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Mineralization of 4-Chlorodibenzofuran by a Consortium Consisting of *Sphingomonas* sp. Strain RW1 and *Burkholderia* sp. Strain JWS

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The dibenzofuran-degrading bacterium *Sphingomonas* sp. strain RW1 (R.-M. Wittich, H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel, Appl. Environ. Microbiol. 58:1005–1010, 1992) attacks 4-chlorodibenzofuran on the unsubstituted aromatic ring via distal dioxygenation adjacent to the ether bridge to produce 3′-chloro-2,2′,3-trihydroxybiphenyl, which was identified by nuclear magnetic resonance spectroscopy and mass spectrometry. The compound is subsequently *meta* cleaved, and the respective intermediate is hydrolyzed to form a C-5 moiety, which is further degraded to Krebs cycle intermediates and to 3-chlorosalicylate. This dead-end product is released into the culture medium. A coculture of strain RW1 and the 3,5-dichlorosalicylate-degrading strain *Burkholderia* sp. strain JWS (A. Schindowski, R.-M. Wittich, and P. Fortnagel, FEMS Microbiol. Lett. 84:63–70, 1991) is able to completely degrade 4-chlorodibenzofuran with concomitant release of Cl^- and formation of biomass.

The polyhalogenated dibenzo-*p*-dioxins (DDs) and dibenzofurans (DFs) generated by combustion processes and as unwanted by-products of the chemical syntheses of several classes of haloaromatics have become widely distributed in the environment and are of public concern because of their high toxicity to animals and humans (1, 10, 18). Although a number of effective physical and chemical technologies to destroy such pollutants have been developed, such expensive and harsh methods are not suitable for many typical contaminant situations in which the pollutant is present at low concentrations and distributed throughout large volumes of soil which should not also be degraded in the treatment. Microbiological treatment processes which are neither harsh nor destructive to sensitive components of the natural environment are thus of considerable interest for the detoxification and degradation of these compounds.

Several laboratory studies of anaerobic cultures and methanogenic consortia have demonstrated their ability to use halogenated compounds such as polychlorinated biphenyls and other haloaromatics as well as haloaliphatics as electron acceptors with concomitant dehalogenation (9, 11). This type of anaerobic respiration leads to less-halogenated compounds and even to the nonhalogenated structures (4), which, however, tend to persist unless they can enter aerobic biological compartments of the ecosystem, where they may be further metabolized by aerobic pathways.

During our investigations of the aerobic degradation of DD, DF, and the structurally related diphenyl ether, we isolated exclusively sphingomonads (6, 16, 21) by standard enrichment procedures, whereas other groups identified isolates with catabolic potentials similar to those of a *Brevibacterium* sp. and a *Staphylococcus* sp. (12, 17), which suggests that a wide spectrum of bacteria harbor the genetic potential for diaryl ether degradation. Recently, we demonstrated that our strain, *Sphingomonas* sp. strain RW1, is capable of co-oxidizing numerous

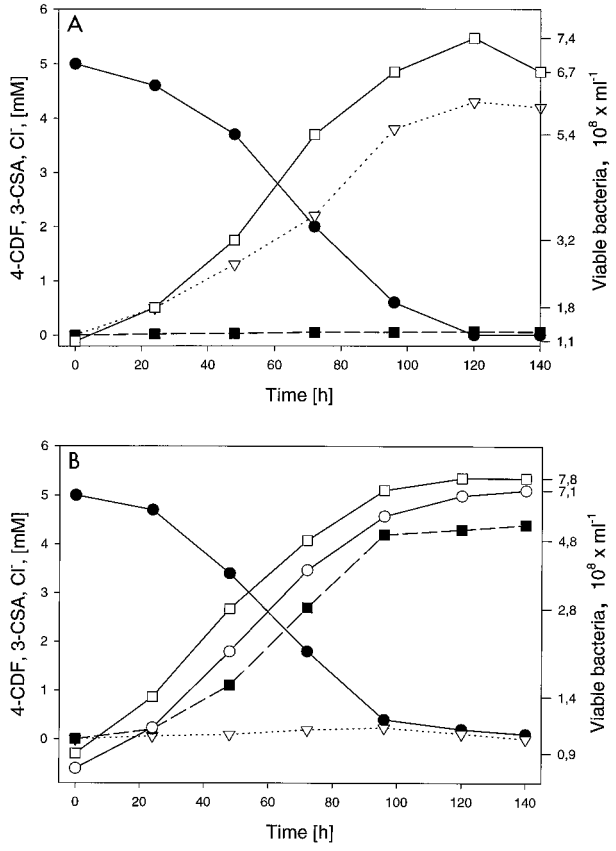

FIG. 1. (A) Utilization of 4-CDF by *Sphingomonas* sp. strain RW1 (□). Parallel sets of Erlenmeyer flasks supplemented with solid 4-CDF (●), corresponding to a 5 mM concentration as the only carbon source, were inoculated with RW1 cells from an active preculture. At the indicated time points, flasks were removed and viable cells were enumerated by being plated on Luria broth and mineral salts agar supplemented with DF or with 3-chlorosalicylate (3-CSA). Substrate depletion and product formation (3-chlorosalicylate [△] and chloride [■]) were determined as described in Materials and Methods. (B) The same experiment was carried out with a consortium consisting of strain RW1 and Burkholderia sp. strain JWS (○).

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TABLE 1. Relative oxygen uptake rates by resting cells of *Sphingomonas* sp. strain RW1 after growth with DF

Assay substrate	Relative oxygen uptake rate[a]
DF	100
4-CDF	83
2,3-Dihydroxybiphenyl	385
2,2′,3-Trihydroxybiphenyl	182
3′-Chloro-2,2′,3-trihydroxybiphenyl	129
Salicylate	56
3-Chlorosalicylate	3
4-Chlorosalicylate	7
5-Chlorosalicylate	5
3,5-Dichlorosalicylate	1
Catechol	142
3-Chlorocatechol	12[b]
4-Chlorocatechol	38[b]

[a] The specific oxidation rate for DF was 311 nmol of O_2 per min per mg of protein. The data represent means of two independently performed experiments. Rates are corrected for endogenous respiration.

[b] Initial rate is given (see text).

low-chlorinated DDs and DFs to chlorocatechols and chlorosalicylates (20). Neither strain RW1 nor several other DF degraders tested in our laboratory express or even possess genes coding for a chlorocatechol pathway needed for the complete mineralization of these intermediates. Here, we demonstrate the mineralization of 4-chlorodibenzofuran (4-CDF) by a coculture of our DF-degrading strain RW1 and a chlorosalicylate-degrading bacterium, *Burkholderia* sp. strain JWS (13, 15).

MATERIALS AND METHODS

Strains and growth conditions. *Sphingomonas* sp. strain RW1 (DSM 6014) (21) and *Burkholderia* sp. strain JWS (DSM 6431; reclassified by 16S ribosomal DNA sequencing) (13) were used. Cells were grown in mineral salts medium (6) at 30°C in Erlenmeyer flasks or culture tubes on an overhead rotating shaker at 80 to 100 rpm to allow permanent contact of the culture with fine mortar-ground substrate crystals. This is in principle not possible on a rotary shaker because of the tendency of the solid substrate to concentrate in an aggregation of biomass and crystals above the liquid phase. Growth experiments were performed in two parallel sets in Teflon-sealed 100-ml Erlenmeyer flasks filled with 20 ml of the mineral salts medium. At the time points given in the figures, one set was worked up for determinations of growth parameters. Determination of residual substrate was carried out as described earlier (16). Active biomass was estimated as CFU by plating of appropriate dilutions on Luria broth plates and on solid mineral salts medium with DF and 5-chlorosalicylate as the selective substrate. Water-soluble metabolites were quantitated by high-performance liquid chromatography (HPLC) analysis.

Determination of enzyme activities, oxygen uptake rates, and chloride release. Enzyme activity of trihydroxybiphenyl 1,2-dioxygenase (7) was assayed with a DU-70 (Fullerton, Calif.) Beckman spectrophotometer. The same molar extinction coefficient was also used for quantification of the product formed from 3′-chloro-2,2′,3-trihydroxybiphenyl and for hydrolysis by HHOPDA hydrolase (3), assuming that the chlorine bound to the aromatic ring would scarcely interfere with the yellow chromophore of the side chain. Preparation of cell extracts was performed as described earlier (6). Oxygen uptake rates and protein estimations were performed as described previously (6). Chloride ions in the culture medium were determined with a chloride-sensitive flow injection electrode developed by M. Otto from the Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik, Stuttgart, Germany (specific information is available from the corresponding author upon request). Because the linear response of the electrode lies in the range of 1 to 250 μM, samples were appropriately diluted with 50 mM acetate running buffer at pH 5.0. The Gilson 231XL system supplied by Abimed Analysentechnik GmbH, Langenfeld, Germany, was used for autoinjection.

Production, isolation, and identification of metabolites. Resting cells of DF-grown strain RW1 (optical density at 600 nm of 2.0) were used for the production of the chlorotrihydroxybiphenyl from 4-CDF in the presence of 1 mM 3-chlorocatechol (initial concentration). The formation of a single product was monitored by analytical HPLC as previously described (21). When the product reached a maximal concentration in the medium, cells and solid residual substrate were removed by centrifugation, and the supernatant fluid was then extracted with ethyl acetate. After evaporation of the organic solvent, the residue was dissolved in methanol and subjected to preparative HPLC on a column (250 by 16 mm) filled with LiChrosorb ODS5 from Merck AG, Darmstadt, Germany, with methanol-water (30:70 [vol/vol]) as the solvent. The eluted fractions were tested by analytical HPLC, pooled, and evaporated to dryness. For elucidation of the structure of the purified metabolite, gas chromatography-mass spectrometry analysis was performed after derivatization with trimethylsulfonium hydroxide (Machery-Nagel GmbH & Co. KG, Düren, Germany) with a Shimadzu GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan) on a 30-m XTI-5 fused silica column (Restek GmbH, Bad Soden, Germany) with helium as the carrier gas. The Shimadzu QP-5000 quadrupole mass spectrometer was operated in the electron impact mode at 70 eV. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of a purified metabolite were recorded on a Bruker CXP 300 (Bruker GmbH, Karlsruhe, Germany) with tetramethylsilane as the internal standard. *d*-Dimethyl sulfoxide (*d*-DMSO) was used as the solvent.

Chemicals. 4-CDF was prepared by application of the Sandmeyer reaction from 4-amino-DF. This compound was synthesized from DF via metalization and subsequent reaction with *O*-methylhydroxylamine (20). 2,2′,3-Trihydroxybiphenyl and 3-chlorocatechol were prepared as previously described (7, 14). All other chemicals were of the highest purity commercially available.

RESULTS AND DISCUSSION

Growth of pure strain and consortium with 4-CDF. *Sphingomonas* sp. strain RW1 grows at the expense of 4-CDF in mineral salts medium and releases almost stoichiometrical

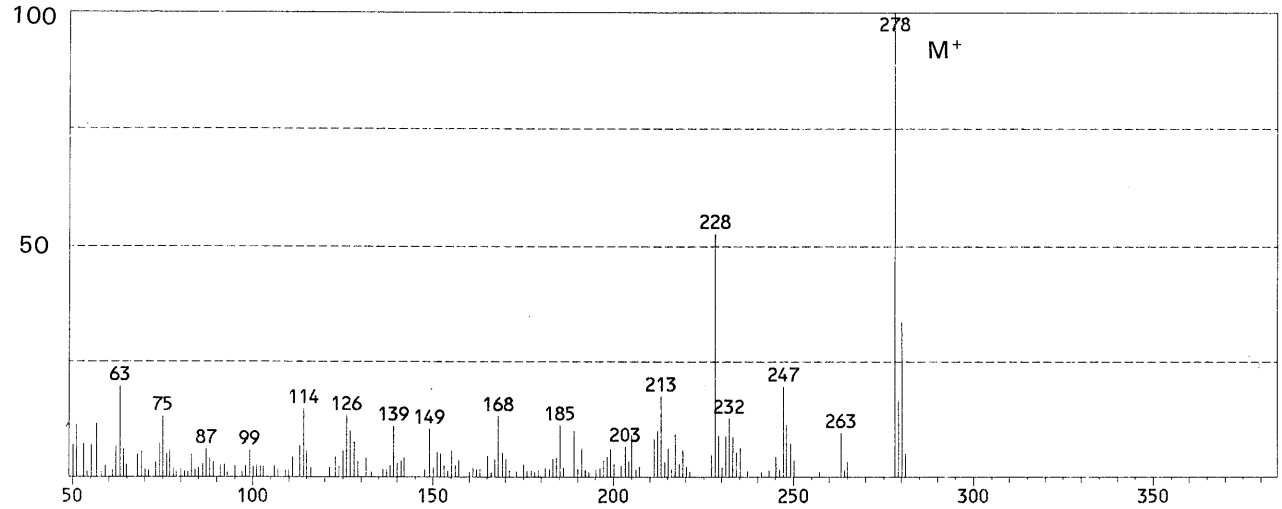

FIG. 2. Mass spectrum (70 eV) of trimethylsulfonium hydroxide-derivatized 3′-chloro-2,2′,3-trihydroxybiphenyl.

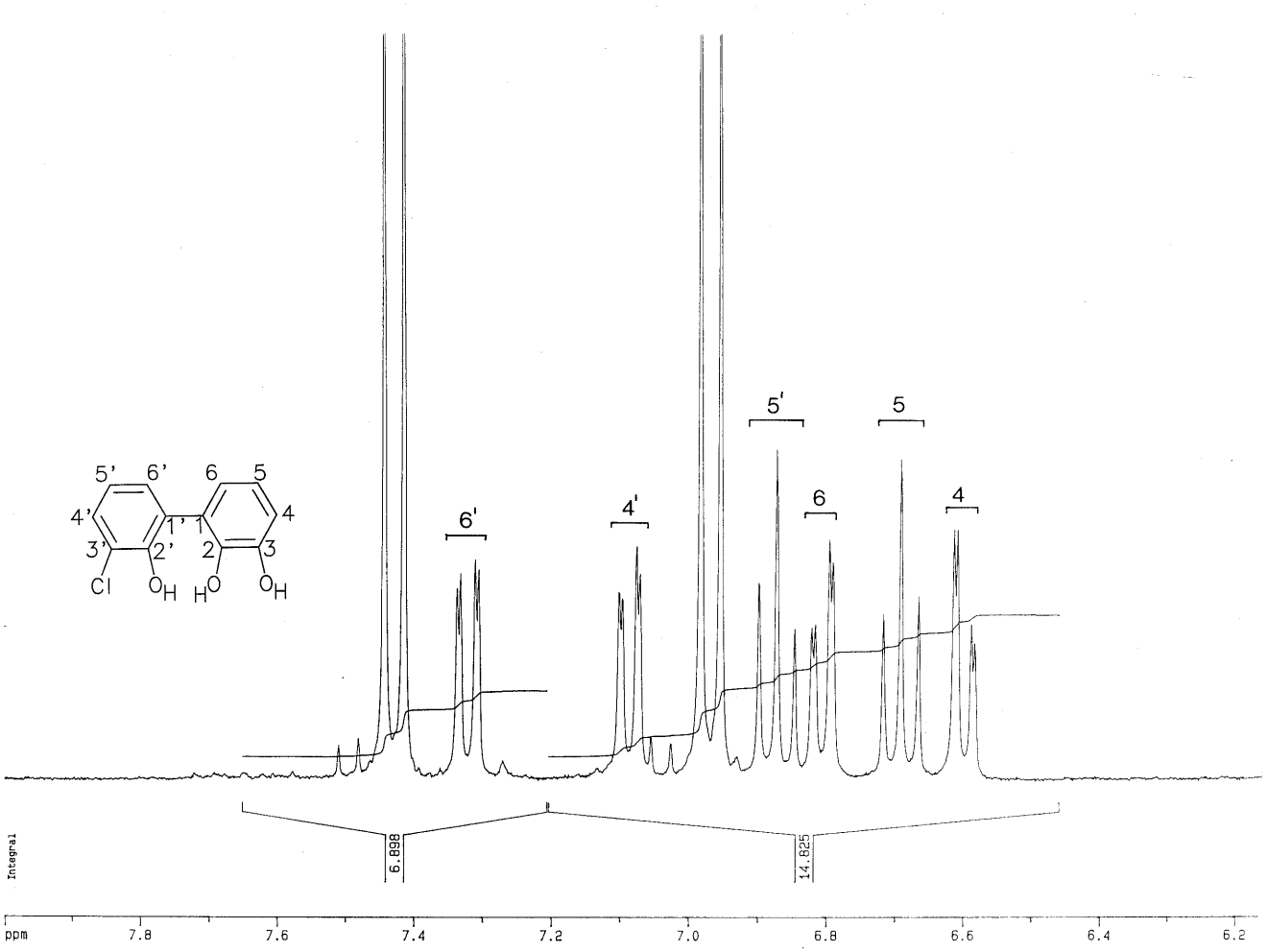

FIG. 3. ^{1}H NMR of 3′-chloro-2,2′,3-trihydroxybiphenyl in *d*-DMSO.

amounts of 3-chlorosalicylate into the culture medium (Fig. 1A). This observation indicates attack exclusively of the nonhalogenated aromatic nucleus of 4-CDF and suggests that the initial dioxygenase is not capable of oxygenolytic dehalogenation, a property exhibited by a few initial dioxygenases (5). Figure 1B demonstrates that a coculture consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS completely mineralizes 4-CDF. The latter organism, which possesses a chlorocatechol pathway not present in strain RW1, is capable of growth on 3-chloro-, 5-chloro-, and 3,5-dichlorosalicylate and therefore scavenges the 3-chlorosalicylate excreted by strain RW1. Chloride release from 4-CDF is almost stoichiometric, and the levels of growth of both members of the coculture were similar.

Determinations of enzyme activities and oxygen uptake rates. From the measurements of specific oxygen uptake rates shown in Table 1, it is clear that the initial attack of 4-CDF by the dioxygenase (2, 19) is only marginally less efficient than that with unsubstituted DF and that the first metabolite, 3′-chloro-2,2′,3-trihydroxybiphenyl, is oxidized at relatively high rate. This conclusion was supported by spectrophotometric assays of the *meta* cleavage enzyme 2,2′,3-trihydroxybiphenyl dioxygenase and the hydrolase, which carries out the next reaction in the pathway: crude cell extracts of DF-grown cells exhibited specific activities of 2.3 and 1.3 μmol/min/mg of protein with 2,3-dihydroxybiphenyl and the chlorinated trihydroxybiphenyl, respectively, and of 0.20 and 0.13 μmol/min/mg of protein, respectively, for hydrolysis of the ring cleavage products (data represent means of two independently performed experiments). The resulting 3-chlorosalicylate, in contrast to salicylate, was oxidized at a negligible rate; a chlorocatechol pathway is absent (21). Initial rates for the oxidation of 3-chloro- and 4-chlorocatechol were stable for no longer than 1.0 to 1.6 min; thereafter, they decreased significantly. The potential of 3-chlorocatechol as a potent inhibitor of *meta*-cleaving enzymes has been used previously for preparative production of 2,2′,3-trihydroxydiphenyl ether from DD (21) and was used as described below for production of the respective chlorotrihydroxybiphenyl.

Isolation and identification of metabolites produced from 4-CDF. The central metabolites of DF and 3-CDF degradation have been previously identified, and the pathways have been proposed (6, 8). An analogous pathway for 4-CDF degradation can be assumed and confirmed by the identification of pathway intermediates. The chlorinated trihydroxybiphenyl was formed from 4-CDF with high yield (>80%) by resting cells within a 6-h incubation period when the subsequently acting *meta*-cleaving dioxygenase was effectively inhibited by 3-chlorocatechol. Only very small amounts of 3-chlorosalicylate were detectable with HPLC after this biotransformation. 3′-Chloro-

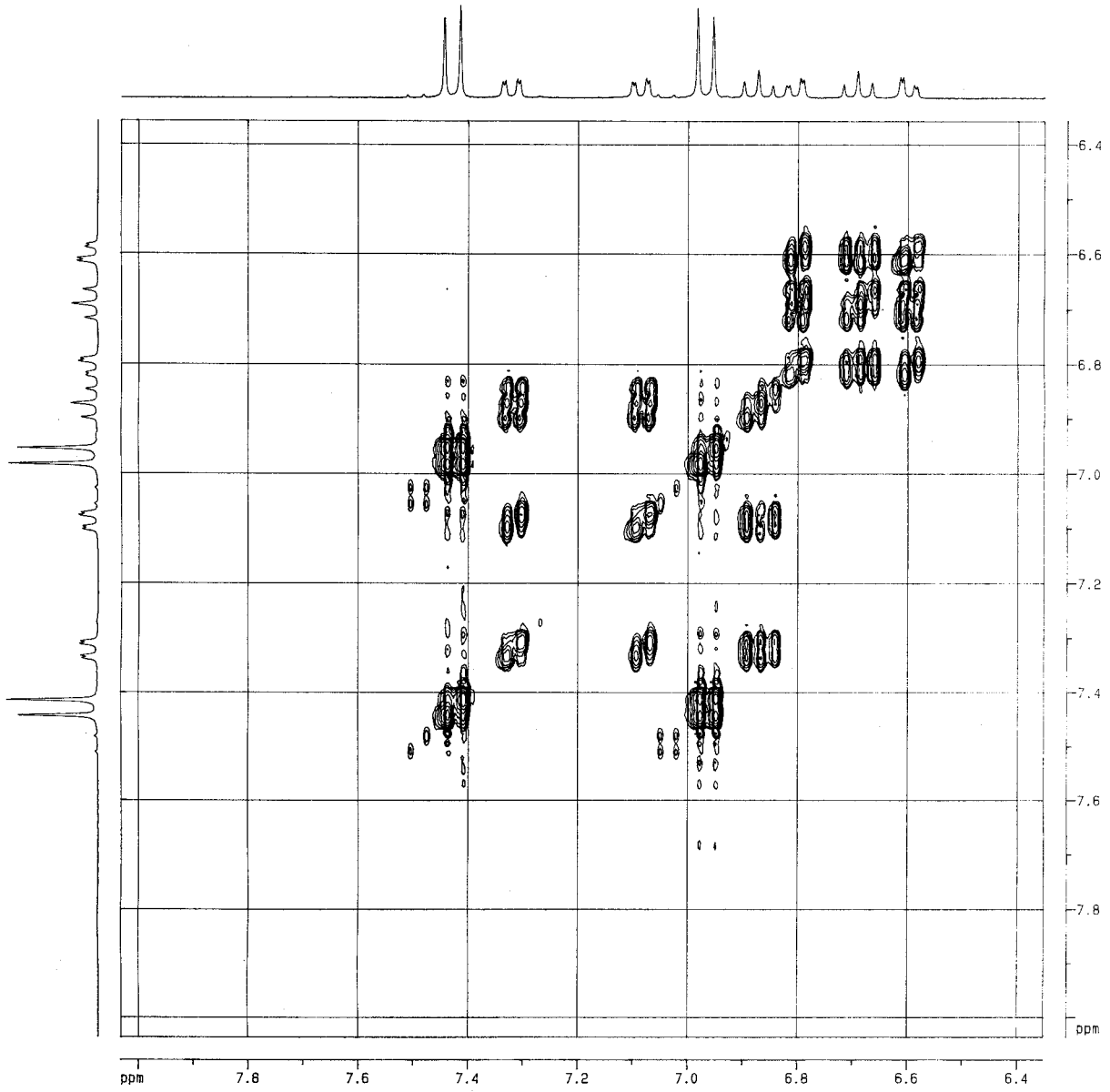

FIG. 4. H,H-COSY contour plot of 3′-chloro-2,2′,3-trihydroxybiphenyl.

2,2′,3-trihydroxybiphenyl, the structure of the major product, was confirmed by mass spectrometry (Fig. 2), and ^{1}H (Fig. 3 and 4) as well as ^{13}C NMR spectroscopy, and its properties are presented here for the first time. In the mass spectrum of 3′-chloro-2,2′,3-trimethoxybiphenyl, the molecular ion is found at m/z = 278.5 (base peak). Data obtained from ^{1}H NMR were as follows (chemical shifts and coupling constants; proton assignment is based on increment calculations as well as on an H,H-correlated spectroscopy [H,H-COSY] three-dimensional contour plot): δ = 6.59 (H-4, $J_{4,5}$ = 8.7 Hz, $J_{4,6}$ = 1.89 Hz), 6.69 (H-5, $J_{5,6}$ = 8.47 Hz), 6.81 (H-6), 6.87 (H-5′, J = 8.42 Hz), 7.08 (H-4′, $J_{4'5'}$ = 7.8 Hz, $J_{4'6'}$ = 1.6 Hz) and 7.26 (H-6′) ppm; the strong signals at δ = 6.97 and 7.43 (J = 8.2 Hz) ppm resulted from coupling of aromatic OH protons with vicinal C-H protons, a common feature in *d*-DMSO due to hydrogen bonding. Recording of spectra in $CDCl_3$ or in CD_3OD-C_6D_6 (100:10 to 15% [vol/vol]) did not allow resolution of, specifically, the a,b,c pattern of the dihydroxylated aromatic ring system. In the H,H-COSY correlation shown in Fig. 4, two independent spin systems of three protons each are depicted; one of them is blotted over by interactions of the above-mentioned OH coupling with H-4′ and H-6′, respectively. The signals of the 5′ proton are easily assigned to the a,c pattern of the chlorinated ring. The three OH protons visible in the ^{1}H NMR spectrum recorded in *d*-DMSO signaled at δ = 8.7 ppm (2,2′-) and at δ = 9.45 ppm (3-), respectively; they are not demonstrated in Fig. 3 and 4. ^{13}C NMR data (recorded at 75.47 MHz; solvent and internal standard as described above) are as follows: δ = 115.8 (C-4), 121.6 (C-5′ or C-3′), 121.8 (C-3′ or C-5′), 123.0 (C-5), 123.8 (C-4′), 127.4 (C-1′), 129.6 (C-6′), 130.4 (C-1), 131.8 (C-6), 143.7 (C-2), 147.3 (C-3), and 152.2 (C-2′) ppm. 3′-Chloro-2,2′,3-trihydroxybiphenyl represents a main component of the pathway; we observed that the compound is used as a carbon source by strain RW1.

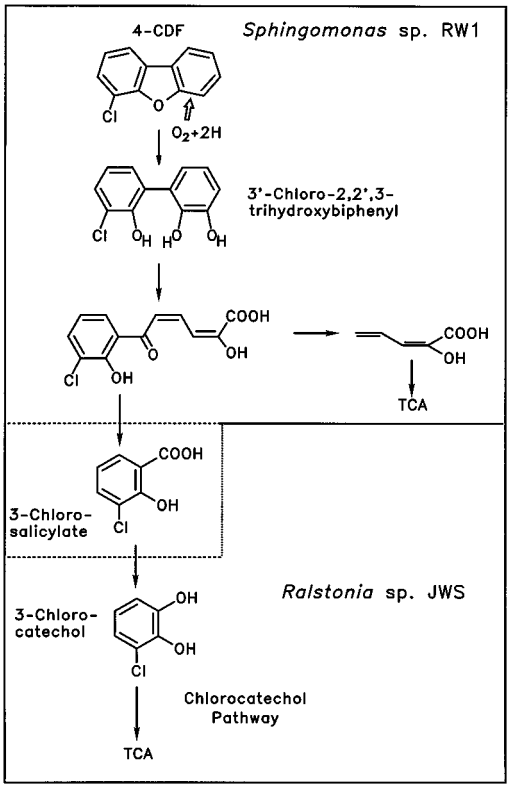

FIG. 5. Proposed pathway for coculture mineralization of 4-CDF by *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. TCA, tricarboxylic acid cycle.

Neither strain RW1 nor the consortium is capable of mineralizing or, at least, partly degrading 3-CDF, 2-CDF, or 1-CDF, utilizing the C-5 moiety for growth as described here for 4-CDF. These three isomers can be broken down by co-oxidation only (8, 20), and the reason for their unproductive breakdown probably is found in the attack of both aromatic rings, leading to metabolic misrouting. A chlorinated C-5 moiety has to be assumed, the metabolic fate of which, however, has not been clear up to now.

Figure 5 summarizes a pathway for the mineralization of 4-CDF by the two-species consortium. Work is currently in progress to combine both parts of the degradative pathway in a single organism to achieve improved performance for field application.

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