; BphC2, 2,3-dihydroxybiphenyl-1,2-dioxygenase C2, subunit molecular weight, 38,000. C23O, catechol-2,3-dioxygenase, subunit molecular weight, 36,500. Enzyme activities for 3-phenylcatechol (PC) and catechol (BC) were determined photometrically; those for 2,2′,3-trihydroxybiphenyl (HPC) were determined polarographically. In general, polarographic determinations of activities gave 67% of the values determined photometrically due to instabilities of enzyme activity in reaction mixtures. Numbers in parentheses are standard errors of the means.

whether *orf1* is part of an active IS element in *Terrabacter* sp. strain DPO360.

DISCUSSION

The mineralization of aromatic compounds is described for a variety of gram-positive genera (e.g., *Rhodococcus*, *Arthrobacter*, *Bacillus*, and *Nocardia*). Previously, Strubel et al. (59) described the mineralization of dibenzofuran by strain DPO1361, which was preliminarily characterized as a *Brevibacterium* species (14). Here, on the basis of 16S rRNA sequence data, we demonstrate that strain DPO1361 and the closely related strain DPO360 belong to the genus *Terrabacter*. These two strains are representatives of two different subgroups of a new species, altogether comprising 13 bacterial isolates (data not shown), as was shown by restriction fragment length polymorphism analysis of rRNA genes and DNA-DNA hybridization. *T. tumescens* and the DPO strains, together with *Kytococcus nishinomiyaensis*, *Kytococcus sedentarius*, and (less significantly) *Dermatophilus congolensis*, represent one of the major phylogenetic clusters of the gram-positive bacteria with a high DNA G+C content. A detailed characterization of this new cluster of strains will be published separately. Although all of these strains are very similar, their distinctiveness indicates that the use of an unusual substrate (here dibenzofuran) in combination with a special growth medium (here a high concentrated phosphate buffer) may select for organisms constituting a unique physiological and taxonomical group.

Generally, in aerobic mineralization of aromatic compounds, the fast and efficient cleavage of toxic catecholic intermediates is a biochemical prerequisite. According to this fact, intradiol and extradiol dioxygenases, cleaving catechol or derivatives thereof, have been a subject of intensive research (25). Four different extradiol dioxygenases were detected in *Terrabacter* sp. strain DPO360, with three of them being involved in dibenzofuran degradation. Based on kinetic constants, BphC1 and BphC2 are responsible for the cleavage of 2,2′,3-trihydroxybiphenyl, the first intermediate in the dibenzofuran pathway. BphC2 is supposed to be active at very low, and BphC1 at rather high, intracellular concentrations of 2,2′,3-trihydroxybiphenyl. Despite its high affinity for 2,2′,3-trihydroxybiphenyl, C23O is supposed to be involved only in the cleavage of catechol, derived from salicylate, which in turn is a degradation intermediate of the dibenzofuran pathway in *Terrabacter* sp. strain DPO360. At present, the function of the silent *bphC3* gene in *Terrabacter* sp. strain DPO360 remains unclear. For the closely related *Terrabacter* sp. strain DPO1361, a 2,3-dihydroxybiphenyl dioxygenase showing a similar substrate spectrum as BphC3 from *Terrabacter* sp. strain DPO360 was detected in crude extracts of biphenyl-grown cells after anion-exchange chromatography (57). By FPLC analysis methods, this enzyme activity could be detected neither in *Terrabacter* sp. strain DPO1361 grown on dibenzofuran nor in *Terrabacter* sp. strain DPO360 grown on dibenzofuran or biphenyl.

In *Terrabacter* sp. strain DPO1361, grown on dibenzofuran, two different extradiol dioxygenase activities could be detected in crude extracts after one anion-exchange chromatography step (59). Interestingly, cleavage of 2,2′,3-trihydroxybiphenyl could be detected only in the pooled fractions showing relative activities for 3-phenylcatechol and catechol of 1:1. This activity was rather tentatively assigned to a catechol 2,3-dioxygenase according to the overall substrate spectrum. Generally, the extradiol cleavage of monocyclic and uncharged catechols is catalyzed by catechol 2,3-dioxygenases, and the cleavage of bicyclic catechols is assigned to different classes of extradiol dioxygenases like BphC- and NahC-like enzymes (25, 26). After anion-exchange chromatography, cell extracts of *Terrabacter* sp. strain DPO1361 grown on dibenzofuran (57) showed the same elution profile of extradiol dioxygenase activities as *Terrabacter* sp. strain DPO360 grown on dibenzofuran. In *Terrabacter* sp. strain DPO360, C23O, which has a very low activity for 2,2′,3-trihydroxybiphenyl and 3-phenylcatechol, coelutes with BphC2 showing a high activity for 2,2′,3-trihydroxybiphenyl and 3-phenyl-catechol in relation to the activity for catechol. If this would hold also for the 2,2′,3-trihydroxybiphenyl-cleaving activity pool of *Terrabacter* sp. strain DPO1361, an explanation for the unusual specificity of the catechol-2,3-dioxygenase activity would be possible. Further investigations will clarify this point. *meta* cleavage of 2,2′,3-trihydroxybiphenyl was also described for the degradation pathway of 2,2′-dihydroxybiphenyl in *Pseudomonas* sp. strain HBP1 (38). After anion-exchange chromatography of crude extracts, this activity was assigned to a metapyrocatechase showing a broad substrate spectrum similar to 2,3-dihydroxybiphenyl-1,2-dioxygenase enzymes like BphC1 or BphC2 of *Terrabacter* sp. strain DPO360. According to amino acid sequence similarities (Fig. 6), BphC1 shows a similar distance to NahC-like enzymes and to BphC-like enzymes from different *Pseudomonas* and *Rhodococcus* species. Highest sequence similarities are observed with PcbC, a 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Pseudomonas* sp. strain DJ-12 (36a). The cleavage of 2,2′,3-trihydroxybiphenyl has further been suggested for BphC from *Pseudomonas paucimobilis* Q1 (19), an enzyme later characterized as a 1,2-dihydroxynaphthalene dioxygenase (40). Other enzymes shown to cleave 2,2′,3-trihydroxybiphenyl (4) have no or only little amino acid sequence similarity to BphC1 from *Terrabacter* sp. strain DPO360, indicating that the cleavage of 2,2′,3-trihydroxybiphenyl is not a unique feature of a closely related group of extradiol dioxygenases but rather a common activity of BphC-type enzymes cleaving the structurally very similar 2,3-dihydroxybiphenyl.

Cleavage of 2,2′,3-trihydroxybiphenyl was also reported for DbfB from *Sphingomonas* sp. strain RW1. This enzyme was

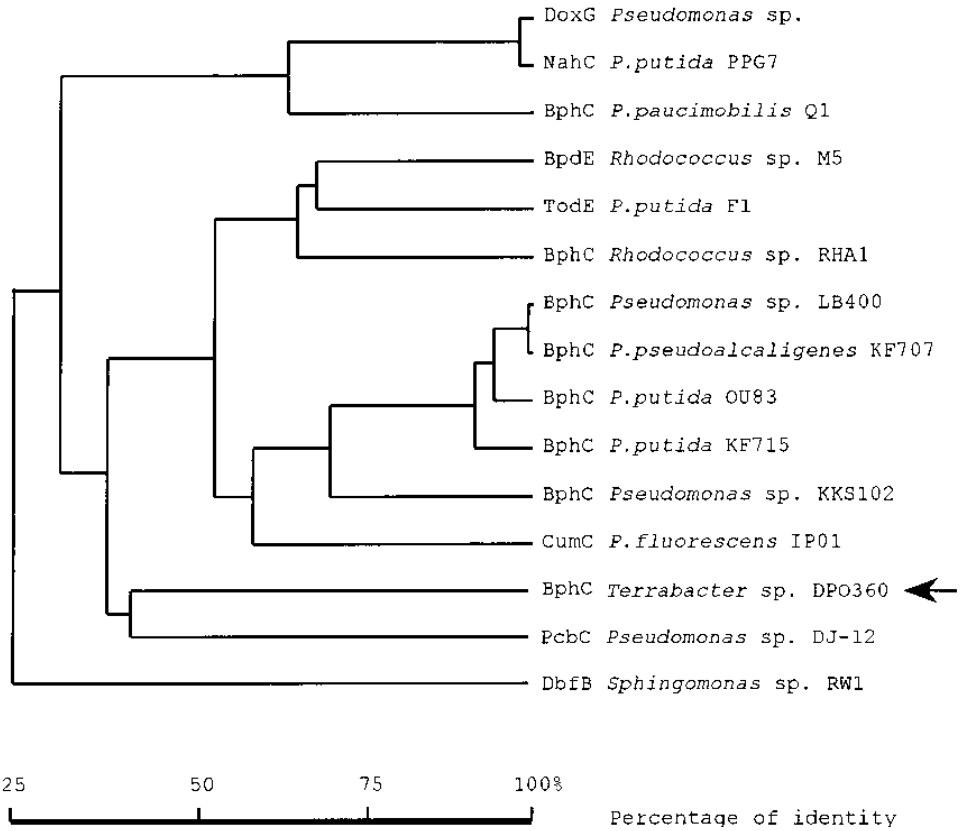


FIG. 6. Dendrogram showing the relationship between the amino acid sequences of different extradiol dioxygenases. The dendrogram was generated with the programs Lineup and Pileup implemented in the Genetics Computer Group DNA analysis software package (13). The sequences are as follows: DoxG, *Pseudomonas* sp. 1,2-dihydroxynaphthalene dioxygenase (12); NahC, *P. putida* PpG7 1,2-dihydroxynaphthalene dioxygenase (26); BphC, *P. paucimobilis* Q1 2,3-dihydroxybiphenyl-1,2-dioxygenase (62); BpdE, *Rhodococcus* sp. strain M5 (64); TodE, *P. putida* F1 3-methylcatechol-2,3-dioxygenase (70); BphC, *Rhodococcus* sp. strain RHA1 2,3-dihydroxybiphenyl-1,2-dioxygenase (46); BphC, *Pseudomonas* sp. strain LB400 2,3-dihydroxybiphenyl-1,2-dioxygenase (30); BphC, *Pseudomonas pseudoalcaligenes* KF707 2,3-dihydroxybiphenyl-1,2-dioxygenase (61); BphC, *P. putida* OU83 2,3-dihydroxybiphenyl-1,2-dioxygenase (33); BphC, *P. putida* KF715 2,3-dihydroxybiphenyl-1,2-dioxygenase (27); BphC, *Pseudomonas* sp. strain KKS102 2,3-dihydroxybiphenyl-1,2-dioxygenase (37); CumC, *Pseudomonas fluorescens* IP01 (3); PcbC, *Pseudomonas* sp. strain DJ-12 2,3-dihydroxybiphenyl-1,2-dioxygenase (36a); DbfB, *Sphingomonas* sp. strain RW1 2,2′3,-trihydroxybiphenyl dioxygenase (24). The 2,3-dihydroxybiphenyl-1,2-dioxygenase (*Terrabacter* sp. strain DPO360, BphC1) described in this work is marked by an arrow.

cloned from *Sphingomonas* sp. strain RW1 and purified and characterized from a corresponding *E. coli* clone (24). It was suggested to be active in the mineralization of dibenzofuran in *Sphingomonas* sp. strain RW1. However, final evidence is missing, since, as in *Terrabacter* sp. strain DPO360, two different extradiol dioxygenases showing activity for 3-phenylcatechol and 2,2′,3-trihydroxybiphenyl and one C23O were detected in *Sphingomonas* sp. strain RW1 growing on dibenzofuran or salicylate (8). In dibenzofuran-grown *Sphingomonas* sp. strain RW1, also two different reductases for the dibenzofuran dioxygenase system (9), as well as two different HOPDA hydrolases (8), were detected. A possible explanation for this fact could be the common expression of two well-separated pathways for dibenzofuran and biphenyl. Growth on biphenyl was reported for *Sphingomonas* sp. strain RW1 (67) and for *Terrabacter* sp. strain DPO360 (57). The existence of multiple genes of extradiol dioxygenase isoenzymes in bacterial genomes has also been described for other gram-positive bacteria (4, 43, 54), as well as for other gram-negative bacteria (28, 31, 32).

In *Terrabacter* sp. strain DPO360, a functional HOPDA hydrolase gene (*bphD*) is located directly downstream of *bphC*. Interestingly, no 2,3-dihydroxy-2,3-dihydrobiphenyl dehydrogenase gene (*bphB*) could be detected upstream of *bphC1*, indicating that *bphC1* is not part of a complete biphenyl-type operon, as found in other bacteria (21, 29, 35). This was also observed in *Beijerinckia* sp. strain B1, in which this gene was supposed to be located over 6 kb downstream of *bphC* (36). As a 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase is not necessary in the degradation of dibenzofuran and is also not induced in dibenzofuran-grown *Terrabacter* sp. strain DPO360 (57), it will be interesting to look for a gene encoding dibenzofuran 4,4a-dioxygenase in the neighborhood of *bphC1*. In *Terrabacter* sp. strain DPO360, a DNA region (*orf1*) showing significant similarity to ISAE1, an insertion element of *Alcaligenes eutrophus* H1, is located 370 bp upstream of *bphC1*. To date, the function of *orf1* is unknown, and the question whether it is part of an active IS element and plays a role in the metabolism of dibenzofuran or biphenyl in *Terrabacter* sp. strain DPO360 will be the subject of further research.

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