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Interaction of *Sphingomonas* and *Pseudomonas* strains in the degradation of chlorinated dibenzofurans

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We have studied the concerted degradation of two monochlorodibenzofurans by a bacterial consortium, consisting of the chlorodibenzofurans-cometabolizing and chlorosalicylates-excreting strain *Sphingomonas* sp RW16, and *Pseudomonas* sp RW10, which mineralized the released chlorosalicylates. Neither of the organisms was able to grow with chlorodibenzofurans alone. Degradation of 2-chloro- and 3-chlorodibenzofuran proceeded to the end products 5-chloro- and 4-chlorosalicylate, respectively, when the initial dioxygenase of *Sphingomonas* sp RW 16 attacked the unchlorinated aromatic ring of the heterocyclic dibenzofuran molecule. 2-Hydroxypenta-2,4-dienoate, formed upon *meta*-cleavage of the intermediary chlorotrihydroxybiphenyls, served as a growth substrate for the sphingomonad. Presumably, most of the chlorosalicylates were excreted and degraded further by *Pseudomonas* sp RW10. Mineralization of both chlorosalicylates proceeded through a converging pathway, via 4-chlorocatechol, and protoanemonin. Chlorosalicylates were mineralized by the pseudomonad only when their concentration in the culture medium was below 1.5 mM. In the case of initial dioxygenation taking place on the chlorinated aromatic ring, salicylate and chlorinated hydroxypentadienoates should be formed. The metabolic fate of putative chlorohydroxypentadienoates is not clear; ie, they may be channeled into unproductive catabolism and, thus, represent the critical point in the breakdown of the carbon of these two chlorodibenzofurans by *Sphingomonas* sp RW16.

Keywords: biodegradation; 2-chlorodibenzofuran; 3-chlorodibenzofuran; *Pseudomonas*; *Sphingomonas*; protoanemonin pathway

Introduction

Highly toxic polyhalogenated dibenzo-*p*-dioxins and dibenzofurans, generated by combustion processes and as unwanted by-products of certain chemical syntheses of haloaromatics, have become widely distributed in the environment [2,9]. Although several other physico-chemical processes apart from high-temperature incineration have been developed for their elimination from environmental compartments [10,36], these methodologies are not economically suitable for treatment of contaminant situations where the pollutants are present at low concentrations. Microbiological treatment processes which are benign towards the environment are more appropriate for detoxification and degradation of pesticides, (poly-)halogenated dioxins, dibenzofurans, biphenyls, phenols, benzenes, and other compounds of environmental concern.

During recent decades, public and scientific interest has focused mostly on environmental contamination by highly toxic 2,3,7,8-chlorinated dibenzo-*p*-dioxin, dibenzofuran and their higher halogenated congeners [2]. Abiotic and anaerobic reductive biodehalogenations during a respiratory process, however, generate lower-halogenated derivatives [1,16,23], which have been detected in ground- and, consequently, in tap water [31]. Such compounds, which are metabolized by higher organisms, exhibit mutagenic activity ie, in rats [15], with 3-chlorodibenzofuran being the most mutagenic in the Ames test [21,22]. Their remediation from the biosphere is, therefore, of interest.

We have recently demonstrated the mineralization of 4-chlorodibenzofuran through 3-chlorosalicylate by a defined consortium, consisting of the dibenzofuran degrader, *Sphingomonas* sp RW1, and the chlorosalicylate degrader, *Burkholderia* sp JWS [4]. The latter organism expresses a chlorocatechol pathway and mineralizes 3-chloro-, 5-chloro- and 3,5-dichlorosalicylate but is unable to degrade 4-chlorosalicylate [29]. The present work demonstrates that two other monohalogenated congeners can be degraded not only through co-oxidation by resting cells of dibenzofuran degraders [14,27] but, through a cometabolic process coupled to mineralization of intermediary chlorosalicylates, via the novel protoanemonin pathway.

Materials and methods

Bacterial strains

Strains RW10, 11 and 16 originated from an enrichment experiment. *Sphingomonas* sp RW16 (DSM 12677) and *Pseudomonas* sp RW10 (DSM 12647) were the bacterial strains used in this study and have been identified by analysis of their 16S rRNA gene sequences, following the methods and conditions described previously for genomic DNA extraction, PCR-amplification and sequencing [17,18,24,25]. *Sphingomonas* sp HH69 (DSM 7135) [12] was used for production of metabolites used as standard compounds.

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General procedures

Cells were grown for production of biomass in mineral salts medium with dibenzofuran (strain RW16) or salicylate and 5-chlorosalicylate (strain RW10), at 30°C, in Erlenmeyer flasks, shaken at 80 rpm on an orbital shaker. For the generation of growth curves, cells were grown in Teflon-sealed, 13-ml culture tubes (1 ml of medium) on an overhead rotating shaker at 100 rpm, to achieve optimal contact of the cells with substrate crystals. Samples were removed from cultures to monitor growth parameters, such as active biomass as colony forming units (CFU). The determination of residual substrate was carried out by HPLC (UV detection) of mineral salts medium upon its five-fold dilution with methanol, and dissolution of crystals therein. Chloride determinations were performed with an FIA electrode, as described earlier [4]. Routine readings of the optical densities of cultures were performed spectrophotometrically at A_{600}. HPLC was also used for the quantification and identification of metabolites [4,6]. Enzyme activities were determined with crude cell extracts, oxygen uptake rates were determined using whole cells and protein estimations were performed as described previously [4,6,7].

Chemicals

2-Chloro- and 3-chlorodibenzofuran were obtained from Dr H Wilkes, Institut für Organische Chemie, Universität Hamburg, and Dr K Figge, NATEC Institut für naturwissenschaftlich-technische Dienste, Hamburg. 2,3-Dihydroxybiphenyl was from Wako Chemicals, Neuss, Germany. 2,2′,3-Trihydroxybiphenyl and 3-chlorocatechol were prepared as described previously [12,13]. *cis*-Acetylacrylic acid was obtained from the *trans* isomer (Lancester Synthesis) by UV radiation [30]. Protoanemonin was produced from 4-chlorocatechol, as described earlier [6], using crude cell extracts of strain RW10 pregrown on salicylate. All other chemicals were of the highest purity commercially available.

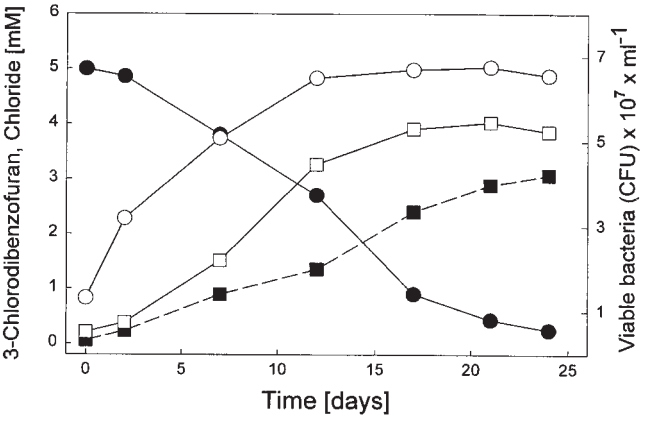

Figure 1 Growth of the consortium. Parallel sets of 13-ml culture tubes supplemented with solid 3-chlorodibenzofuran, which corresponded to a 5-mM concentration, were inoculated with cells from a mid-phase preculture. Viable cells were enumerated by plating on LB plates and mineral salts agar supplemented with dibenzofuran or with 5-chlorosalicylate. Other parameters were monitored as described in the Materials and Methods section. —●— 3-Chlorodibenzofuran; —■— chloride; —□— CFU RWIO; —○— CFU RW16.

Results

A 3-chlorodibenzofuran-degrading consortium was obtained from an enrichment culture inoculated with aerobic upper sediment samples from the River Elbe, downstream of Hamburg harbor, after approximately 4 months of cultivation in mineral salts medium supplemented with the target carbon source. During prolonged subcultivation, the medium turned brownish, after a transient yellow color which lasted for about 2–4 weeks. After several subcultures, a stable culture was obtained from which three strains were isolated and termed RW10, RW11, and RW16.

Growth of the consortium with chlorodibenzofurans

None of the isolated strains, alone, was found to be able to grow with 3-chlorodibenzofuran as the carbon source. Studies of various combinations of the three strains revealed that a co-culture of two strains, RW10 and RW16, was able to form a slow-growing, 3-chlorodibenzofuran-degrading consortium. Re-addition of strain RW11 to the RW10/RW16 pair did not result in significant effects; a previous investigation of the flora of the consortium had shown that the abundance of strain RW11 in the late enrichment culture was always less than 10% of the total CFUs. As can be seen in Figure 1, 3-chlorodibenzofuran was almost completely depleted from the RW10/RW16 co-culture, although the release of chlorine as chloride ion was not quantitative, only ca 60% of the theoretical amount. The co-culture was also able to grow on 2-chlorodibenzofuran and released about 60% of the chlorine. No further experiments were carried out with 2-chlorodibenzofuran as substrate because of the very small amount available. With both substrates, the consortium developed a stable yellow color, indicative of the accumulation of a *meta*-cleavage product. The consortium tolerated high amounts of 3-chlorodibenzofuran, at least 10 g L^{-1}. Strain RW10, alone, grew well at 1.0 and 1.5 mM 4-chloro- or 5-chlorosalicylate in batch culture; above the latter concentration the medium turned brownish and cells died off. Strain RW10 also grew on salicylate at 5 and 10 mM concentrations, but not on dibenzofuran. Dibenzofuran and and salicylate were utilized by strain RW16. Neither chlorosalicylates nor chlorocatechols were used for growth by strain RW16.

Taxonomic assignment of bacterial strains

PCR-amplification and sequencing of the 16S rRNA genes of strains RW10 and RW16 allowed the determination of 1492 and 1428 nucleotide positions, respectively, corresponding to approximately 97% of the complete genes. Cluster analyses demonstrated that the 16S rRNA gene sequence of strain RW10 grouped with those of bacteria of the γ-subclass of Proteobacteria [32] and most closely with the 16S rRNA gene sequences of species of the genus *Pseudomonas* [3,25]. Strain RW16 clustered with those of bacteria of the α-subclass of Proteobacteria and most closely with the 16S rRNA gene sequences of the genus *Sphingomonas* [5,17,18,24,26,33–35,40]. From 16S rRNA gene sequence comparisons with validly published species of the genera *Pseudomonas* and *Sphingomonas*, strain RW10 was most closely related to *P. pavonaceae* (98.8% sequence similarity), *P. agarici* (98.0% sequence

similarity) and *P. putida* (97.9% sequence similarity). Strain RW16 was most closely related to *S. yanoikuyae* (94.9% sequence similarity) and *S. chlorophenolicus* (94.7% sequence similarity). Such levels of 16S rRNA gene sequence similarities (ie, less than 99%) suggest that strains RW10 and RW16 comprise heretofore undescribed species of *Pseudomonas* and *Sphingomonas*, respectively. The sequence of isolate RW11 showed high similarity with *Bradyrhizobium elkanii* (99.5%; van Berkum PB, D Prevost and G Laguerre, USDA, ARS, Soybean and Alfalfa Research Laboratory, Beltsville, MD, USA: Phylogeny of *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and several bradyrhizobial strains originating from the nodules of *Astragalus* species, unpublished) a member of the α-subdivision of the Proteobacteria and was, therefore, assumed to belong to this species.

Identification of metabolites

Turnover experiments with resting cells of dibenzofuran-grown strain RW16 produced non-stoichiometric amounts of 4-chloro- and 5-chlorosalicylate, and traces of salicylate, from the conversion of 3-chloro- and 2-chlorodibenzofuran, respectively. Identification was made from HPLC retention times and *in situ* recorded UV spectra, by comparison with authentic standards. The conversion of 3-chlorodibenzofuran in the presence of 3-chlorocatechol, as an inhibitor of *meta*-cleavage of chlorotrihydroxybiphenyl(s) [4], led to accumulation of two catabolites with a peak ratio of about 1.2 : 1 (HPLC). Their UV spectra and retention times were identical to those obtained from a parallel turnover experiment with *Sphingomonas* sp strain HH69 [14] and, therefore, they are likely to be 4-chloro-2,2′,3-trihydroxybiphenyl and 4′-chloro-2,2′,3-trihydroxybiphenyl, according to our earlier work [14], and analogous to the compound identified from our earlier transformation of 4-chlorodibenzofuran [4]. Protoanemonin was identified by HPLC in the supernatant of strain RW10 when incubated in the presence of 4-chlorosalicylate (5 or 10 mM). The conversion of 4-chlorocatechol (0.1 mM) by a cell-free extract of chlorosalicylate-grown strain RW10 yielded almost stoichiometric amounts of protoanemonin. We could not identify *cis*-acetylacrylic acid as the next potential catabolite when working with whole cells or crude extracts. This compound served as a good carbon source for strain RW10, in contrast to the *trans* isomer, which was not degraded. The doubling time on the *cis* isomer was approximately 2.8 h in mineral salts medium.

Table 1 Relative oxygen uptake rates by resting cells of *Sphingomonas* sp RW16 after growth with dibenzofuran

Assay substrate	Specific oxygen uptake rate
Dibenzofuran	742
2-Chlorodibenzofuran	399
3-Chlorodibenzofuran	457
2,3-Dihydroxybiphenyl	10329
2,2′,3-Trihydroxybiphenyl	8352
Salicylate	666
3-Chlorosalicylate	114
4-Chlorosalicylate	162
5-Chlorosalicylate	114
3,5-Dichlorosalicylate	6
Catechol	228
3-Chlorocatechol	56
4-Chlorocatechol	194

The specific oxidation rates are given in nmoles of O_2 per min per mg of protein. Data represent means of two independent experiments. Rates are corrected for endogenous respiration.

Table 2 Specific activities of degradative enzymes of *Pseudomonas* sp RW10

Enzyme	Substrate	Specific activity after growth with	
		Salicylate	5-Chlorosalicylate
Gentisate dioxygenase	Gentisate	<1	<1
Salicylate hydroxylase	Salicylate	326	nd
	4-Chlorosalicylate	160	nd
	5-Chlorosalicylate	46	nd
	3,5-Dichlorosalicylate	23	nd
Catechol 2,3-dioxygenase	Catechol	<1	<1
Catechol 1,2-dioxygenase	Catechol	403	525
	3-Chlorocatechol	1	2
	4-Chlorocatechol	34	80
Muconate cycloisomerase	*cis*,*cis*-Muconate	251	250
	3-Chloromuconate	180	160
(Diene-) lactone hydrolase	*trans*-Dienelactone	128	250
	cis-Dienelactone	12	20
	Protoanemonin	2	2

Activities of salicylate hydroxylase were assayed with resting cells, using HPLC for determination of substrate consumption rates, and assuming that the uptake of the substrate is not limiting. All other activities were determined spectrophotometrically. Specific activities are given in nmoles of substrate converted per min per mg of protein. nd, Not determined.

Enzyme activities and oxygen uptake rates of strains RW16 and RW10

The oxidative potential of strain RW16 towards dibenzofuran, its monohalogenated derivatives and potential degradation products, such as salicylate, chlorosalicylates and catechols was determined (Table 1). The relative oxidative activities for the chlorinated derivatives of the growth substrate, dibenzofuran, were over 55% and, thus, relatively high. The rates for the oxidation of monochlorinated salicylates were significant and 4-chlorocatechol was oxidized with a rate similar to that observed for the oxidation of catechol. During this oxidation, a yellow color, typical of *meta* cleavage activity, developed and accumulated in the medium. Although 3-chlorocatechol was oxidized, the rate was significant only during the initial seconds and subsequently dropped to zero, which is indicative of the inactivation of many *meta*-cleavage enzymes by this substrate.

Specific enzyme activities of the chlorosalicylate-degrading strain RW10 were measured with whole cells grown on salicylate and 5-chlorosalicylate. As can be seen in Table 2, strain RW10 did not exhibit any detectable gentisate dioxygenase or catechol 2,3-dioxygenase activities. The relative activities of catechol 1,2-dioxygenase for catechol and chlorinated substrates were typical of those featured by a catechol dioxygenase and not of those of a chlorocatechol dioxygenase, with little but significant activity for 4-chloro-

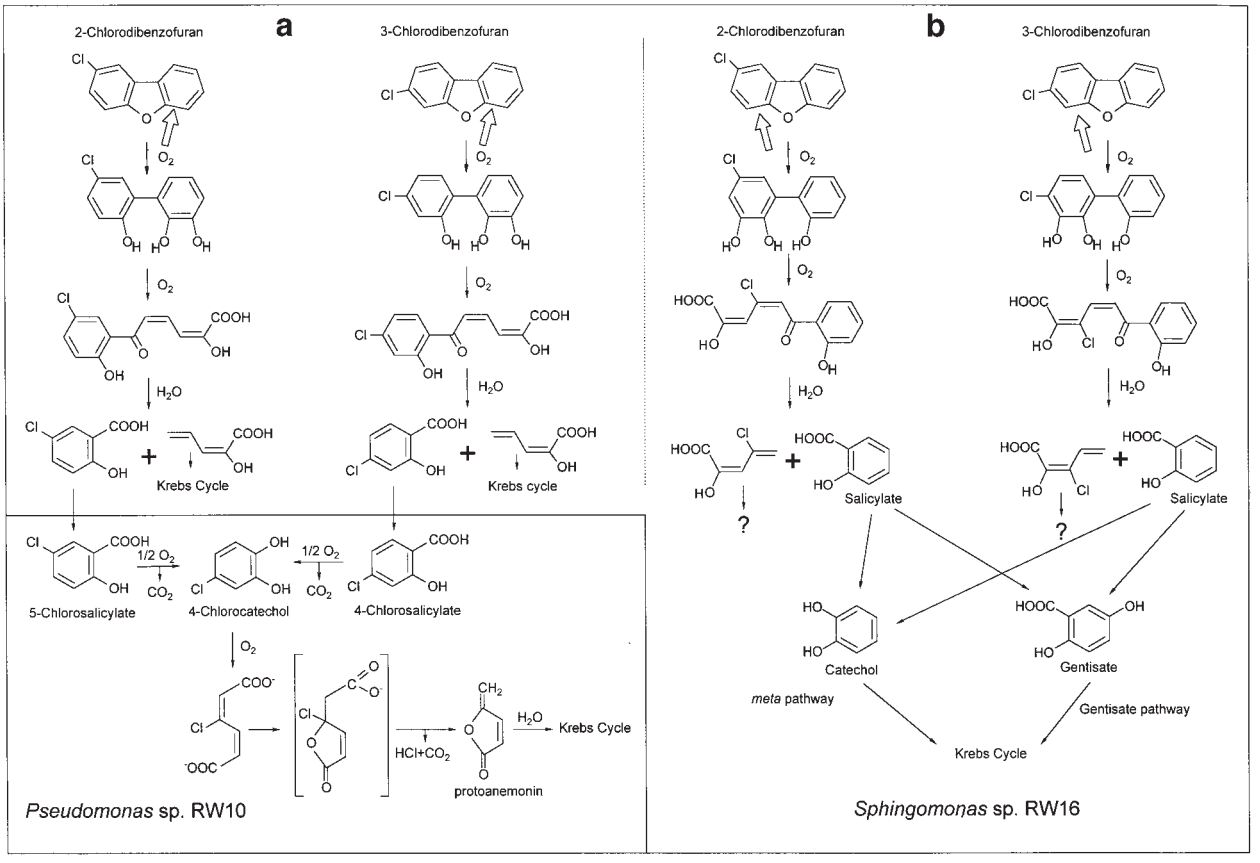

Figure 2 Proposed pathway for the concerted co-culture degradation of 2-chloro- and 3-chlorodibenzofuran by *Sphingomonas* sp RW16 and *Pseudomonas* sp RW10 consortium. The fate of chlorinated hydroxypentadienoates is unknown. Chlorosalicylates excreted by strain RW16 were taken up by strain RW10 and mineralized via 4-chlorocatechol and protoanemonin. The oxidative attack takes place at the unchlorinated ring (a), or at the chlorinated ring (b).

catechol and no detectable activity for 3-chlorocatechol. Muconate cycloisomerase transformed *cis,cis*-muconate and 3-chloro-*cis,cis*-muconate at significant rates. In the dienelactone hydrolase assay, the specific activity for the hydrolytic cleavage of the *trans* isomer was one order of magnitude higher than that for the *cis* isomer and two orders of magnitude higher than that for protoanemonin. The measured enzyme activities are in agreement with results from substrate-utilization experiments, which showed that strain RW10 grows with 4-chloro- and 4-fluorocatechol, and 4-chloro- and 5-chlorosalicylate, but not with 3-fluoro- and 3-chlorocatechol, nor with 3-chlorosalicylate.

Discussion

The fact that the chlorosalicylate-mineralizing member of the consortium, strain RW10, grows on these compounds only at substrate concentrations not higher than 1.0–1.5 mM, may be explained by the observed accumulation of protoanemonin when chlorosalicylates were supplied at high concentrations. The intrinsic toxicity of protoanemonin towards several taxa of bacteria, fungi and yeast has been well established [6,11,19,20]. Consequently, this new type of chlorosalicylate-degrader may have developed in an environmental niche characterized by very low substrate concentration which, indeed, is provided by the dibenzofuran mineralizer, strain RW16, when it slowly converts 3-chlorodibenzofuran to 4-chlorosalicylate as the end product during its growth in the enrichment culture and, later on, in the defined consortium. Recently, we have reported a *Pseudomonas* sp MT1, which also uses a pathway via 4-chlorocatechol, 3-chloromuconate and protoanemonin for degradation of 4-chlorosalicylate and which also had developed under the conditions of a carbon-limited chemostat inoculated with highly polluted river sediment [28].

Metabolism of protoanemonin by bacteria is not very well understood. Brückmann *et al* demonstrated hydrolysis of protoanemonin by the dienelactone hydrolase of *Pseudomonas* sp B13 [8]. It is feasible that the dienelactone hydrolase activity of strain RW10, which we have shown to hydrolyse protoanemonin at very low rates, is responsible for the turnover of this compound in this new chlorosalicylate pathway. Further work is necessary to clarify whether this activity represents a new type of enzyme, since dienelactone hydrolase is typical of a chlorocatechol pathway; the catechol (3-oxoadipate) pathway contains no such enzyme type [6].

Our observation that protoanemonin is not toxic to strain RW10 when growing on 4-chloro- or 5-chlorosalicylate at concentrations below 1.5 mM clearly indicates that the intermediary formation of protoanemonin is not responsible

for the observed depression of growth of the consortium on the two chlorodibenzofurans. Evidence from earlier work implies that 2-chloro- and 3-chlorodibenzofuran are attackable at both aromatic rings [14,38], in contrast to 4-chlorodibenzofuran [4]. Upon attack on the non-halogenated ring of 2-chloro- or 3-chlorodibenzofuran, the corresponding chlorosalicylate and 2-hydroxypentadienoate are formed in all cases, and the latter compound represents the carbon source for strain RW16. The strain is capable of fortuitously oxidizing, to a significant extent, chlorinated salicylates and catechols. Since strain RW16 cannot use them for growth, they may be mischanneled into less productive pathways eg, the catechol *meta*-pathway and/or the gentisate pathway. Thus, the low growth rate of the mixed culture and the yellow color of the medium may be explained.

Attack on the chlorinated ring would supply strain RW16 with salicylate, as is the case during degradation of unchlorinated dibenzofuran. The chlorohydroxypentadienoates also formed in this degradation (Figure 2) may be channeled slowly into an unproductive pathway not allowing their dehalogenation. The assumption is consistent with the finding that only about 60% of the chlorine is released as chloride. The putative accumulation of such degradation products probably slows down the overall growth rate of the consortium on these two chlorodibenzofurans; for the mineralization of 4-chlorodibenzofuran we have previously observed a much faster growth rate [4]. The same, or similar chloro-2-hydroxypentadienoates, depending on the congener to be converted, should also be formed during the degradation of chlorinated biphenyls although they have not been detected to date. Consequently, the fate of such intermediates is unknown and deserves much more attention. It is evident that these postulated intermediates could function as inhibitors of enzymes catalyzing the breakdown of the unchlorinated hydroxypentadienoate or they are metabolized to toxic end products which negatively interfere with other systems of bacterial metabolism. A pathway for degradation of 2-chloro- and 3-chlorodibenzofuran by the two-species consortium is proposed in Figure 2.

It is interesting that none of the numerous diaryl ether-degrading *Sphingomonas* isolates obtained thus far [39] harbor a functioning chlorosalicylate and/or chlorocatechol pathway, although sphingomonads with capabilities for the breakdown of polychlorophenols and halogenated benzenes have been described [26,37]. Our results could account for the tendency to isolate, thus far, only interactive bacterial consortia capable of sharing the carbon of much more complex substrate molecules, such as halogenated heterocyclic compounds eg, chlorinated dibenzofurans, rather than pure cultures.

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