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Biodegradation and Transformation of 4,4′- and 2,4-Dihalodiphenyl Ethers by *Sphingomonas* sp. Strain SS33

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The bacterium *Sphingomonas* sp. strain SS33, obtained from parent diphenyl ether-mineralizing strain SS3 (S. Schmidt, R.-M. Wittich, D. Erdmann, H. Wilkes, W. Francke, and P. Fortnagel, Appl. Environ. Microbiol. 58:2744–2750, 1992) after several weeks of adaptation on 4,4′-difluorodiphenyl ether as the new target compound, also utilized 4,4′-dichlorodiphenyl ether for growth. Intermediary halocatechols were also mineralized via the *ortho* pathway by type I enzymes. 4,4′-Dibromodiphenyl ether was not used as a carbon source although transformation by resting cells yielded mononuclear haloaromatic compounds, such as 4-bromophenol and 4-bromocatechol. The same was true for the conversion of 2,4-dichlorodiphenyl ether, which yielded the respective (halo-) phenols and (halo-) catechols.

In recent years there has been interest in using polyhalogenated diphenyl ethers as chemicals to replace the polyhalogenated biphenyls formerly utilized as flame retardants. 4,4′-Dibromodiphenyl ether (44DBDE) is the lowest halogenated commercial congener belonging to this class of compounds of environmental concern. Hydroxy and/or nitro derivatives of diphenyl ethers have also been produced in bulk and have been used as pesticides in agriculture. During the last few decades these compounds have been detected in environmental samples and have entered the food chain (4, 8). They are subject to microbial and mammalian catabolism depending on their structure (9, 10). In this paper we describe the extension of the catabolic potential of *Sphingomonas* sp. strain SS33 to dihalogenated diphenyl ethers.

The growth conditions used, the methods used for preparation of resting cells and extracts, oxygen uptake measurements, enzyme assays, and analytical procedures, and the chemicals used have been described previously (5, 7). 2,4-Dichloro-4′-nitrodiphenyl ether (NITROFEN) was obtained from Wako Chemicals GmbH, Neuss, Germany. 4,4′-Difluorodiphenyl ether (44DFDE) was obtained from Alfa Products and was supplied by Johnson Matthey, Karlsruhe, Germany. 44DBDE was obtained from Aldrich Chemie, Steinheim, Germany. A crude preparation of 2,4-dichlorodiphenyl ether was kindly supplied by K. Figge, Natec Institute, Hamburg, Germany, and was purified by preparative high-performance liquid chromatography (HPLC). 4,4′-Dichlorodiphenyl ether (44DCDE) was prepared from 4,4′-diaminodiphenyl ether (Fluka GmbH, Neu-Ulm, Germany) by chlorinating its diazonium salt, using the Sandmeyer reaction (2).

We were not successful in our attempts to screen directly for potent bacterial strains capable of utilizing dihalogenated diphenyl ethers for growth. However, we succeeded in adapting *Sphingomonas* sp. strain SS3, an organism that mineralizes diphenyl ether and several 4-halodiphenyl ethers, to the utilization or transformation of some dihalogenated diphenyl ethers; *Sphingomonas* sp. strain SS3 utilized diphenyl ether, 4-fluorodiphenyl ether, and (to a considerably lesser extent) 4-chlorodiphenyl ether for growth (7) but did not grow at the expense of dihalogenated congeners. An adaptation period of several weeks was needed to achieve growth with 44DFDE; the doubling times of our adapted strain, designated SS33, were about 19 h (Fig. 1) in the systems that we used for growing the organisms, as described previously (5, 7). During growth with 44DCDE the doubling times were considerably greater (more than 40 h), and 44DBDE was not utilized at all. The levels of halide ions released from 44DFDE and 44DCDE when endpoint determinations were made were 91 and 93%, respectively; no bromide ions were detected after conversion of 44DBDE. The pesticides NITROFEN (2,4-dichloro-4′-nitrodiphenyl ether) and TRICLOSAN (2,4,4′-trichloro-2-hydroxydiphenyl ether) were not transformed by resting cells.

The addition of an excess of substrate to cell suspensions maintained in Erlenmeyer flasks equipped with baffle plates led to the accumulation of metabolites in the medium. 4-Fluorophenol and 4-fluorocatechol were produced from 44DFDE, whereas the conversion of 44DCDE yielded the respective chloro derivatives, which were identified by HPLC analysis by using authentic reference samples as described previously (7). Although 44DBDE was not utilized for growth, it was accessible to dioxygenolytic attack by the first enzyme, as shown by the oxygen uptake rates of resting cells pregrown with 44DFDE (Table 1) and the detection and identification of catabolites; the respective bromo derivatives were found as described above. Similar observations were made during the conversion of 2,4-dichlorodiphenyl ether. In this case phenol, 2,4-dichlorophenol, catechol, and 3,5-dichlorocatechol were identified. A scheme for the turnover of dihalogenated and possibly higher halogenated diphenyl ethers is proposed in Fig. 2.

The results described above, together with those obtained from oxygen uptake experiments (Table 1), clearly indicate that the conversion of dihalogenated diphenyl ethers strongly depends on steric effects, such as the bulkiness of the halo substituents, rather than the degree of substitution by halogens. Further conversion seemed to be restricted by

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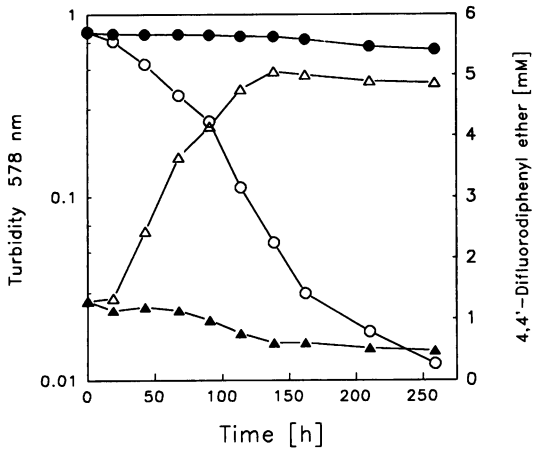

FIG. 1. Growth of *Sphingomonas* sp. strain SS33 with 44DFDE as the carbon source. Experiments were performed in parallel sets of closed Erlenmeyer flasks containing 44DFDE dispersed in mineral salts medium. The flasks were inoculated from a just outgrown preculture (inoculum, about 1%). Batches were worked up after the times indicated. The concentrations of 44DFDE were determined by HPLC. Growth of the culture (△) and consumption of 44DFDE (○) are plotted against time together with the results for controls grown in the absence of 44DFDE (▲) and for experiments performed with heat-inactivated inocula (●).

the limited substrate range of phenol hydroxylase and catechol 1,2-dioxygenase, which in this study exhibited substantially the same activities as they did in parent strain SS3 (7) and therefore are not described here, and probably by the subsequent enzymes. Some explanations for the restricted biodegradation of halocatechols have been given previously by Dorn and Knackmuss (3).

Work is in progress to extend the substrate range (i.e., the specificity of the initial dioxygenase having an apparently rather low regio-selectivity [11] and representing the first bottleneck in the pathway for the turnover and utilization of the polyhalogenated substrates) by adaptation experiments under selective pressure in the presence of our target compounds and to achieve short generation times of the organism. Furthermore, we will produce hybrid strains that harbor the genes for production of the type II enzymes which are responsible for the turnover of halogenated substrates, such as a halophenol hydroxylase and the chlorocatechol 1,2-dioxygenase, and the subsequent enzymes contained in halophenol- and halocatechol-degrading strains (6) or in the 3,4,6-trichlorocatechol-mineralizing organism *Pseudomonas* sp. strain PS12/14 (5) in order to circumvent this bottleneck in the lower diphenyl ether pathway. Hybrid strains should offer a nice opportunity to mineralize higher halogenated diphenyl ethers without formation of critical intermediates and therefore should help avoid those problems that occur during the nonproductive co-oxidation of the structurally analogous polyhalogenated biphenyls (1).

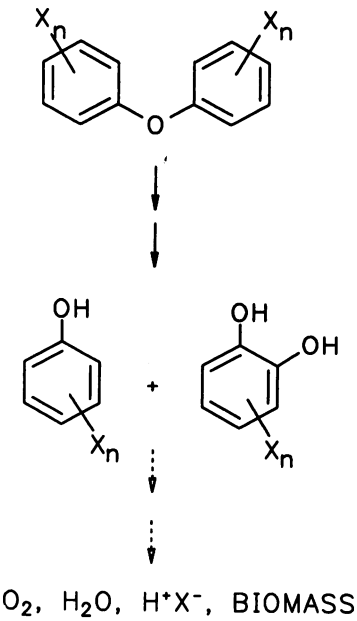

FIG. 2. Proposed pathway for the degradation and/or transformation of dihalogenated diphenyl ethers by *Sphingomonas* sp. strain SS33. Abbreviations: x, F, Cl, or Br; n, 0, 1, or 2.

TABLE 1. Relative oxygen uptake rates with halogenated diphenyl ethers by resting cells of *Sphingomonas* sp. strain SS33 pregrown with 44DFDE[a]

Assay substrate	Relative activity (%)
Diphenyl ether	82
4-Fluorodiphenyl ether	123
4-Chlorodiphenyl ether	58
4-Bromodiphenyl ether	41
44DFDE	100
44DCDE	35
44DBDE	15
2,4-Dichlorodiphenyl ether	18

[a] Results are the means of at least three independently performed experiments. The specific rate for the oxidation of 44DFDE was 127 nmol of O_2 per min per mg of protein.

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