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Metabolism of Dibenzo-*p*-Dioxin by *Sphingomonas* sp. Strain RW1

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In the course of our screening for dibenzo-*p*-dioxin-utilizing bacteria, a *Sphingomonas* sp. strain was isolated from enrichment cultures inoculated with water samples from the river Elbe. The isolate grew with both the biaryl ethers dibenzo-*p*-dioxin and dibenzofuran (DF) as the sole sources of carbon and energy, showing doubling times of about 8 and 5 h, respectively. Biodegradation of the two aromatic compounds initially proceeded after an oxygenolytic attack at the angular position adjacent to the ether bridge, producing 2,2',3-trihydroxydiphenyl ether or 2,2',3-trihydroxybiphenyl from the initially formed dihydrodiols, which represent extremely unstable hemiacetals. Results obtained from determinations of enzyme activities and oxygen consumption suggest *meta* cleavage of the trihydroxy compounds. During dibenzofuran degradation, hydrolysis of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate yielded salicylate, which was branched into the catechol *meta* cleavage pathway and the gentisate pathway. Catechol obtained from the product of *meta* ring fission of 2,2',3-trihydroxydiphenyl ether was both *ortho* and *meta* cleaved by *Sphingomonas* sp. strain RW1 when this organism was grown with dibenzo-*p*-dioxin.

Polychlorinated dibenzo-*p*-dioxins (DDs) and dibenzofurans (DFs) are unintentionally formed as contaminating by-products during the manufacture of pesticides, incineration of halogen-containing chemicals and of industrial and domestic waste, and bleaching of paper pulp (3, 5, 31) and thus have become widespread contaminants of the environment.

Dibenzo-*p*-dioxin, its monochloroderivatives, and dibenzofuran were shown to be cooxidized to *ortho*-dihydroxylated compounds by bacterial strains utilizing naphthalene or biphenyl for growth (4, 18, 19). Cleavage of the aromatic nuclei of the target compounds, however, did not occur. During our investigations of the microbial degradation of diaryl ethers, a *Pseudomonas* sp., strain HH69, which utilized DF as its sole source of carbon and energy and showed cometabolic activity toward DD and 3-chlorodibenzofuran (8, 12, 13), was isolated. The degradation of DF was initiated by an angular attack of a dioxygenase enzyme system followed by cleavage of the resulting unstable phenolic hemiacetal to yield 2,2',3-trihydroxybiphenyl, the latter product being degraded in a manner similar to that of the classical biphenyl pathway. Recently, cooxidation of some low-chlorinated DDs and DFs by a biphenyl-utilizing *Alcaligenes* sp. strain was reported (24, 25). However, complete biodegradation of DD has not yet been shown. Here, we describe for the first time the properties of a bacterium that mineralizes DD.

MATERIALS AND METHODS

Isolation, identification, and growth of bacteria. For enrichment and growth, a mineral salts medium which has been previously described was used (8). The enrichment cultures were inoculated with water samples, supplemented with DD (0.5 g/liter) as the substrate and actidione (0.1 g/liter) to suppress growth of protozoa, and incubated aerobically at 28°C on a rotary shaker at 150 rpm. Aliquots were transferred weekly from the culture to fresh medium. Subcultures were streaked onto nutrient medium, and after incubation at 28°C, single colonies were transferred onto mineral salts agar plates containing the above carbon source. Well-grown colonies (plates without DD were used as controls) were retransferred into selective liquid medium, and after growth, stock cultures were stored at −70°C until used for experiments.

The identification of the strain was carried out by standard laboratory procedures and those described in *Bergey's Manual* (23); the guanine-plus-cytosine content of bacterial DNA was determined as described previously by Frank-Kamenetskii (10). The isolate is deposited at the German Collection of Microorganisms and Cell Cultures (no. 6014; Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSM], Braunschweig, Germany).

Growth experiments with and without (control) DD or DF were performed with 1-liter screw-cap, polytetrafluoroethylene-sealed Erlenmeyer flasks containing the mineral salts medium (10% the nominal volume) and the appropriate amount of substrate crystals as described above. At time points given in Fig. 1 and 2 (experimental section), flasks were sacrificed from two parallel batches. For the estimation of the cell number (CFU), 10-μl aliquots were plated on solid acetate medium (10 mM) after appropriate dilution and counted after about 1 week. The determinations of further growth parameters such as turbidity and protein content of the bacterial culture were performed as described previously (8). For the estimation of the total amount of the carbon source, including dissolved material, undissolved crystals, and the residue adsorbed on the cell surface and glass walls, the entire contents of the Erlenmeyer flasks were extracted with an equal volume of chloroform. After drying with anhydrous sodium sulfate, quantification was carried out by high-performance liquid chromatography (HPLC). The recovery rate was always quantitative, as had been determined in preceding experiments.

Determination of oxygen uptake rates and enzyme activities.

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Oxygen uptake rates of resting cell suspensions with DD, structurally related compounds and their (potential) metabolites, and the activities of enzymes present in cell-free preparations were determined as previously described (8). The *meta*-cleaving activities of crude cell extracts for the oxidation of 2,3-dihydroxybiphenyl and 2,2',3-trihydroxybiphenyl and the hydrolysis of their reaction products were estimated by the procedures reported by Furukawa et al. (11), Ishigooka et al. (15), and Omori et al. (22). For calculation of the formation of the assumed metabolite 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate from 2,2',3-trihydroxybiphenyl (8), the molar absorption coefficient (11.100 cm^2 mol^{-1} at 432 nm) of the *meta* cleavage product of 2,3-dihydroxybiphenyl (15) had to be used because no reference sample was available. The conversion of 2,2',3-trihydroxydiphenyl ether by cell extracts was monitored by following substrate consumption with HPLC analysis of the assay medium described above for the biphenyl 2,3-dioxygenase activity, since the formation of a yellow product suitable for optical enzyme assays was not detectable. Estimations of salicylate hydroxylase, catechol oxygenases, and gentisate dioxygenase were performed as previously described (8). Assays were performed with an Uvikon 930 spectrophotometer (Kontron Instruments, Eching, Germany) at 25°C.

Isolation of metabolites. Metabolites which were excreted into the medium by growing or cometabolizing cultures were extracted and purified as previously described (8). For the preparation of 2,2',3-trihydroxydiphenyl ether or the respective trihydroxybiphenyl, DF-grown resting cells were incubated in the presence of a 0.1 mM concentration of the potent *meta* cleavage inhibitor 3-chlorocatechol (2) and a large excess (about 5 g/liter) of mortar-ground, crystalline DD or DF to ensure the sufficient dissolution kinetics needed for good bioavailability of the substrates. Product formation was followed by HPLC.

Analytical methods. Monitoring of culture supernatants by HPLC, gas chromatographic analyses of extracted metabolites, and identification of purified compounds by mass spectrometry and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy were carried out as previously described (8).

Chemicals. 2,3-Dihydroxybiphenyl was from Wako Chemicals, Neuss, Germany. DD, 2-hydroxydibenzo-*p*-dioxin, 2-acetoxydibenzo-*p*-dioxin, 2-acetoxydibenzofuran, 3-chlorocatechol, 4-chlorocatechol, 2,2',3-trihydroxybiphenyl, and 2-(2-hydroxyphenoxy)-muconate were prepared as described previously (8, 13, 27). All other chemicals were of the highest purity commercially available.

RESULTS

Enrichment, isolation, and characterization of the DD-utilizing organism. With water samples from the Elbe River collected downstream from the city of Hamburg as the inoculum, enrichment cultures were started by mixing 100 ml of the samples with 300 ml of a mineral salts medium supplemented with 0.1 g of DD crystals as the substrate. After about 5 weeks, utilization of the substrate became visible by the increasing turbidity of the enrichment culture. Strain RW1 was isolated from subcultures and initially identified as a *Pseudomonas* species on the basis of the classification schemes of *Bergey's Manual*. The strain was gram negative and catalase and oxidase positive and grew under strictly aerobic conditions. The guanine-plus-cytosine content of bacterial DNA was estimated to be 67 mol%. The

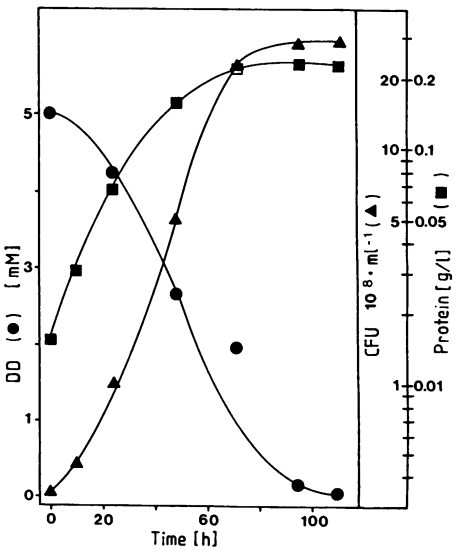

FIG. 1. Growth of *Sphingomonas* sp. strain RW1 with DD. A mineral salts medium was supplemented with DD crystals as the sole source of carbon and energy at a concentration of 5 mM. Two parallel sets of Erlenmeyer flasks were inoculated with an exponentially growing preculture at late log phase which was freed from substrate crystals by filtration. After an appropriate time, parallel batches were worked up for determination of substrate consumption and formation of biomass, as described in Materials and Methods.

short rods (about 1 by 3 μm) were immotile, as judged by microscopic investigations; however, less than 1% of cells were found to be motile when the organism was grown in nutrient broth. Further investigations (which were performed by DSM) allowed assignment to the *Pseudomonas paucimobilis* group upon determination of the ubiquinone and fatty acid patterns. Recently, this group was transferred to the new genus *Sphingomonas* by Yabuuchi et al. (35). Further work by DSM on definite assignment to a more distinct species is in progress.

Growth of bacteria with aromatic compounds. The following compounds were utilized by *Sphingomonas* sp. RW1 for growth: DD, DF, 2-hydroxydibenzofuran, 2-acetoxydibenzofuran, 2-hydroxybiphenyl, 2,3-dihydroxybiphenyl, 2,2'-dihydroxybiphenyl, 2,2',3-trihydroxybiphenyl, 2,2',3-trihydroxydiphenyl ether, benzoate, salicylate, gentisate, 5-methylsalicylate, phenylacetate, phenylmalonate, DL-mandelate, phenylglyoxylate, 2-hydroxyphenylglyoxylate, and catechol. Benzene, naphthalene, biphenyl, 4-hydroxybiphenyl, diphenyl ether, fluorene, fluoren-9-one, xanthene, xanthen-9-one, isomeric hydroxy- and methylbenzoates or methylsalicylates other than the above, 2-hydroxyphenylacetate, phenol, the isomeric methylphenols, phenoxyacetate, and 2-(2-hydroxyphenoxy)-muconate, the *ortho* cleavage product of 2,2',3-trihydroxydiphenyl ether (13), were not used for growth. However, spontaneous mutants able to utilize biphenyl were isolated after 4 weeks of incubation in the presence of this compound at a rate of about 10^{-9}.

Generation times during growth with DD (Fig. 1) were 8 h at 28°C during the early exponential phase, and those with DF were 5 h when the substrate was at a concentration of 5 mM. Significantly shorter generation times were obtained for both substrates when these carbon sources were offered in excess.

Figure 2 shows the simultaneous degradation of DD and

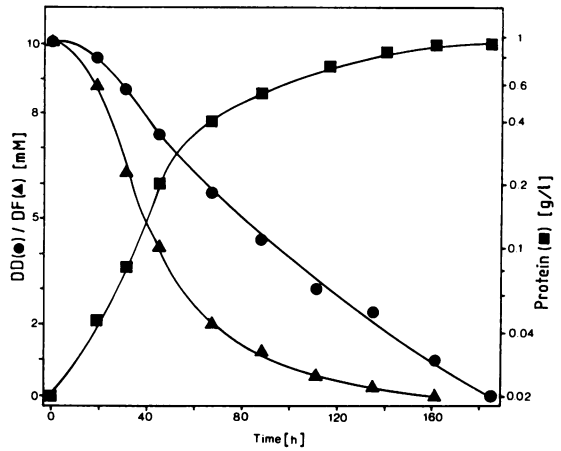

FIG. 2. Simultaneous degradation of DD and DF by *Sphingomonas* sp. strain RW1. Equal amounts of DD and DF, each at a concentration of 10 mM, were added to a mineral salts medium. Inoculation of the medium with an acclimated, late-exponential-phase culture pregrown with this substrate mixture and work-up of the parallel batches were carried out as described in the legend to Fig. 1.

DF by an acclimated culture of *Sphingomonas* sp. RW1 and shows that DF seemed to be the preferred substrate. This observation will depend on the degree of the bioavailability of the substrates; the concentrations of dissolved DD and DF were about 0.4 and 5 mg/liter of the mineral salts medium at 28°C, as was determined by HPLC.

Oxygen uptake rates by resting cells. Washed cell suspensions of strain RW1 grown on acetate, DD, and DF were used for oxygen uptake rate studies. Growth-supporting compounds and their substituted derivatives, some potential metabolites, and structurally related compounds were tested. Results are shown in Table 1. DD- and DF-grown cells showed relatively high activities during the oxidation of their aromatic substrates; of their respective acetoxy derivatives; and to a lesser extent, of the corresponding hydroxy derivatives. Very high activities were found during the oxidation of 2,3-dihydroxybiphenyl, 2,2',3-trihydroxybiphenyl, and even 2,2',3-trihydroxypdiphenyl ether. The intense yellow color which developed during oxidation of the di- and trihydroxylated biphenyls and which was stable for more than 15 min, however, was not observed during oxidation of the above ether.

Acetate-grown cells, used as the control, exhibited relatively low but significant levels during the oxidation of the aromatic heterocycles DD, DF, and most of their above derivatives. Activities during the oxidation of 2,3-dihydroxybiphenyl, 2,2',3-trihydroxybiphenyl, catechol, salicylate, and some of their derivatives were as high as those found for cells grown on selective aromatics. These results gave evidence of inducible initial enzymes present at significant constitutive levels, whereas the sequence of the consecutive enzymes seemed to be constitutive. Benzoate-grown resting cells exhibited high rates for these substrates and for catechol but not during the oxidation of DD, DF, 2,3-dihydroxybiphenyl, 2,2',3-trihydroxybiphenyl, and the respective trihydroxydiphenyl ether, providing evidence of repression or elimination of the degradative pathway for both diaryl ether compounds (data not shown).

Determination of enzyme activities in crude extracts. The enzyme activities of (i) the catabolic *meta*-cleaving enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase, (ii) that responsible for hydrolysis of its reaction product [2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate hydrolase (HOPDA hydrolase)], (iii) catechol 2,3-dioxygenase, and (iv) catechol 1,2-dioxygenase were determined with cell extracts of DD-, DF-, and acetate-grown cells. Activities of salicylate hydroxylases (forming catechol and gentisate) were not detectable in crude extracts.

TABLE 1. Specific rates of oxygen uptake with aromatic compounds by resting cells of *Sphingomonas* sp. strain RW1[a]

Compound	Specific oxygen uptake rate (nmoles of O_2/min/mg of protein) after growth on:		
	Acetate	DD	DF
DD	87	273	312
2-Hydroxydibenzo-*p*-dioxin	29	90	118
2-Acetoxydibenzo-*p*-dioxin	83	213	230
DF	99	302	338
2-Hydroxydibenzofuran	106	119	120
2-Acetoxydibenzofuran	113	217	206
2-Methoxydibenzofuran	83	100	89
Biphenyl	43	14	49
2,3-Dihydroxybiphenyl	840	920	1,128
2,2',3-Trihydroxybiphenyl	359	478	553
2,2',3-Trihydroxydiphenyl ether	122	198	212
Diphenyl ether	56	37	49
Naphthalene	31	18	22
Anthraquinone	5	<2	4
Fluoren-9-one	46	32	51
Dibenzothiophene	45	22	41
Xanthene	41	22	48
Xanthen-9-one	48	49	55
Benzoate	7	<2	<2
Catechol	438	451	621
Salicylate	152	174	188
Gentisate	25	29	28

[a] Cells were grown in the presence of the above carbon sources, harvested by centrifugation, washed with 55 mM phosphate buffer (pH 7.2), and resuspended in this buffer (E_{578} = 1). Stock solutions of water-insoluble compounds were made up with dimethyl sulfoxide. The final concentration in the assay was always 1 mM; the concentrations of hydroxy derivatives of DD, DF, biphenyl, and diphenyl ether were 0.1 mM (each) to prevent toxic effects to cells. Rates represent the means of at least two independently performed experiments and are corrected for endogenous respiration.

Results shown in Table 2 indicate that catechol 1,2-dioxygenase was induced solely during growth with DD as the substrate. This enzyme also showed minute activity during the cleavage of both isomeric methylcatechols. Additionally, significant levels of catechol 2,3-dioxygenase were present, showing high rates during the oxidation of catechol and 3-methylcatechol; 4-methylcatechol was cleaved at a low rate.

Activities of catechol 2,3-dioxygenase found in DF-grown cells were only slightly higher than those found in DD-grown cells. The activity of this enzyme was also present in acetate-grown cells. Very high activities during the *meta* cleavage of 2,3-dihydroxybiphenyl and 2,2',3-trihydroxybiphenyl and during hydrolysis of the respective reaction products by HOPDA hydrolase were found. No activity, however, and no development of a yellow color caused by the accumulation of *meta* cleavage products were detectable when 2,2',3-trihydroxydiphenyl ether was used as the substrate in this spectrophotometric assay. The activity for this substrate was therefore determined by HPLC, following the decline of the substrate concentration. The estimation of

TABLE 2. Specific activities of catabolic enzymes in cell extracts of *Sphingomonas* sp. strain RW1[a]

Enzyme and substrate	Sp act (μmol/min/mg of protein) after growth on:		
	Acetate	DD	DF
Catechol 1,2-dioxygenase			
Catechol	<0.002	0.143	<0.002
3-Methylcatechol	<0.002	0.048	<0.002
4-Methylcatechol	<0.002	0.041	<0.002
Catechol 2,3-dioxygenase			
Catechol	0.065	0.127	0.215
3-Methylcatechol	0.087	0.194	0.428
4-Methylcatechol	0.006	0.014	0.018
2,3-Dihydroxybiphenyl 1,2-dioxygenase			
2,3-Dihydroxybiphenyl	1.292	1.981	2.238
2,2',3-Trihydroxybiphenyl	0.417	0.639	0.873
2,2',3-Trihydroxydiphenyl ether[b]	ND	0.018	0.020
HOPDA hydrolase			
2-Hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoate	0.256	0.729	0.672
2-Hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate	0.189	0.431	1.184
Gentisate dioxygenase	ND	0.279	0.432

[a] Enzyme activities of crude cell extracts were recorded by spectrophotometrical standard methods. Data represent means of at least 2 experiments. ND, not determined.

[b] Conversion of the trihydroxydiphenyl ether by crude extracts was determined by HPLC.

oxygen uptake rates of cell extracts with 2,2',3-trihydroxybiphenyl and the respective ether as the enzyme substrates, however, yielded specific rates of 460 versus 122 nmol/min/mg of protein. Experiments performed with whole cells and extracts of strain RW1 with 2-(2-hydroxyphenoxy)-muconate, the *ortho* cleavage product of 2,2',3-trihydroxydiphenyl ether, showed no activity.

3-Chlorocatechol was an efficient competitive inhibitor of the catechol 2,3-dioxygenase of *Sphingomonas* sp. strain RW1 (K_m = 0.83 mM for catechol); the inhibitor constant (K_i) with crude extracts of DD- and DF-grown cells for determinations of enzyme activity was always 30 μM. 4-Chlorocatechol exhibited a less inhibitory character (K_i = 74 μM).

Isolation and identification of metabolites from DD. Trihydroxydiphenyl ether and catechol were identified as metabolites in ethyl acetate extracts of the spent supernatant of a DD-grown culture by gas chromatography-mass spectrometry analysis. For large-scale production of the trihydroxydiphenyl ether, washed cells of DD- or DF-grown cultures were incubated in the presence of excess DD (5 g/liter) to ensure sufficient bioavailability of the dissolved substrate and 3-chlorocatechol (0.1 mM) to achieve inhibition of the 2,3-dihydroxybiphenyl 1,2-dioxygenase (3-phenylcatechol 2,3-dioxygenase). The formation of polar products, one of which was easily identified as catechol, and the maintenance of 3-chlorocatechol concentration were monitored by HPLC. When the rate of product formation decreased, DD crystals were removed by filtration, and after further incubation for 1 h, cells were pelleted by centrifugation. The supernatant was extracted with ethyl acetate, dried over Na_2SO_4, and concentrated to dryness. The residue was dissolved in methanol and purified by preparative HPLC.

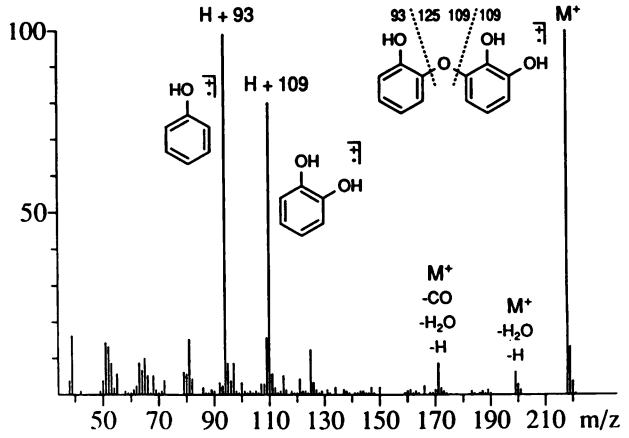

FIG. 3. 70 eV mass spectrum of 2,2',3-trihydroxydiphenyl ether. The *y* axis represents the relative percent.

The pure product was subsequently analyzed by mass and NMR spectroscopy. In the mass spectrum of 2,2',3-trihydroxydiphenyl ether (Fig. 3), the molecular ion at m/z = 218 (base peak) is accompanied by two major fragment ions at m/z = 94 and m/z = 110, formed upon stabilization of the respective α-cleavage products by hydrogen transfer. Two minor fragment ions at m/z = 199 and m/z = 171 are formed upon successive loss of 18 (H_2O), 1 (H), and 28 (CO) amu. ^1H NMR data (400.13 MHz, CD_3OD/C_6D_6 = 100:15; tetramethylsilane as internal standard) (Fig. 4) were as follows: δ = 6.36 (H-4, $J_{4,5}$ = 8.0 Hz, $J_{4,6}$ = 1.6 Hz), 6.60 (H-5, $J_{5,6}$ = 8.0 Hz), 6.66 (H-6), 6.73 (H-5', $J_{5',6'}$ = 8.0 Hz), 6.83 (H-6'), 6.93 (H-4', $J_{4',5'}$ = 7.1 Hz, $J_{4',6'}$ = 1.5 Hz), and 6.98 (H-3', $J_{3',4'}$ = 8.0 Hz, $J_{3',5'}$ = 1.8 Hz) ppm. The following ^{13}C NMR data (100.62 MHz, solvent and internal standard as above) were obtained: δ = 111.1 (C-4), 111.9 (C-6), 117.7 (C-3'), 119.9 (C-6'), 120.1 (C-5), 120.9 (C-5'), 125.0 (C-4'), 137.5 (C-2), 146.1 (C-1'), 146.7 (C-1 or C-3), 147.9 (C-1 or C-3), and 149.1 (C-2') ppm. The assignment of protons and carbons is based on increment calculations as well as on H,H-COSY (1) (Fig. 5), H,C-COSY (33), and H,C-COLOC (17) correlations. The coupling pattern of the protons is confirmed by the H,H-COSY correlation showing two independent spin systems consisting of three and four protons, respectively. The

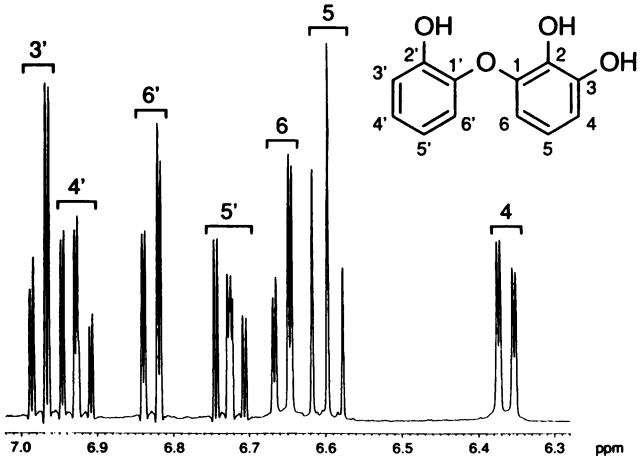

FIG. 4. ^1H NMR spectrum of 2,2',3-trihydroxydiphenyl ether.

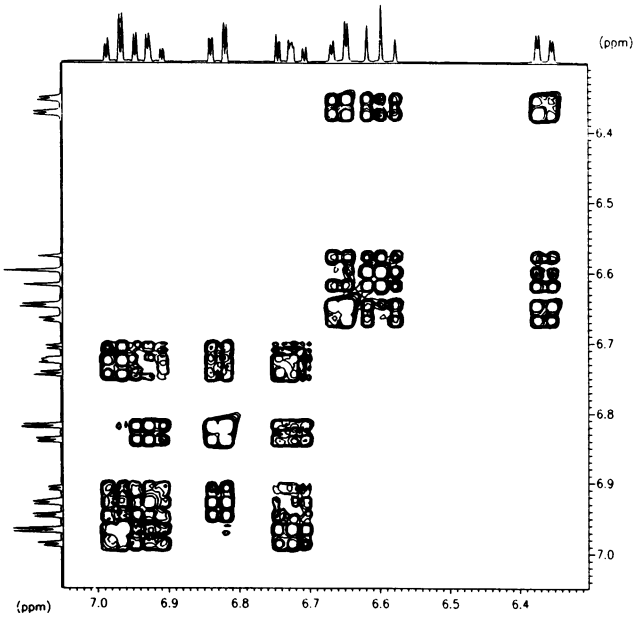

FIG. 5. H,H-COSY contour plot of 2,2',3-trihydroxydiphenyl ether.

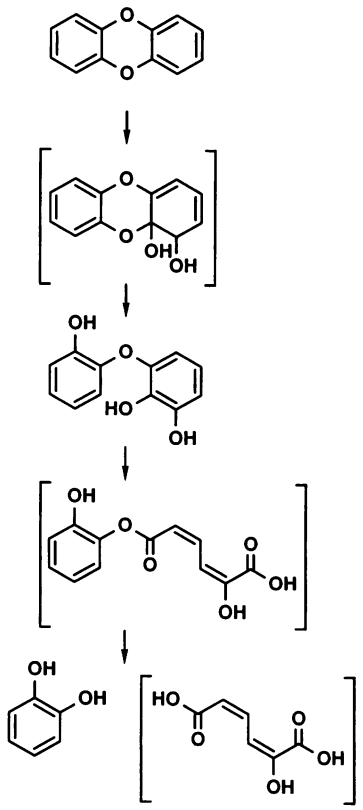

FIG. 6. Proposed pathway for the degradation of DD by *Sphingomonas* sp. strain RW1. Catechol is *ortho* as well as *meta* cleaved.

determined coupling constants indicate the presence of a 1,2-di- and a 1,2,3-trisubstituted aromatic nucleus. The higher extent of high field shift observed for all protons of the trisubstituted nucleus corresponds with the expected shielding effect of oxygen substituents. With respect to the ^{13}C NMR experiment, data for C-1 and C-3 are interchangeable because of the symmetry of the 1,2,3-trisubstituted aromatic nucleus.

Experiments performed with the structurally related compound fluoren-9-one yielded the known angular dihydrodiol (6, 9) irrespective of whether cells were grown with DF or DD.

DISCUSSION

Bacterial strains of the *P. paucimobilis* group now transferred to the new genus *Sphingomonas* (35) are potent microorganisms degrading aromatic compounds of environmental concern (12, 16, 21). Our DF-utilizing *Pseudomonas* sp. strain HH69 (8) was also assigned to this new genus after recent investigations of its patterns of fatty acids and ubiquinone (performed by DSM). Studies of RNA of the latter organism, however, should provide evidence of its relationship to the gram-positive *Brevibacterium* family (14). Bacteria of both systematic groups have been shown to degrade DF by an oxygenolytic attack at the angular position (6–9, 13, 30, 34) and diphenyl ether by a similar reaction (28). Biodegradation of diphenyl ether by a *Pseudomonas cepacia* strain, however, was shown to be initiated by 2,3-dioxygenation, following the classical biphenyl pathway (26). Furthermore, angular dioxygenation for effective dibenzothiophene degradation was demonstrated for a *Brevibacterium* sp. (32). Metabolism or cooxidation of the heterocycles dibenzothiophene (20), DF (4), DD (18, 19), and low-chlorinated congeners (19, 24, 25) followed classical schemes and never led to complete degradation or mineralization. The pathway postulated for DD degradation by our newly isolated *Sphingomonas* sp. strain, shown in Fig. 6, demonstrates ring cleavage of the substrate and suggests *meta* cleavage of the isolated 2,2',3-trihydroxydiphenyl ether. Our suggestion is based on the fact that 2-(2-hydroxyphenoxy)-muconate, the product of *ortho* ring fission, was neither utilized for growth nor oxidized at all. A further indication of *meta* cleavage was the inhibitory action of 3-chlorocatechol. This inhibitor was also utilized for the preparation of 2,2',3-trihydroxybiphenyl by Strubel et al. (29). *meta* cleavage of analog 2,3-dihydroxydiphenyl ether has been shown by Pfeifer et al. (26), proceeding by hydrolysis and intramolecular transesterification, forming phenol and the lactone of 2-hydroxymuconate. *meta* cleavage of 2,2',3-trihydroxydiphenyl ether may occur at either side of the catechol structure between C-1 and C-2, yielding the 6-(2-hydroxyphenyl) ester of 2-hydroxymuconic acid, or between C-3 and C-4, producing 2-hydroxy-3-(2-hydroxyphenoxy)muconic acid semialdehyde. Neither compound has yet been described; their chemical structures indicate considerable thermodynamic instability. The ester may be easily saponified and does not necessarily cause any yellow color, which is generally regarded as an indication of *meta* cleavage and which could not be observed during our experiments. Since the aldehyde should show a yellow color, we, like Pfeifer et al. (26), consider the ester to be a good candidate for the initial product of the degradation of 2,2',3-trihydroxydiphenyl ether. Details of initial reactions of the degradation of (halogenated) diaryl ethers will be published separately.

ACKNOWLEDGMENTS

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REFERENCES

1. **Aue, W. P., E. Bartholdi, and R. R. Ernst.** 1975. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. J. Chem. Phys. **64:**2229–2246.
2. **Bartels, I., H.-J. Knackmuss, and W. Reineke.** 1984. Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. **47:**500–505.
3. **Buser, H.-R.** 1986. Polybrominated dibenzofurans and dibenzo-*p*-dioxins: thermal reaction products of polybrominated diphenyl ether flame retardants. Environ. Sci. Technol. **20:**404–408.
4. **Cerniglia, C. E., J. C. Morgan, and D. T. Gibson.** 1979. Bacterial and fungal oxidation of dibenzofuran. Biochem. J. **180:**175–185.
5. **Czuczwa, J. M., and R. A. Hites.** 1984. Environmental fate of combustion-generated polychlorinated dioxins and furans. Environ. Sci. Technol. **18:**444–450.
6. **Engesser, K. H., V. Strubel, K. Christoglou, P. Fischer, and H. G. Rast.** 1989. Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. FEMS Microbiol. Lett. **65:**205–210.
7. **Fortnagel, P., H. Harms, R.-M. Wittich, S. Krohn, H. Meyer, and W. Francke.** 1989. Cleavage of dibenzofuran and dibenzo-*p*-dioxin ring systems by a *Pseudomonas* bacterium. Naturwissenschaften **76:**222–223.
8. **Fortnagel, P., H. Harms, R.-M. Wittich, S. Krohn, H. Meyer, V. Sinnwell, H. Wilkes, and W. Francke.** 1990. Metabolism of dibenzofuran by *Pseudomonas* sp. strain HH69 and the mixed culture HH27. Appl. Environ. Microbiol. **56:**1148–1156.
9. **Fortnagel, P., R.-M. Wittich, H. Harms, S. Schmidt, S. Franke, V. Sinnwell, H. Wilkes, and W. Francke.** 1989. New bacterial degradation of the biaryl ether structure. Naturwissenschaften **76:**523–524.
10. **Frank-Kamenetskii, M. D.** 1971. Simplification of the empirical relationship between melting temperature of DNA, its GC content, and concentration of sodium ions in solution. Biopolymers **10:**2623–2624.
11. **Furukawa, K., J. R. Simon, and A. M. Chakrabarty.** 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154:**1356–1362.
12. **Harms, H., H. Wilkes, V. Sinnwell, R.-M. Wittich, K. Figge, W. Francke, and P. Fortnagel.** 1991. Transformation of 3-chlorodibenzofuran by *Pseudomonas* sp. HH69. FEMS Microbiol. Lett. **81:**25–30.
13. **Harms, H., R.-M. Wittich, V. Sinnwell, H. Meyer, P. Fortnagel, and W. Francke.** 1990. Transformation of dibenzo-*p*-dioxin by *Pseudomonas* sp. strain HH69. Appl. Environ. Microbiol. **56:** 1157–1159.
14. **Höfle, M. (Gesellschaft für Biotechnologische Forschung, Braunschweig).** Personal communication.
15. **Ishigooka, H., Y. Yoshida, T. Omori, and Y. Minoda.** 1986. Enzymatic dioxygenation of biphenyl-2,3-diol and 3-isopropylcatechol. Agric. Biol. Chem. **50:**1045–1046.
16. **Katayama, Y., S. Nishikawa, A. Murayama, M. Yamasaki, N. Morohoshi, and T. Haraguchi.** 1988. The metabolism of biphenyl structures in lignin by the soil bacterium (*Pseudomonas paucimobilis* SYK-6). FEBS Lett. **233:**129–133.
17. **Kessler, H., C. Griesinger, J. Zarbock, and H. R. Loosli.** 1984. Assignment of carbonyl carbons and sequence analysis in peptides by heteronuclear shift correlation via small coupling constants with broad decoupling in t^1(COLOC). J. Magn. Res. **57:**331–336.
18. **Klecka, G. M., and D. T. Gibson.** 1979. Metabolism of dibenzo-*p*-dioxin by a *Pseudomonas* species. Biochem. J. **180:**639–645.
19. **Klecka, G. M., and D. T. Gibson.** 1980. Metabolism of dibenzo-*p*-dioxin and chlorinated dibenzo-*p*-dioxins by a *Beijerinckia* species. Appl. Environ. Microbiol. **39:**288–296.
20. **Laborde, A. L., and D. T. Gibson.** 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. Appl. Environ. Microbiol. **34:**783–790.
21. **Mueller, J. G., P. J. Chapman, B. O. Blattmann, and P. H. Pritchard.** 1990. Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl. Environ. Microbiol. **56:**1079–1086.
22. **Omori, T., K. Sugimura, H. Ishigooka, and Y. Minoda.** 1986. Purification and some properties of a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolyzing enzyme from *Pseudomonas cruciviae* S93 B1 involved in the degradation of biphenyl. Agric. Biol. Chem. **50:**931–937.
23. **Palleroni, N. J.** 1984. *Pseudomonas*, p. 141–198. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, 9th ed. The Williams and Wilkins Co., Baltimore.
24. **Parsons, J. R., C. Ratsak, and C. Siekerman.** 1990. Biodegradation of chlorinated dibenzofurans by an Alcaligenes strain, p. 377–380. *In* O. Hutzinger and H. Fiedler (ed.), Dioxin '90, Organohalogen Compounds 1, EPRI-Seminar, Bayreuth, Germany.
25. **Parsons, J. R., and M. C. M. Storms.** 1989. Biodegradation of chlorinated dibenzo-*p*-dioxins in batch and continuous cultures of strain JB1. Chemosphere **19:**1297–1308.
26. **Pfeifer, F., S. Schacht, J. Klein, and H. G. Trüper.** 1989. Degradation of diphenylether by *Pseudomonas cepacia*. Arch. Microbiol. **152:**512–519.
27. **Sander, P., R.-M. Wittich, P. Fortnagel, H. Wilkes, and W. Francke.** 1991. Degradation of 1,2,4-trichloro- and 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. **57:**1430–1440.
28. **Schmidt, S. (Universität Hamburg).** Personal communication.
29. **Strubel, V., K.-H. Engesser, P. Fischer, and H.-J. Knackmuss.** 1991. 3-(2-Hydroxyphenyl)catechol as substrate for proximal *meta* ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. J. Bacteriol. **173:**1932–1937.
30. **Strubel, V., H. G. Rast, W. Fietz, H.-J. Knackmuss, and K. H. Engesser.** 1989. Enrichment of dibenzofuran utilizing bacteria with high cometabolic potential towards dibenzodioxin and other anellated aromatics. FEMS Microbiol. Lett. **58:**233–238.
31. **Swanson, S. E., C. Rappe, J. Malmström, and K. P. Kringstad.** 1988. Emissions of PCDDs and PCDFs from the pulp industry. Chemosphere **17:**681–691.
32. **van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper.** 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. **153:**324–328.
33. **Wilde, J. A., and P. H. Bolton.** 1984. Suppression of homonuclear couplings in heteronuclear two-dimensional spectroscopy. J. Magn. Res. **59:**343–346.
34. **Wittich, R.-M., S. Schmidt, and P. Fortnagel.** 1990. Bacterial degradation of 3- and 4-carboxybiphenyl ether by *Pseudomonas* sp. NSS2. FEMS Microbiol. Lett. **67:**157–160.
35. **Yabuuchi, E., I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki, and H. Yamamoto.** 1990. Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. Microbiol. Immunol. **34:**99–119.