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Biodegradation of Diphenyl Ether and its Monohalogenated Derivatives by *Sphingomonas* sp. Strain SS3

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The bacterium *Sphingomonas* sp. strain SS3, which utilizes diphenyl ether and its 4-fluoro, 4-chloro, and (to a considerably lesser extent) 4-bromo derivatives as sole sources of carbon and energy, was enriched from soil samples of an industrial waste deposit. The bacterium showed cometabolic activities toward all other isomeric monohalogenated diphenyl ethers. During diphenyl ether degradation in batch culture experiments, phenol and catechol were produced as intermediates which were then channeled into the 3-oxoadipate pathway. The initial step in the degradation follows the recently discovered mechanism of 1,2-dioxygenation, which yields unstable phenolic hemiacetals from diphenyl ether structures. Oxidation of the structure-related dibenzo-*p*-dioxin yielded 2-(2-hydroxyphenoxy)-muconate upon *ortho* cleavage of the intermediate 2,2′,3-trihydroxydiphenyl ether. Formation of phenol, catechol, halophenol, and halocatechol from the conversion of monohalogenated diphenyl ethers gives evidence for a nonspecific attack of the dioxygenating enzyme system.

The diaryl ether linkage represents a common structural feature of a group of compounds of environmental concern like polyhalogenated diphenyl ethers (DEs), dibenzo-*p*-dioxins, and dibenzofurans (36). Since the 1930's, bulk amounts of DE have been produced industrially at rates of several thousand tons per year (23, 30). Meanwhile, this persistent compound was detected in samples of drinking water (17) and of sea water (1). Halogenated derivatives of DEs (59) widely used as pesticides have been detected in environmental samples (31, 45) and, like DE itself, are subject to vertebrate metabolism (5, 7, 9, 49). Recently, a biogenic halo DE, 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol, was isolated from cultures of marine bacteria (11).

The first report on the biodegradation of DE was from Takase et al. (47), who isolated a *Pseudomonas cruciviae* strain that utilized DE, biphenyl, and a few more biphenyl-related compounds. DE and biphenyl were both dioxygenated at the 2,3 position. Whereas 2,3-dihydroxybiphenyl was degraded by the classical *meta* pathway (6, 28), the corresponding 2,3-dihydroxydiphenyl ether furnished phenol and 2-phenoxymuconic acid, giving strong indications for *ortho* cleavage of the aromatic diol structure. Minute amounts of this *ortho* ring fission product were also identified as a probable by-product of DE degradation by *Pseudomonas cepacia* (33). The breakdown by this organism was also initiated by 2,3-dioxygenation, but, after *meta* cleavage, the intramolecular transesterification of 2-hydroxy-6-phenoxymuconic semialdehyde furnished 2-pyrone-6-carboxylic acid accumulating in the culture medium as the predominant dead-end product. Only phenol was utilized for growth (34).

The mineralization of a carboxyl-substituted DE, 3-phenoxybenzoate, has been reported by Topp and Akhtar (48), who identified phenol as the product utilized by one of the organisms of their mixed culture. Degradation of 3- and 4-carboxyl-substituted DEs was also shown by Fortnagel et al. (15), Engesser et al. (12), and Wittich et al. (55), who identified phenol and 3,4-dihydroxybenzoic acid as metabolites, indicating the direct dioxygenolytic cleavage of the ether bond. Unsubstituted DE and the 4-hydroxy-, 4-nitro-, and 4-chloro-substituted DEs should be cometabolically degraded or cleaved by an enzyme of an *Erwinia* strain; the gene responsible for this reaction was cloned in *Escherichia coli* (26). A salicylic acid-utilizing *Acinetobacter* sp. also should cleave the ether linkage of above compounds (27). The transformation of the 3-formyl derivative of DE, 3-phenoxybenzaldehyde, to 3-hydroxybenzoic acid and to conjugated derivatives by chicken metabolism has been reported (2). The oxidation of DE to 4-hydroxy- and 4,4′-dihydroxydiphenyl ether recently was shown to be carried out by the fungus *Cunninghamella echinulata* (44). Biotransformations of chlorinated hydroxydiphenyl ethers by *Rhodococcus chlorophenolicus* have been described; the corresponding anisols obtained with these reactions, however, remained undegraded (51).

Here, we report for the first time the complete mineralization of DE and even of some of its monohalogenated derivatives by a newly isolated bacterium.

MATERIALS AND METHODS

Enrichment, isolation, and identification. Starting with soil samples from the Hamburg-Georgswerder industrial waste deposit as the inoculum, enrichment cultures were established with 4-fluorodiphenyl ether as the sole source of carbon and energy. Aliquots were transferred every second week into fresh medium. The identification of the isolated bacterium was based on the classification schemes of *Bergey's Manual of Systematic Bacteriology* (32); the identification was performed by standard laboratory tests and those described in the manual. For further investigations of cell morphology, standard laboratory techniques were applied for electron microscopic investigations on a model 201 transmission electron microscope (Philips Co., Eindhoven, The Netherlands). Determination of the guanine-plus-cytosine content of DNA (melting point) was performed by the method of Frank-Kamenetskii (16); the ubiquinone pattern was estimated by the method of Yamada et al. (58) with

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high-performance liquid chromatography (HPLC) for separation and identification of these compounds. The organism (DSM 6432) is deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

Culture conditions. The mineral salts medium used for enrichment and growth of the organism, standard culture conditions, and determination of growth parameters used throughout our work were described previously (14, 37). Culture media were supplemented with DE or the halogenated derivatives over the gas phase or, for production of large amounts of biomass, by direct addition of the substrates to the hot, freshly autoclaved mineral salts medium as described by Sander et al. (37). Cells in general were grown in polytetrafluoroethylene-sealed Erlenmeyer flasks up to the late-exponential growth phase and separated by decantation from residual amounts of substrate. Since it was impossible to separate cells from DEs still attached to the cell surface, cultures were shaken for about two additional hours to allow complete substrate consumption before utilization for experiments.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared from exponentially growing cultures as previously described (14, 37). The assays of catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) were performed as described by Pieper et al. (35). The extinction coefficients of the ring fission products were those reported by Dorn and Knackmuss (10). The method of Dorn and Knackmuss was also applied for the estimation of the ε value of 2-(2-hydroxyphenoxy)muconic acid (11,000 mol cm^{-1}). The activity of 2,3-dihydroxybiphenyl-1,2-dioxygenase was assayed by the method of Ishigooka et al. (20). The activities of the phenol-hydroxylating enzyme were determined by monitoring the decrease of the substrate with HPLC and/or by the estimation of oxygen consumption as described by Pieper et al. (35). Soluble protein was determined by the Bradford method (4).

Activities with whole cells. Determinations of specific oxygen uptake rates were also applied for the estimation of O_2 consumption during the oxidation of carbon sources, structurally related compounds, and metabolites. The assays were performed in the 1-ml cell of a Clark-type oxygen electrode, and determinations of whole-cell protein were done as previously described (14).

Analytical methods. HPLC, gas chromatography, gas chromatography-mass spectroscopy, mass spectroscopy, proton nuclear magnetic resonance spectrometry, and thin-layer chromatography were performed as previously described (14, 37). Determinations of bromide, chloride, and fluoride ions were made by using ion-sensitive electrodes (37). HPLC was also used for the determination of the amount of substrate dispersed and/or dissolved in bacterial cultures. DE and its halo derivatives were extracted with 0.1 volume of chloroform and quantified by HPLC. Previous experiments had shown that recoveries were quantitative in the range from 5 to 1,000 mg of DE per liter of medium.

Chemicals. DE; 4-chloro-, 4-hydroxy-, and 4-nitrodiphenyl ether; phenol; 2-fluoro-, 2-chloro-, 3-chloro-, 4-chloro-, and 4-bromophenol; catechol; and 3-fluoro- and 4-chlorocatechol were from Aldrich-Chemie GmbH & Co. KG (Steinheim, Germany). 4-Bromodiphenyl ether and 3-fluoro-, 3-bromo-, and 4-bromophenol were from Janssen Chimica (Brüggen, Germany). 4-Fluorodiphenyl ether was from Riedel-de Haën AG (Seelze, Germany). 4-Fluorophenol and benzophenone were from Fluka (Neu-Ulm, Germany). 2,3-Dihydroxybiphenyl was purchased from Wako Chemicals (Neuss, Germany). 3,4-Dihydroxybiphenyl was a generous gift from D. D. Focht, University of California, Riverside.

Dibenzo-*p*-dioxin was prepared as described previously (14). 3-Chlorocatechol was prepared from the corresponding salicyl aldehyde by published methods (8); 4-fluoro- and 4-bromocatechol were synthesized from the corresponding veratroles by the method of McOmie et al. (29) for the cleavage of aryl methyl ethers by the action of boron tribromide. 2-Fluoro-, 3-fluoro-, 2-chloro-, 3-chloro-, 2-bromo-, and 3-bromodiphenyl ether were synthesized through condensation of phenol with the corresponding haloiodobenzenes by application of the Ullmann ether synthesis (50). 4-Carboxymethylenbut-2-en-4-olide was prepared as described by Kaschabek (22).

2,2',3-Trihydroxydiphenyl ether was produced biologically from dibenzo-*p*-dioxin by inhibition of the *meta* cleaving dioxygenase of *Sphingomonas* sp. strain RW1 (DSM 6014) (56) with 3-chlorocatechol (3, 24, 46). 3-Bromocatechol was also produced biologically from bromobenzene (E. Merck, Darmstadt, Germany) with *Pseudomonas* sp. strain PS14 (DSM 6434) as described previously (37). Ubiquinones were from Sigma Chemie GmbH (Deisenhofen, Germany). All other chemicals were of the highest purity commercially available.

RESULTS

Isolation and identification of the organism. From the enrichment cultures supplemented with 4-fluorodiphenyl ether as the sole source of carbon and energy after several passages, a pure bacterial culture was obtained in less than 3 months without further purification procedures. The following morphological and physiological characteristics of the isolated organism allowed the tentative assignment to the genus *Pseudomonas*. The strictly aerobic organism was gram negative and oxidase and catalase positive. Electron microscopic investigations of the rods (about 2.5 by 0.9 μm) and flagellum staining procedures clearly showed a single polarly inserted flagellum. The G+C content of the DNA was 66 mol% on the basis of melting point determinations. The predominant ubiquinone was ubiquinone Q_{10}; small amounts of Q_9 and traces of Q_8 were also present. Further investigations performed very recently by the German Collection of Microorganisms and Cell Cultures (DSM) resulted in the assignment to the new genus *Sphingomonas* (57) because of the organism's lipid, fatty acid, and ubiquinone patterns.

The isolate grew well with DE, 4-fluorodiphenyl ether, phenol, 4-fluorophenol, catechol, benzoate, and 4-hydroxybenzoate. After a short acclimation period of about 1 month, 4-chlorodiphenyl ether and, to a considerably lesser extent, 4-bromodiphenyl ether were also utilized by the organism. Utilization of 4-fluoro-, 4-chloro, and 4-bromocatechol was only observed when cells were grown on solid gradient plates: about 10^6 cells from early-stationary-phase cultures were plated on solid mineral salts medium, and substrate crystals were placed near the edge of the petri dishes. When there was growth (dishes without substrate served as the control), it became visible in the diffusion gradient, leaving a clear halo around the crystals. Biphenyl, 2,3-dihydroxybiphenyl, 3,4-dihydroxybiphenyl, naphthalene, dibenzofuran, dibenzo-*p*-dioxin, 3-carboxy- and 4-carboxydiphenyl ether, 4-hydroxy- and 4-nitrodiphenyl ether, salicylic acid, gentisic acid, and protocatechuic acid were not utilized for growth. Some nonaromatic compounds like several sugars and tri-

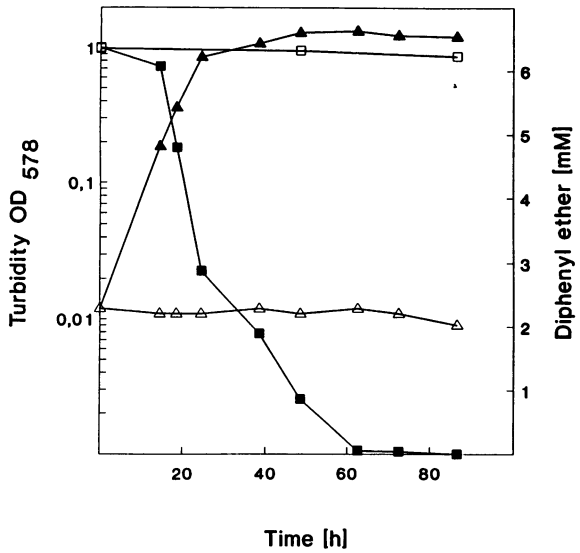

FIG. 1. Growth of *Sphingomonas* sp. strain SS3 with DE as the carbon source. The experiment was performed in parallel sets of closed Erlenmeyer flasks containing DE dispersed in the mineral salts medium. The flasks were inoculated from a recently outgrown preculture; batches were worked up after the appropriate times as indicated. The concentration of DE was determined by HPLC as described in Materials and Methods. Growth of the culture (▲) and consumption of DE (■) are plotted against time together with controls in the absence of DE (△) and those performed with heat-inactivated inocula (□). OD_{578}, optical density at 578 nm.

TABLE 1. Specific catabolic enzyme activities of *Sphingomonas* sp. strain SS3[a]

Enzyme and substrate	Sp act after growth with:			
	DE	4-Fluoro-diphenyl ether	4-Chloro-diphenyl ether	Acetate
Catechol 1,2-dioxygenase[b]				
Catechol	182	201	209	1.5
4-Fluorocatechol	55	57	60	0.5
3-Fluorocatechol	1.3	1.5	1.8	<0.1
4-Chlorocatechol	17	22	24	0.3
3-Chlorocatechol	0.2	0.3	0.3	<0.1
4-Bromocatechol[c]	0.1	0.2	0.2	<0.1
3,5-Dichlorocatechol	0.2	0.3	0.3	<0.1
Phenolhydroxylase[d]				
Phenol	35	36	38	1.0
4-Fluorophenol	25	30	29	<0.5
3-Fluorophenol	23	26	26	<0.5
2-Fluorophenol	9	11	9	<0.5
4-Chlorophenol	10	13	15	<0.5
3-Chlorophenol	11	12	14	<0.5
2-Chlorophenol	2	3	4	<0.5
4-Bromophenol	7	9	9	<0.5
3-Bromophenol	5	7	8	<0.5
2-Bromophenol	<0.5	<0.5	<0.5	<0.5

[a] Results represent means of at least three independently performed experiments. Absolute specific activities are expressed in nanomoles per minute per milligram of protein.
[b] Activity was determined with cell extracts.
[c] Activity is expressed as the change in A_{260} per minute per milligram of protein, since no ε is known for the respective muconate.
[d] Activity was determined with whole cells.

carboxylic acid cycle intermediates were also used as carbon and energy sources.

Growth with DE and halogenated derivatives. Figure 1 demonstrates the growth of *Sphingomonas* sp. strain SS3 with DE as the substrate. During the early growth phase, the doubling time of the culture was about 4.5 h. The doubling time increased markedly when the bioavailability of the substrate, depending on dissolution kinetics, became the growth-limiting factor. The solubility of DE in the saturated mineral salts medium under growth conditions was 141 μM (corresponding to 24 mg/liter in HPLC determinations). The protein yield of the culture was 31 g per mol of substrate utilized.

The doubling times were significantly longer during growth with halogenated DEs. The doubling times and solubilities of respective substrates were as follows: 4-fluorodiphenyl ether, 7.5 h, 104 μM; 4-chlorodiphenyl ether, 30 h, 28 μM; and 4-bromodiphenyl ether, about 60 h, 14 μM. Protein yields also decreased markedly. Cultures grown with 4-fluoro- and 4-chlorodiphenyl ether produced 29 and 24 g/mol, correlated with the almost stoichiometric release of halogenid ions (95 and 92%, respectively). From 4-bromodiphenyl ether the very slowly growing culture obviously utilized minute amounts of the brominated ring; the protein yield was only 12 g/mol, and only 35% of the theoretical amounts of bromide ions were detectable in the dark-brown culture supernatant after complete consumption of the substrate.

Enzyme activities and oxygen uptake rates. The catabolic enzyme activities of *Sphingomonas* sp. strain SS3 after growth with DE, 4-fluoro- and 4-chlorodiphenyl ether, and acetate are demonstrated in Table 1. Catechol 1,2-dioxygenase activities furnishing the respective muconic acids were determined with crude cell extracts; catechol 2,3-dioxygenase activities were absent. Activities of the catechol 1,2-dioxygenase significantly decreased with increasing atom radii of the halogen substituents at carbon 4. The K_m values determined with cell extracts for catechol (3.6 μM), 4-fluorocatechol (4.7 μM), and 4-chlorocatechol (9.0 μM) remained unchanged, irrespective of whether cells were grown with DE or the respective halo derivatives.

Since phenol hydroxylase activities were not detectable in crude extracts, activities were estimated by HPLC measuring substrate consumption by resting cells. Activities again decreased with increasing radii of the halo substituents and the position of substitution in the order 4 > 3 ⩾ 2.

Specific rates of oxygen uptake by resting cells for the oxidation of various aromatic substrates are shown in Table 2; Fig. 2 also shows the results for the investigated DEs. Results concerning DE oxidation again clearly show that specific oxidation rates correlate with radii and positions of the halo substituents, although the unsubstituted aromatic ring is still accessable for oxidative or oxygenolytic attack. Neither catechol nor phenol nor any of their respective halo derivatives induced activities for the oxidation of (halogenated) DEs. Enzyme activities of acetate-grown cells were not induced for the oxidation of aromatic compounds or showed very low levels.

In the *ortho* pyrocatechase assay, a significant activity was determined for the oxidation of 2,2',3-trihydroxydiphenyl ether with a rate of 37 nmol/min/mg of protein, whereas no indications were found for the *ortho* or *meta* cleavage of 2,3-dihydroxy- and 2,2',3-trihydroxybiphenyl.

Isolation and identification of metabolites and oxidation products. Metabolites accumulating during incubation of

TABLE 2. Specific oxygen uptake rates with aromatic compounds by resting cells of *Sphingomonas* sp. strain SS3[a]

Assay substrate	Specific oxygen uptake rate after growth with:			
	DE	4-Fluoro-diphenyl ether	4-Chloro-diphenyl ether	Acetate
Diphenyl ether	231	212	155	13
4-Fluorodiphenyl ether	202	221	161	10
3-Fluorodiphenyl ether	160	181	107	7
2-Fluorodiphenyl ether	157	180	97	<5
4-Chlorodiphenyl ether	147	157	193	6
3-Chlorodiphenyl ether	111	145	126	<5
2-Chlorodiphenyl ether	91	130	106	<5
4-Bromodiphenyl ether	91	116	110	<5
3-Bromodiphenyl ether	85	106	97	<5
2-Bromodiphenyl ether	61	80	69	<5
Catechol	312	313	286	26
4-Fluorocatechol	109	110	107	8
3-Fluorocatechol	24	29	23	<5
4-Chlorocatechol	77	96	88	<5
3-Chlorocatechol	19	26	23	<5
4-Bromocatechol	43	54	50	<5
Phenol	108	130	96	6
4-Fluorophenol	63	93	69	<5
3-Fluorophenol	47	54	48	<5
2-Fluorophenol	24	30	23	<5
4-Chlorophenol	29	60	42	<5
3-Chlorophenol	18	37	39	<5
2-Chlorophenol	<5	9	9	<5
4-Bromophenol	22	33	33	<5
3-Bromophenol	11	13	12	<5
2-Bromophenol	<5	<5	<5	<5
Benzophenone	40	38	29	8
Dibenzo-*p*-dioxin	29	27	25	<5
2,2',3-Trihydroxydiphenyl ether	50	54	58	<5

[a] Results represent means of at least three independently performed experiments. Oxygen uptake rates are expressed as specific activities (nanomoles of O_2 consumed per minute per milligram of protein) and are corrected for endogenous respiration.

resting cells of *Sphingomonas* sp. strain SS3 in the presence of an excess of DE or one of its halogenated derivatives were detected by HPLC. The phenols and catechols formed from these substrates were analyzed by HPLC and/or, after extraction, by gas chromatography-mass spectroscopy and identified by comparison with authentic samples.

4-Carboxymethylenbut-2-en-4-olide was always identified as the main product by comparison with an authentic standard from upscaled catechol 1,2-dioxygenase assays performed in the absence of EDTA with 4-fluoro-, 4-chloro-, and 4-bromocatechol as the substrates. From 4-bromocatechol a second butenolide, 3-bromo-4-carboxymethylbut-2-en-4-olide, was also isolated and identified by mass spectroscopy and nuclear magnetic resonance spectrometry (data not shown).

From cooxidation experiments performed with DE-grown resting cells and dibenzo-*p*-dioxin and 2,2',3-trihydroxydiphenyl ether as the substrates, 2-(2-hydroxyphenoxy)-*cis*,*cis*-muconate was identified as the only product. The mass spectrum is shown in Fig. 3; the signal at *m/z* 232 results from loss of 18 atomic mass units (H_2O) from the molecular ion. The following ^1H nuclear magnetic resonance data (400.13 MHz, acetone-d_6, tetramethylsilane as the internal standard) were obtained: δ = 5.74 (H-5), 6.83 (H-4', $J_{4',5'}$ = 7.2 Hz, $J_{4',6'}$ = 1.7 Hz), 6.95 (H-3', $J_{3',4'}$ = 8.3 Hz, $J_{3',5'}$ = 1.7 Hz), 6.98 (H-6'), 7.04 (H-5', $J_{5',6'}$ = 8.3 Hz), 7.37 (H-3, $J_{3,4}$ = 11.5 Hz, $J_{3,5}$ = 1.2 Hz), and 7.66 (H-4, $J_{4,5}$ = 11.5 Hz) ppm. The ^{13}C nuclear magnetic resonance data (100.62 MHz, acetone-d_6, tetramethylsilane as the internal standard) were as follows: δ = 114.9 (CH), 118.2 (CH), 120.5 (CH), 120.9 (CH), 121.5 (CH), 126.8 (CH), 138.8 (CH), 142.8 (C), 149.2 (C), 150.5 (C), 163.5 (COOH), and 167.1 (COOH) ppm. The spectroscopic properties of the corresponding dimethyl ester, obtained upon methylation with diazomethane, were very similar to that previously reported for the 2-(2-hydroxyphenoxy)-*cis*,*trans*-muconic acid dimethyl ester (19). However, the coupling constant of $J_{4,5}$ = 11.5 Hz indicates a *cis* configuration of the C-4=C-5 double bond of the muconate formed by strain SS3.

DISCUSSION

A pathway for DE degradation by our *Sphingomonas* sp. strain SS3, proposed on the basis of our results, is shown in Fig. 4; it closely resembles the pathway for the degradation of the diaryl ethers dibenzofuran (13, 14) and dibenzo-*p*-dioxin (56), carboxylated DEs (12, 55), and the thio ether dibenzothiophene (52). Upon attack of a novel, highly regioselective dioxygenase system at the 1,2-position, i.e., the carbon carrying the ether bridge and the adjacent unsubstituted carbon of DE an unstable phenolic hemiacetal, 1,2-dihydroxy-1,2-dihydrodiphenyl ether is formed and then rearranged to yield phenol and catechol. In addition, 2-(2-hydroxyphenoxy)-*cis*,*cis*-muconate was identified as the end product of the cooxidation of dibenzo-*p*-dioxin by our DE-grown organism, providing evidence for the postulated unstable intermediate hemiacetal, 1,10*a*-dihydroxy-1,10*a*-dihydrodibenzo-*p*-dioxin (Fig. 5). The above muconate, also obtained from the conversion of 2,2',3-trihydroxydiphenyl ether [3-(2-hydroxyphenoxy)-catechol], therefore, might have been produced by the action of a catechol 1,2-dioxygenase of broad substrate specificity of our strain SS3. A similar observation has been made by Takase et al. (47) for the unproductive *ortho* cleavage of 2,3-dihydroxydiphenyl ether. Pfeifer (33) has shown that the activity of the purified catechol 1,2-dioxygenase of a DE-degrading *P. cepacia* strain was also responsible for the intradiolic cleavage of 2,3-dihydroxydiphenyl ether.

The dioxygenolytic cleavage of ether linkages, which is completely different from that reported by Pfeifer et al. (34), has been established unambiguously by isotope labeling experiments performed in an $^{18}O_2$ atmosphere with 4-chlorodiphenyl ether, dibenzofuran, and dibenzo-*p*-dioxin as the substrates. Detailed results are published elsewhere (54). Cooxidation of several structure-related benzophenones furnished the respective stable 1,2-dihydrodiols (53).

Generally, decreasing specific oxygen uptake rates were observed for the halo DEs carrying halogen substituents of increasing atomic radii (F > Cl > Br) or in which the position of substitution was changed from *para* via *meta* to *ortho* (Fig. 2). A significant exception, however, was observed when cells were grown with 4-chlorodiphenyl ether: in this case the highest rates were determined for the respective chloro derivatives of all three substituent positions. Thus, especially for the oxidation of *ortho*-substituted DEs, steric effects may play an important role, hindering dioxygenolytic attack even at the nonhalogenated nucleus. A general scheme demonstrating product formation from halogenated

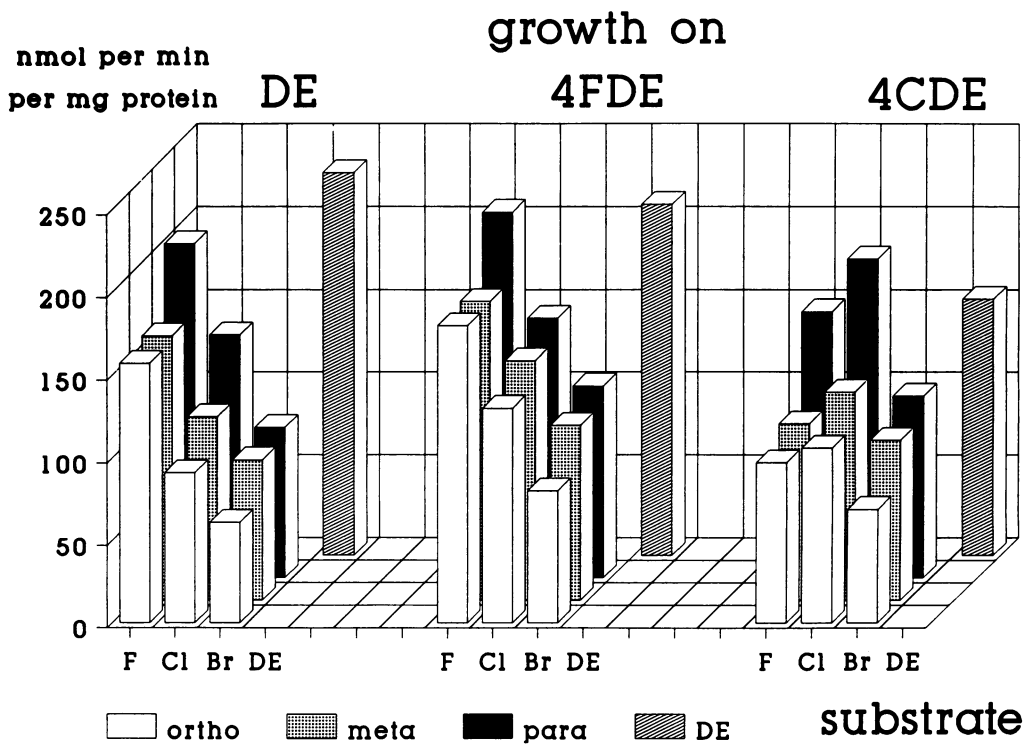

FIG. 2. Specific oxygen uptake rates with DEs by resting cells of strain *Sphingomonas* sp. strain SS3 after growth with DE, 4-fluorodiphenyl ether (4FDE), or 4-chlorodiphenyl ether (4CDE).

DEs is presented in Fig. 6. This figure also demonstrates that the halogenated and the nonhalogenated aromatic rings of the molecule are attacked by the initial dioxygenase, as was clearly confirmed by our $^{18}O_2$ studies (54). Recently, we also showed that the cooxidation of 3-chlorodibenzofuran by the dibenzofuran-oxidizing mutant strain HH69/II obtained from the dibenzofuran-mineralizing and dibenzo-*p*-dioxin-cooxidizing bacterium *Sphingomonas* sp. strain HH69 (earlier identified as a *Pseudomonas* sp. [14, 19]) yielded equal amounts of 4'-chloro-2,2',3-trihydroxybiphenyl and 4-chloro-2,2',3-trihydroxybiphenyl (18).

Determinations of enzyme activities for the oxidation of phenol and catechol and for their halogenated derivatives give indications that our *Sphingomonas* sp. strain SS3 only induces type I enzymes. Type I enzymes allow the efficient mineralization of nonhalogenated and fluorinated catechols, as has been shown by Schlömann et al. (38, 39); chlorocatechols, however, are much more slowly converted (10, 42). These problems, which arise during the conversion of halophenols and their halogenated metabolites by type I enzymes of nonadapted or poorly adapted strains, have been

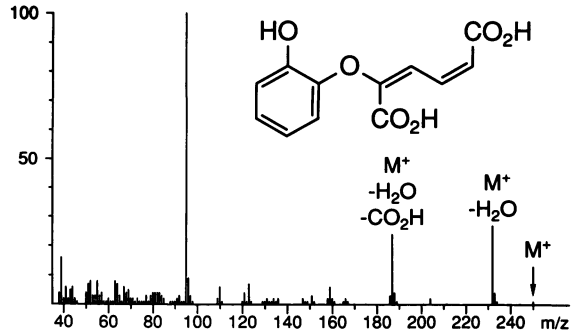

FIG. 3. Mass spectrum (EI, 70 eV) of 2-(2-hydroxyphenoxy)-*cis*,*cis*-muconate.

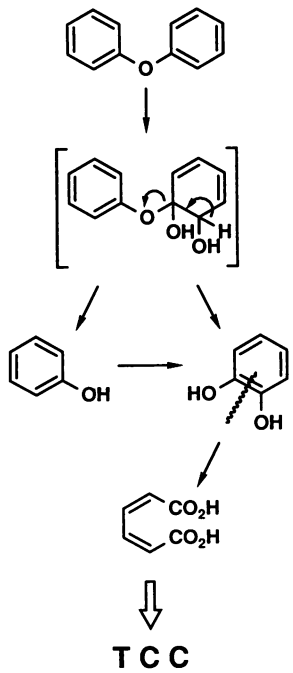

FIG. 4. Proposed pathway for the degradation of DE by *Sphingomonas* sp. strain SS3. TCC, tricarboxylic acid cycle.

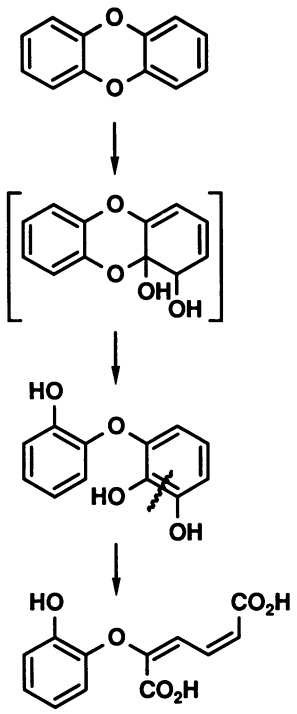

FIG. 5. Proposed pathway for dibenzo-*p*-dioxin cooxidation.

investigated and reported in detail (21, 25, 40, 43) and are clearly demonstrated by the identification of 3-bromo-4-carboxymethylbut-2-en-4-olide accumulating as the dead-end metabolite during bromocatechol degradation.

Identification of 4-carboxymethylenebut-2-en-4-olide formed from the halocatechols substituted in the 4-position, on the other hand, confirms a known and common pathway of the biodegradation of these compounds. Schmidt and Knackmuss (41) have shown that both type I and type II muconate cycloisomerases of the 3-chlorobenzoate-degrading strain *Pseudomonas* sp. B13 convert 3-chloromuconate into the above-mentioned dienelactone.

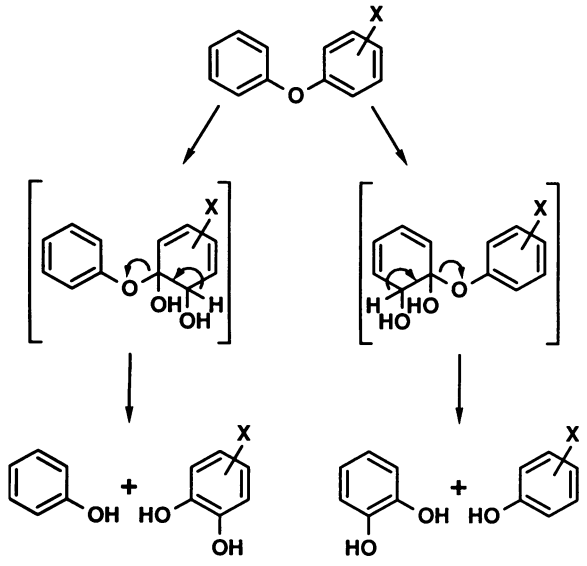

FIG. 6. Nonspecific attack of the dioxygenase on halodiphenyl ethers. X indicates F, Cl, or Br; positions: *ortho*, *meta*, and *para*.

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