Degradation of Chlorinated Dibenzofurans and Dibenzo-*p*-Dioxins by *Sphingomonas* sp. Strain RW1

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The ability of the dibenzofuran- and dibenzo-*p*-dioxin-mineralizing bacterium *Sphingomonas* sp. strain RW1 (R.-M. Wittich, H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel, *Appl. Environ. Microbiol.* 58:1005–1010, 1992) to oxidize chlorinated derivatives of dibenzofuran and dibenzo-*p*-dioxin was analyzed. Strain RW1 degraded several mono- and dichlorinated dibenzofurans and dibenzo-*p*-dioxins, but it did not degrade more highly chlorinated congeners. Most mono- and dichlorinated dibenzofurans and dibenzo-*p*-dioxins investigated in this study were degraded to the corresponding mono- and dichlorinated salicylates and catechols, respectively, together with salicylate and catechol. This indicates an initial dioxygenolytic attack on the substituted as well as on the unsubstituted aromatic nucleus of most of the target compounds. Strain RW1 could not grow at the expense of monochlorinated dibenzo-*p*-dioxins and dibenzofurans as carbon sources, with the exception of 4-chlorodibenzofuran, which was stoichiometrically converted to 3-chlorosalicylate.

In recent years, a number of studies of the aerobic bacterial degradation of the diaryl ethers dibenzo-*p*-dioxin (DD), dibenzofuran (DF), and diphenyl ether, as well as some of their less highly halogenated derivatives, have been performed (8, 13–16, 20, 22–24, 26, 29–31, 34–37, 40, 41). Several key enzymes of the degradative pathway have been investigated in detail (6, 7, 10, 19). Studies on the anaerobic dechlorination of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) showed that they are converted by methanogenic consortia to less highly chlorinated derivatives (1, 38), similar to the well-known anaerobic dechlorination of polychlorinated biphenyls and other chloroaromatics (25).

Until now, no aerobic enrichment cultures capable of utilizing chlorinated DFs and/or DDs for growth have been obtained. However, the cooxidation of chlorinated DDs and DFs is also of interest in terms of the possible fate of PCDFs and PCDDs in the environment. In this paper, we describe the cooxidative potential of *Sphingomonas* sp. strain RW1 toward some mono- and dichlorinated DFs and DDs used as model compounds for the more highly chlorinated and more toxic congeners.

MATERIALS AND METHODS

Growth conditions, preparation of resting cells, oxygen uptake measurements, and analytical procedures. Experimental procedures were as previously described (15, 32, 41). A Shimadzu class LC-10 system equipped with a diode array detector was used for further high-pressure liquid chromatography (HPLC) analysis. Retention volumes (in milliliters) for the separation of chlorinated DDs and DFs on a 12-cm octadecyl silane 5 column (with an eluent of methanol-water, 90:10 [vol/vol], and a flow rate of 1 ml min^{−1}) were as follows: DD, 2.15; 1-chlorodibenzo-*p*-dioxin (1-CDD), 2.87; 2-CDD, 3.65; 2,7-dichlorodibenzo-*p*-

dioxin (2,7-DCDD), 5.10; 2,3-DCDD, 5.63; 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), 7.35; 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD), 11.80; DF, 2.05; 4-chlorodibenzofuran (4-CDF), 2.81; 2-CDF, 3.06; 3-CDF, 3.33; 2,8-dichlorodibenzofuran (2,8-DCDF), 4.83; 2,3-DCDF, 5.08; 3,7-DCDF, 5.62; 2,4,8-trichlorodibenzofuran (2,4,8-TrCDF), 8.43.

Setup of depletion and oxidation experiments. Cells of strain RW1 were grown on DF until they reached an optical density (E_{578}) of about 1.5. They were then separated from substrate crystals by filtration and, after about 30 min of vigorous agitation to allow residual substrate to be consumed, were washed twice with 25 mM phosphate buffer (pH 7.3).

Turnover rates (depletion experiments) were determined at 20°C on an overhead rotating mixer in 12-ml glass tubes closed with Teflon seal screw caps. The tubes contained 1 ml of fresh resting-cell suspension (optical density, 0.9) and the target substrate corresponding to a 250 μ M concentration. Stock solutions of chlorinated DFs and DDs were made up to 12.5 mM in acetone. To maximize the bioavailability of the compounds, just prior to starting the experiments the solvent was allowed to evaporate from the tubes by rolling them on a solid surface in the fume hood to obtain a thin but loose film of crystals which was washed from the wall upon addition of the cell suspension and whirl-mixing. At time points indicated in the figures, reactions in a set of two parallel tubes were stopped by immediate chilling on ice and addition of 5 ml of hexane for extraction. Control experiments were performed with heat-inactivated cells (55°C for 30 min) and only DF or DD as the substrate. Each set of experiments was performed in duplicate. Tubes were maintained at 4°C until analysis of substrate concentration by HPLC was carried out.

For production of metabolites, about 48 h before starting the experiment 50 ml of 50 mM phosphate buffer (pH 7.3) was added to 300-ml baffled Erlenmeyer flasks containing 25 mg of the target compound to be oxidized, in order to obtain a saturated solution. This crystal suspension received a concentrated suspension of resting cells to a final optical density of 2.0, corresponding to about 0.42 mg of protein per ml. The flasks were sealed and incubated at 30°C on a rotary shaker at 150 rpm. The formation of products was monitored by HPLC as previously described (32). Experiments were terminated when product formation reached a maximum, and the batches were worked up as previously described (41). For elucidation of the structures of intermediates, the ethyl acetate extracts were analyzed by HPLC and/or gas chromatography-mass spectrometry (GC-MS) (32).

Chemicals. The synthesis of DD and 2-methyl-4*H*-chromen-4-one, as well as 3-CDF, 2,3-DCDF, and 3,7-DCDF, has been described previously (13, 22, 39). 2-CDF and 4-CDF were prepared from the corresponding aminodibenzofurans by the Sandmeyer reaction (9). 2-Aminodibenzofuran was obtained from 3-acetamidodibenzofuran (4) by nitration with nitronium tetrafluoroborate by the method of Oliver (27) followed by deacetylation, deamination, and reduction by standard procedures. 4-Aminodibenzofuran was prepared by nucleophilic amination of DF with *O*-methylhydroxylamine (18). 4,5-Dichloro-2-methoxybenzoic acid was prepared from 5-amino-4-chloro-2-methoxybenzoic acid (Aldrich GmbH, Steinheim, Germany) by a Sandmeyer reaction. This compound served as the starting material for preparation of the corresponding methyl ester (dimethylmethane), as well as for preparation of 4,5-dichlorosalicylic acid (demethyla-

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tion with boron tribromide). In a manner analogous to that for 2-methyl-4H-chromen-4-one (15), 6-chloro-2-methyl-4H-chromen-4-one was prepared starting from 5-chloro-2-hydroxyacetophenone, which had been synthesized from 4-chloroanisole by Friedel-Crafts acylation with simultaneous cleavage of the methyl aryl ether bond. 4,5-Dichlorocatechol was a gift from Holger Meyer, Institute of Organic Chemistry, University of Hamburg. All other samples of chlorinated DDs and DFs were from AccuStandard Inc., New Haven, Conn., and were supplied in Germany by Restek GmbH, Sulzbach, DF and 2,4,8-TrCDF were from Aldrich GmbH. Our synthetic standards served as authentic reference samples for structural proof of identified metabolites. All other chemicals were of the highest purity commercially available.

RESULTS AND DISCUSSION

Cooxidation of chlorinated dibenzofurans. As can be seen in Fig. 1A, *Sphingomonas* sp. strain RW1 converted 2-CDF and 3-CDF to 5-chloro- and 4-chlorosalicylate, respectively, and salicylate. The two previously described monochlorinated trihydroxybiphenyls (20) could be obtained from 3-CDF in the presence of 3-chlorocatechol (3, 37). HPLC analysis of the reaction mixture supernatant fluid and GC-MS analysis of the acidic ethyl acetate extract also demonstrated the formation of traces of 2-methyl-4H-chromen-4-one from 2-CDF. Assuming that these products were generated by the previously elucidated DF degradation pathway (15), the occurrence of both salicylate and its corresponding chloro derivative constitutes evidence for dioxygenation of both the substituted and the nonsubstituted aromatic nuclei of the parent compound. The nonselective dioxygenation of halogenated diaryl ethers has been reported previously (20, 34, 35, 40) and was reconfirmed by the present findings. In contrast, dioxygenation of structurally related monohalogenated fluoren-9-ones and benzophenones is characterized by high regioselectivity (17).

When 4-CDF and 2,3-DCDF were offered as substrates to resting cells, 3-chlorosalicylate and 4,5-dichlorosalicylate, respectively, were the only products detected by HPLC (Fig. 1A). In the case of 4-CDF, dioxygenation of the substituted aromatic nucleus is hindered by the chloro substituent adjacent to the carbon carrying the oxygen bridge, which explains why salicylate was not detected in the conversion experiment and why 3-chlorosalicylate accumulated quantitatively in the medium. 4,5-Dichlorosalicylate accumulation was also nearly stoichiometric, and the compound was also identified by GC-MS as methyl 4,5-dichloro-2-methoxybenzoate upon diazomethane derivatization of the acidic ethyl acetate extract of the culture broth (Fig. 2A). The lack of dioxygenation of the substituted nucleus of 2,3-DCDF might be due to electronic influences or to steric hindrance by two chloro substituents on the same aromatic nucleus. Alternatively, salicylate might be completely consumed by such cells, thus preventing detection of this metabolite.

Strain RW1 transformed 2,8-DCDF into 6-chloro-2-methyl-4H-chromen-4-one (Fig. 2B), which accumulated in large amounts as the only product. This compound was identified by comparison with a synthetic standard. The corresponding nonchlorinated 2-methyl-4H-chromen-4-one, as shown in Fig. 1A,

FIG. 1. (A) Products formed from 2-CDF (structure 1), 3-CDF (structure 5), 4-CDF (structure 7), 2,3-DCDF (structure 9), and 2,8-DCDF (structure 11) by resting cells of *Sphingomonas* sp. strain RW1: 2, salicylic acid; 3, 2-methyl-4H-chromen-4-one; 4, 5-chlorosalicylic acid; 6, 4-chlorosalicylic acid; 8, 3-chlorosalicylic acid; 10, 4,5-dichlorosalicylic acid; 12, 6-chloro-2-methyl-4H-chromen-4-one. (B) Products from the turnover of 1-CDD (structure 13), 2-CDD (structure 17), and 2,3-DCDD (structure 19): 14, catechol; 15, 3-chlorocatechol; 18, 4-chlorocatechol; 20, 4,5-dichlorocatechol; 16, 2-chloromuconic acid. Attack by the initial dioxygenase is indicated by A onto the halogenated ring and by B onto the nonhalogenated ring (with the exception of no. 11), furnishing the respective products.

FIG. 2. (A) Mass spectrum (70 eV) of 4,5-dichloro-2-methoxybenzoic acid methyl ester. (B) Mass spectrum (70 eV) of 6-chloro-2-methyl-4H-chromen-4-one.

has been previously identified as a by-product of DF degradation by *Sphingomonas* sp. strain HH69 (15). This observation additionally supports the proposed successive side chain shortening of *meta*-cleavage products of substituted 2,3-dihydroxybiphenyls. The proposed pathway would also provide a plausible explanation for the formation of several products of DF degradation (15) and for some observations made on 2-hydroxydibenzofuran degradation by *Sphingomonas* sp. strain HH69 (21). For the formation of the above chromenones, a mechanism involving addition of a phenolic OH group to the double bond of the side chain with concomitant release of HCl and subsequent shortening of the residual side chain also seems feasible.

Incubation of RW1 resting cells with 3,7-DCDF and 2,4,8-TrCDF did not yield any products detectable by HPLC in culture supernatants, nor were any found in the acidic ethyl acetate extracts analyzed by GC. It remains unclear whether this recalcitrance is due to steric hindrance caused by the bulkiness of the chloro substituents or to their electronic effects. As shown in Fig. 3, at least the initial conversion rates for the chlorinated DFs decrease significantly with increasing chloro substitution; they also seem to depend on the position of the chloro substituents. The specific turnover rates (in micromoles per hour per gram of protein) for DF and the chloro derivatives by resting-cell suspensions were as follows: DF, 563; 2-CDF, 348; 3-CDF, 274; 4-CDF, 352; 2,3-DCDF, 165; 2,8-DCDF, 83; 3,7-DCDF, 41; 2,4,8-TrCDF, <5. Heat-inactivated cells transformed neither DF nor DD (controls not shown in Fig. 3 and 4) and, consequently, should not degrade the chloro derivatives. Similar results were obtained by Parsons et al., who tentatively identified by HPLC 5-chlorosalicylate as

a product of 2-CDF oxidation by *Alcaligenes* sp. strain JB1 (30). This organism, however, did not utilize either DF or DD for growth (28).

Cooxidation of chlorinated dibenzo-*p*-dioxins. Resting cells of DF-grown *Sphingomonas* sp. strain RW1 converted 1-CDD and 2-CDD to 3-chloro- and 4-chlorocatechol, respectively, and catechol (Fig. 1B). The GC-MS analysis additionally revealed two chlorinated trihydroxydiphenyl ethers from both substrates and the corresponding dimethyl esters of 2-chloromuconic acid and two chlorinated (2-hydroxyphenyl)muconic acids from 1-CDD transformation (data not shown). These results clearly indicate that dioxygenation of monochlorinated DDs also occurs on the halogen-substituted aromatic nucleus as well as on the nonsubstituted aromatic nucleus. In contrast to DFs, which exhibit only two sites for the initial enzymatic attack, DDs feature four possible sites for the initial dioxygenation because of the presence of two ether linkages in the symmetric molecule. During incubation of 2,3-DCDD, only 4,5-dichlorocatechol and no catechol were detected by HPLC analysis (Fig. 1B). As in the case of 2,3-DCDF, this might be explained by a rapid turnover of intermediary catechol. No products of 2,7-DCDD and 1,2,4-TCDD transformation experiments were detected. Determination of the specific turnover rates (in micromoles per hour per gram of protein) gave the following trend: DD, 280; 1-CDD, 188; 2-CDD, 63; 2,3-DCDD, 38; 2,7-DCDD, 6; 1,2,4-TrCDD, 8.

The accumulation of intermediates of 3-chlorocatechol and 4,5-dichlorocatechol confronts bioconversion of chlorinated DFs and DDs with a problem: 3-chlorocatechol is known to be a potent inhibitor of *meta*-cleaving enzymes of catechol, di- and trihydroxybiphenyls, and trihydroxydiphenyl ether (3, 36,

FIG. 3. Depletion of DF and chlorinated DFs by resting cells of DF-grown *Sphingomonas* sp. strain RW1. Symbols: ●, DF; ■, 2-CDF; ▲, 3-CDF; ◆, 4-CDF; ○, 2,3-DCDF; □, 2,8-DCDF; △, 3,7-DCDF; ◇, 2,4,8-TrCDF.

41), and 4,5-dichlorocatechol has been reported to be a very strong inhibitor of chlorocatechol 1,2-dioxygenase (5, 11), probably because of its iron-chelating property. Therefore, these compounds represent critical recalcitrant intermediates in the aerobic mineralization of 2,3-DCDF, 2,3-DCDD, and possibly also 2,3,7,8-TCDD and 2,3,7,8-TCDF and more highly chlorinated congeners. As a consequence, their further degradation demands special attention. Chlorocatechols in anaerobic environments do not pose such fundamental problems (2).

These results and the turnover experiments with DF-grown

FIG. 4. Depletion of DD and chlorinated DDs by resting cells of DF-grown *Sphingomonas* sp. strain RW1. Symbols: ●, DD; ■, 1-CDD; ▲, 2-CDD; ▼, 2,3-DCDD; ◆, 2,7-DCDD; ●, 1,2,4-TrCDD.

resting cells (Fig. 4) (DD-grown cells exhibited similar activities [data not shown]) clearly demonstrate decreasing turnover rates with increasing chloro substitution, depending on substituent positions, as was found with chlorinated DFs and described for the cooxidation of halogenated diphenyl ethers by the diphenyl ether-mineralizing strain *Sphingomonas* sp. strain SS33 (34). Comparable results were also obtained by Parsons and Storms (31), who found some depletion of 2-CDD, 1,3-DCDD, 2,7-DCDD, 2,8-DCDD, and 1,2,4-TrCDD by the biphenyl-degrading *Alcaligenes* sp. strain JB1 in batch and chemostat cultures fed with benzoate and 3-methylbenzoate, respectively.

Work is in progress to extend the capacity of the lower catabolic pathway by recruitment of type II enzymes (33), such as the chlorocatechol 1,2-dioxygenase and the three subsequent enzymes responsible for the turnover of halogenated substrates, from halocatechol-degrading strains to produce hybrid strains, which should mineralize more highly halogenated diaryl ethers. Additionally, extension of the substrate tolerance of the initial dioxygenase of diaryl ether-oxidizing bacteria by site-directed mutagenesis, as has recently been demonstrated for the degradation of polychlorinated biphenyls (12), may be a useful approach for reduction of recalcitrance of this class of compounds.

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