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Dechlorination of Chlorobenzenes by a Culture Containing Bacterium DF-1, a PCB Dechlorinating Microorganism

QINGZHONG WU,[†] CHARLES E. MILLIKEN,[†] G. PATRICK MEIER,[‡] JOY E. M. WATTS,[§] KEVIN R. SOWERS,[§] AND HAROLD D. MAY*,[†]

Department of Microbiology and Immunology and Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, South Carolina, and Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland

Polychlorinated benzenes were reductively dechlorinated by an enrichment culture containing the polychlorinated biphenyl (PCB) dechlorinating bacterium DF-1. The culture dechlorinated hexachlorobenzene (hexa-CB) → pentachlorobenzene (penta-CB) → 1,2,3,5-tetrachlorobenzene (1,2,3,5-CB) → 1,3,5-trichlorobenzene (1,3,5-CB) and did not dechlorinate other tetrachlorobenzenes or any trichlorobenzenes. This restricted series of reactions is the most predominant and frequently reported pathway for the dechlorination of hexa-CB and penta-CB by enrichment cultures inoculated with either freshwater or estuarine sediments. The culture did not dechlorinate hydroxylated and methoxylated polychlorinated benzenes or a hydroxylated PCB. Bacterium DF-1 was detected by PCR/DGGE analysis following dechlorination of penta-CB but was not detected when a chlorinated benzene (CB) was not dechlorinated; detection of other members in the community was unaffected by the presence or absence of CB dechlorination. This is the first report of a bacterium that reductively dechlorinates both PCBs and CBs and the first identification of an organism that can dechlorinate a CB with more than four chlorines.

Introduction

Chlorobenzenes (CBs) are ubiquitous in the environment with all 12 isomers reported to occur in coastal marine sediments (*1*, *2*), freshwater lake sediments (*3*, *4*), soils (*5*), sewage sludge (*6*, *7*), wastewater (*3*), groundwater (*8*), and rivers and estuaries (*9*−*12*). Similar to other persistent and hydrophobic chlorinated compounds, e.g. polychlorinated biphenyls (PCBs), CBs are frequently sequestered in anaerobic regions of sediments. They have been widely used for industrial and domestic purposes such as a moth repellent and toilet deodorant (1,4-CB) and as a sewer and septic tank cleaner (1,2-CB) (*6*, *13*). CBs have also been used as industrial solvents and as feedstocks (or produced as intermediates) in the synthesis of some dyes and pesticides (*6*, *7*, *13*−*15*). The fully halogenated hexachlorobenzene (hexa-CB) has been used extensively as a solvent and as a fungicide (*15*, *16*). Although the commercial production of hexa-CB was banned in the United States in 1982, it continues to be produced as a byproduct from the synthesis of solvents and several currently manufactured pesticides (*16*). Due to its persistence and potential toxicity the U.S. EPA has added hexa-CB to its priority list of persistent bioaccumulative toxic (PBT) compounds (http://www.epa.gov/pbt/cheminfo.htm).

Microorganisms involved in the global cycling of halogenated organic compounds are capable of transforming CBs even though these compounds persist in the environment. The less chlorinated benzenes are amenable to oxidative degradation, and organisms that catalyze aerobic CB degradation have been isolated and studied in pure culture (*17*−*20*). In general, the more extensively chlorinated CB isomers, tetra-CBs through hexa-CB, tend to resist aerobic degradation but are susceptible to anaerobic dechlorination (*21*−*25*). The CB dechlorinating anaerobic microorganisms have been difficult to obtain in pure culture, but Adrian et al. recently reported the isolation of a microorganism capable of dechlorinating 1,2,3-CB, 1,2,4-CB, and all three tetrachlorinated benzenes (*26*). Anaerobic microorganisms capable of dechlorinating pentachlorobenzene (penta-CB) or hexa-CB have not been isolated or identified.

The microbial dechlorination of PCBs is similar to that of CBs in that both groups of compounds tend to accumulate in anaerobic sediments and the more extensively chlorinated congeners exhibit greater susceptibility to anaerobic dechlorination (for reviews of PCB dechlorination see refs *27* and *28*). The PCB dechlorinating bacteria have not been grown in pure culture, but two such organisms were recently identified by comparative sequence analysis of 16S rDNA (*29*, *30*). Molecular screening techniques are routinely used in the absence of isolation to identify and monitor difficult to grow microorganisms in situ or within enrichment cultures (*31*−*37*). Using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA from microbial communities enriched in the presence of PCBs Cutter et al. (*29*) and Wu et al. (*30*) were able to identify two distinct PCB dechlorinating bacteria. A similar approach was applied here to investigate the dechlorination of extensively chlorinated CBs by a microorganism reported previously to dechlorinate polychlorinated biphenyls (PCBs), bacterium DF-1.

Materials and Methods

Chemicals. All CBs, pentachlorophenol, and tetrachlorohydroquinone were purchased from Aldrich. All PCBs and 2-hydroxy-2′,3′,4′,5′-tetrachlorobiphenyl were purchased from Accustandard. The chlorinated hydroquinone metabolites (CHMs) produced by fungi, 2,3,5,6-tetrachloro-1,4-dimethoxybenzene (also known as drosophilin A methyl ether or DAME) and 2,3,5,6-tetrachloro-4-methoxyphenol (also known as drosophilin A or DA), were synthesized according to the methods of Vygas and Shah (*38*) by treating tetrachlorohydroquinone (TCHQ) with potassium hydride to make the monoanion, which was then alkylated with methyl iodide to give the monomethyl ether, or DA. Repeating the reaction sequence using potassium hydride and methyl iodide resulted in the production of the dimethyl ether, or DAME. The compounds were purified by flash SiO_2 chromatography

* Corresponding author phone: (843)792-7140; fax: (843)792-2464; e-mail: mayh@musc.edu. Corresponding address: Department of Microbiology and Immunology, Medical University of South Carolina, 173 Ashley Avenue, 225 BSB, P.O. Box 250504, Charleston, SC 29425-2230.

[†] Department of Microbiology and Immunology, Medical University of South Carolina.

[‡] Department of Pharmaceutical Sciences, Medical University of South Carolina.

[§] University of Maryland Biotechnology Institute.

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(DAME, 5% ethyl acetate/hexane; DA, 10% ethyl acetate/hexane) and checked for purity by 1H NMR and GC-MS and were found to be greater than 95% pure.

Culture Conditions. A culture containing bacterium DF-1 was grown under anaerobic conditions as described previously (*30*, *39*). The culture had been transferred sequentially (0.1−1.0% vol/vol) in E-Cl medium with 2,3,4,5-PCB more than 17 times over 4 years before it was used in the present studies. Stock cultures with bacterium DF-1 were maintained with 350 μM 2,3,4,5-PCB, which was added in acetone (10 μL acetone to 10 mL of medium). Congener 2-hydroxy-2′,3′,4′,5-PCB was tested at 162 μM, again added in acetone. Experiments with CBs were carried out with 176 μM of each CB also added in acetone (10 μL acetone to 10 mL of medium); hexa-CB was added at final concentration of 35 μM due to its lower solubility in acetone. CHMs and pentachlorophenol were added at concentrations ranging from 50 μM to 1 mM and were delivered in acetone (10 μL acetone to 10 mL of medium). Sterile controls were prepared by autoclaving cultures for 30 min at 121 °C. All cultures were prepared in duplicate and inoculated with 0.1% (vol/vol) DF culture.

Analysis of Chlorinated Aromatic Compounds. PCBs were analyzed according to the methods of Berkaw et al. (*39*) except that the copper-florisil treatment was eliminated from the procedure since the cultures were free of sediment contamination. CBs were extracted into ethyl acetate (1 mL of culture to 5 mL of ethyl acetate). The organic phase was analyzed using a Hewlett-Packard 6890 series gas chromatography (GC) equipped with a HP-1 methyl siloxane capillary column. The detector temperature was 325 °C, and the injector temperature was 250 °C. The initial oven temperature was 80 °C and was held for 3 min. This was followed by an increasing temperature gradient (15 °C per minute) to 180 °C and a 3 min hold at that temperature. A postrun at 280 °C was held for 3 min. Identification of CBs was made by matching GC retention times with those of authentic standards (99% purity). Mass selective analysis was performed with a Hewlett-Packard 6890 series gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Hewlett-Packard, Atlanta, GA) and a Hewlett-Packard 6890 series mass selective detector (MS). Congeners 1,2,3,5-CB and 1,2,4,5-CB were not resolved on the HP-1 column with the GC-ECD. When necessary the HP-5MS column with the GC-MS was used to identify these two tetrachlorobenzenes. In addition to identification by retention time, the dechlorination products 1,3,5-CB and 1,2,3,5-CB were confirmed by their mass spectra including respective molecular ions (m/z 180 and 214).

Samples with CHMs, pentachlorophenol, and 2-OH-2′,3′,4′,5′-PCB were acidified to pH 2 before extraction with ethyl acetate. The organic phase was analyzed using a Hewlett-Packard 6890 series gas chromatograph (GC) equipped with a HP-1 methyl siloxane capillary column and ECD (described above). The detector temperature was 325 °C, and the injector temperature was 240 °C. The initial oven temperature was 80 °C and was held for 3 min. This was followed by an increasing temperature gradient (5 °C per minute) to 110 °C and a 0.5 min hold at that temperature. A second gradient followed (10 °C per minute) to 180 °C and a hold for 4 min. A postrun at 280 °C was held for 3 min. CHMs were identified by matching GC retention times with those of authentic standards (99% purity).

Analysis of 16S rDNA by DGGE. Genomic DNA was extracted from 1.5 mL culture samples using InstaGene matrix (Bio-Rad, Hercules, CA) according to the manufacturers' instructions or by bead beating the cells (*34*). PCR amplification with primers specific to the Bacteria domain (*29*) and DGGE analysis of bacterial 16S rDNA was carried out as described by Wu et al. (*30*). Samples from duplicate cultures were analyzed a minimum of 3 times. Following DGGE gel purification (1 to 6 additional purifications), DNA fragments at unique migration distances were sequenced and analyzed as described by Watts et al. (*36*).

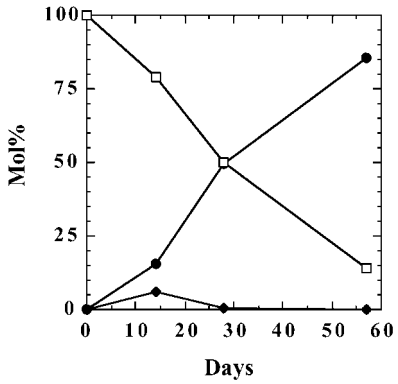

FIGURE 1. Dechlorination of penta-CB (□) to 1,2,3,5-CB (◆) and 1,3,5-CB (●) by the enrichment culture containing bacterium DF-1. Data represents average from duplicate cultures.

Results and Discussion

Dechlorination of Chlorobenzenes. A 2,3,4,5-PCB dechlorinating culture containing bacterium DF-1 was transferred into E-Cl medium with 176 μM penta-CB. Within 14 days more than 20% of the penta-CB was dechlorinated to 1,2,3,5-CB and 1,3,5-CB (Figure 1). As dechlorination continued 1,3,5-CB accumulated. No dechlorination of penta-CB was observed in sterile controls. The lag time for the PCB-grown cells to begin CB dechlorination was less than the lag observed for PCB-grown cells to dechlorinate PCBs (*30*, *36*, *40*, *41*). Therefore, cultures containing bacterium DF-1 either readily adapt to penta-CB or require no adaptation. To determine if PCB dechlorination is sustained following incubation with penta-CB, a penta-CB dechlorinating culture with bacterium DF-1 was transferred (0.1% vol/vol) into E-Cl medium with penta-CB (176 μM) or 2,3,4,5-PCB (350 μM). After 41 days, the subculture with penta-CB dechlorinated 82 mol % penta-CB, and the subculture with 2,3,4,5-PCB dechlorinated 23 mol % 2,3,4,5-PCB to 2,3,5-PCB. After 63 days, 65 mol % 2,3,4,5-PCB was dechlorinated. This demonstrated that the ability to dechlorinate PCBs was not lost following one sequential 0.1% vol/vol transfer into medium containing penta-CB.

Within 56 days the culture with bacterium DF-1 dechlorinated 100% of hexa-CB, or penta-CB at equivalent concentration, to 1,3,5-CB through 1,2,3,5-CB (data not shown). In relation to penta-CB dechlorination, a significant lag time preceded the dechlorination of hexa-CB with only 5% of that congener being dechlorinated to penta-CB after 28 days. Isomers 1,2,3-CB, 1,2,4-CB, 1,3,5-CB, 1,2,3,4-CB, or 1,2,4,5-CB were not dechlorinated within 56 days of incubation. The dechlorinating activity is very restricted and is only directed at selected double flanked chlorines, which is consistent with the catalytic specificity that bacterium DF-1 exhibits with PCBs. Although dechlorination did not occur in the absence of double flanked chlorines, not all CB congeners with double flanked chlorines were dechlorinated. For example, 1,2,3-CB and 1,2,3,4-CB, which share structural similarities with 2,3,4-PCB, 3,4,5-PCB, and 2,3,4,5-PCB, all dechlorinated by DF-1, were not dechlorinated.

In contrast with PCBs and chlorobenzenes, chlorinated phenolic compounds were not dechlorinated by bacterium DF-1 within 62 days. Compounds tested included the hydroxylated PCB, 2-OH-2′,3′,4′,5′-PCB at 162 μM, pentachlorophenol at 50 μM to 1 mM, and chlorinated hydroquinone metabolites or CHMs that are produced by fungi (*42*, *43*) at 50 μM to 1 mM. The fungal compounds included

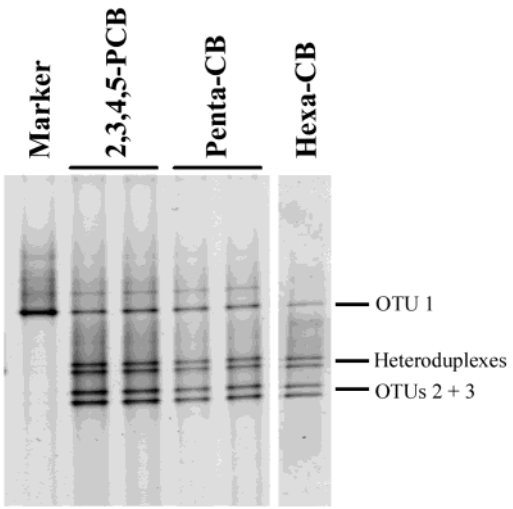

FIGURE 2. DGGE resolution of PCR amplified 16S rDNA fragments from the DF-1 containing culture incubated with 2,3,4,5-PCB and penta-CB in E-Cl medium. A sample from a culture incubated with hexa-CB is presented in a separate gel. Marker is PCR amplified DNA from OTU 1 (bacterium DF-1). The duplicate lanes represent individual samples from duplicate cultures.

2,3,5,6-tetrachloro-1,4-diemethoxybenzene (DAME) and 2,3,5,6-tetrachloro-4-methoxyphenol (DA). DAME and DA are demethylated to tetrachlorohydroquinone (TCHQ) under anaerobic conditions in estuarine sediments (unpublished results). Therefore TCHQ (at 50 μM to 1 mM) was also tested with bacterium DF-1. Considering all of the compounds tested, the dechlorinating activity of bacterium DF-1 was restricted to a select group of PCB and CB compounds, while hydroxylated chlorinated aromatic compounds remained intact.

DGGE Profiles. The culture containing DF-1 is non-methanogenic, and archaeal DNA was not detected by PCR amplification *(30)*. DGGE analysis with primer sequences specific for the bacteria domain showed six DNA bands for the microbial community when grown with 2,3,4,5-PCB (Figure 2). The same overall DGGE profile is observed with DNA from the culture during active dechlorination of penta-CB and hexa-CB (Figure 2). Three of the bands represent operational taxonomic units (OTUs) 1, 2, and 3. The DNA sequence of OTU 1 matches that of bacterium DF-1 (100% similarity), the organism that catalyzes double flanked PCB dechlorination (*30*). The 16SrDNA of bacterium DF-1 is most similar in sequence with *Dehalococcoide*s spp. (89% similarity with *Dehalococcoides ethenogenes*) (*30*). Another band of DNA migrated above OTU 1. Following excision, reamplification and a second DGGE analysis, this band was no longer observed, but a DNA fragment was present at the position of OTU 1. In addition, this band is visible in the cloned and amplified 16S rDNA of DF-1. Therefore this band was an artifact of OTU 1. OTUs 2 and 3 are nearly identical in sequence (99.6% similarity with 221 bp) and are most similar to "*Desulfovibrio caledoniensis*". The two remaining bands were previously reported to be heteroduplex DNA of OTUs 2 and 3 (*30*). All four of the "*Desulfovibrio*" bands belong to a sulfate-reducing organism previously isolated from the DF culture (*30*). The isolated sulfate reducer did not dechlorinate penta-CB when tested as a pure culture. Bacterium DF-1 (OTU 1) was not detected by PCR-DGGE when the culture was grown with CBs that are not dechlorinated (Figure 3).

Past results with a less enriched DF culture growing with PCBs demonstrated that PCR-DGGE detects the same groupings of organisms in the DF culture that PCR-ARDRA or PCR-tRFLP (*36*). Here and in the study identifying bacterium DF-1 as a PCB dechlorinating bacterium (*30*), only two organisms were detected and only two cellular morphologies were observed (a vibrio and a very small irregular coccus similar to *Dehalococcoides* spp.). As reported before (*30*), the 16S rDNA sequence of DF-1 is most similar to that of the PCE dechlorinating *D. ethenogenes* (*45*) and the chlorobenzene dechlorinating bacterium CBDB1 (*26*). Overall the results indicate that the PCB-dechlorinating organism in the culture, bacterium DF-1, is responsible for dechlorination of hexa-CB, penta-CB, and 1,2,3,5-CB.

Implications. Several pathways of CB reductive dechlorination have been observed, but the hexa-CB → penta-CB → 1,2,3,5-CB → 1,3,5-CB pathway is the most predominant pathway reported (*21*−*25*, *44*). Until recently the microorganisms capable of catalyzing these reactions were not known. The PCB-dechlorinating bacterium DF-1, which is indigenous to estuarine sediment, is the first organism identified that performs all of the reactions of the predominant pathway, and only that pathway. Figure 4 shows the different reductive dechlorinations of CBs reported in the literature and those performed by DF-1. Most reports on microbial CB dechlorination have been done with microorganisms from aquatic sediments or sewage sludge, and all have exhibited the predominant pathway with 1,2,3,5-CB as an intermediate (*21*−*24*, *44*). An exception to this observation was reported in a study of soil incubated under anaerobic conditions in which the dechlorination of hexa-CB and penta-CB to 1,2,3,5-CB and 1,3,5-CB was not observed (*25*). Instead, reductive dechlorination of penta-CB →1,2,3,4-CB → 1,2,3-CB + 1,2,4-CB occurred. The organisms responsible for these reactions have not been identified.

Middledorp et al. (*44*) described a methanogenic consortium, originating from a mixture of wastewater sludge and freshwater sediment, enriched with penta-CB and 1,2,4-CB. This consortium exhibited two distinct patterns of CB dechlorination. The first was the dechlorination of CBs with at least one unchlorinated carbon atom and occurs almost immediately. The second was the dechlorination of double flanked chlorines of CBs or PCBs, which started after a lag time of approximately 2 weeks. Although Middledorp et al. did not identify the dechlorinating microbes responsible for these pathways, they hypothesized that the two patterns resulted from the metabolism of at least two different groups of microorganisms. Results from the present study suggest that bacterium DF-1 catalyzes a dechlorination pathway that is similar to that of one of the microorganisms associated with the second pattern of dechlorination reported by Middledorp et al. Dechlorination of the CBs and PCBs with double flanked chlorines by an organism such as DF-1 would explain the observations made by Middledorp et al. However, there are some differences between the CB dechlorination pathway of bacterium DF-1 and the methanogenic consortium. First, the consortium dechlorinated tri-CBs including those with double flanked chlorines, but bacterium DF-1 did not dechlorinate any tri-CBs. Second, dechlorination of 1,2,3,5-CB to 1,3,5-CB by the methanogenic consortium reported by Middledorp et al. was a minor pathway, whereas it was the only pathway observed with bacterium DF-1.

Adrian et al. isolated for the first time a CB dechlorinating bacterium, strain CBDB1 (*26*). This organism is capable of coupling its growth to the dechlorination of several CBs including 1,2,3,4-CB or 1,2,4,5-CB → 1,2,4-CB → 1,3-CB + 1,4-CB and 1,2,3,5-CB → 1,3,5-CB. The isolate does not dechlorinate penta-CB or hexa-CB. Although bacterium DF-1 has not been isolated, the combination of enrichment culture and reductive analysis of the microbial community by DGGE has demonstrated here that the growth of this organism is linked to the dechlorination of double flanked PCBs and polychlorinated benzenes. Therefore, this is the first microorganism identified with the capability to dechlorinate CBs with more than four chlorines. The 16S rDNA of bacterium

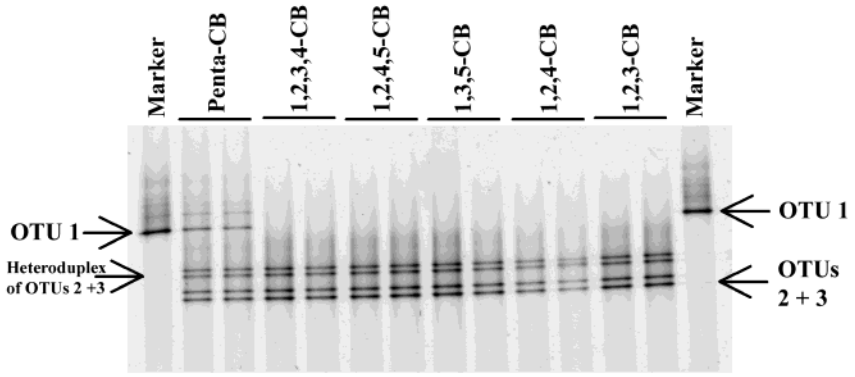

FIGURE 3. DGGE resolution of PCR amplified 16S rDNA fragments from the DF-1 containing culture incubated with various tri- through pentachlorobenzenes in E-CI medium. Marker is PCR amplified 16S rDNA from OTU 1 (bacterium DF-1). The duplicate lanes represent individual samples from duplicate cultures.

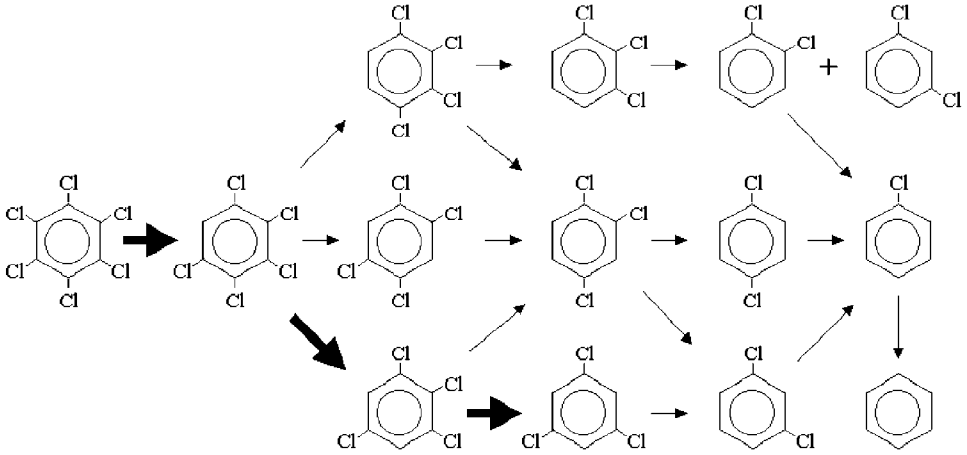

FIGURE 4. Pathways of chlorobenzene reductive dechlorination reported in the literature (*21*−*25*, *44*, *47*). The most frequently cited predominant pathway, and the path performed by DF-1, is marked with large arrows.

DF-1 is distinct from that of strain CBDB1 (89%, 1100 bp comparison) but is most similar to dechlorinating bacteria that branch deeply with CBDB1, the tetrachloroethene-dechlorinating *Dehalococcoides ethenogenes* (*30*, *45*) and a trichloroethene-dechlorinating *Dehalococcoides* sp. FL2 (*46*). Other related organisms in this group include another PCB dechlorinating microorganism, bacterium *o*-17 (*29*), and a number of uncultured organisms detected in a tri-CB-dechlorinating consortium maintained in a fluidized bed reactor (*35*). Such organisms have proven difficult to grow in high numbers or in pure culture. It is possible that this is due to the low solubility of CBs in water and their potential toxicity. For example Holliger et al. (*24*) detected no dechlorination with concentrations of 1,2,3-CB above 40 μM or of 1,3-CB above 70 μM. Adrian and colleagues never observed dechlorination with 1,2,3-CB and 1,2,4-CB above 30 μM (*47*). Adrian et al. (*26*) were able to supply more CB to the isolated CBDB1 by using hexadecane as a CB reservoir. Penta-CB was delivered in acetone directly to aqueous cultures with bacterium DF-1 at 176 μM. The majority of the penta-CB was dechlorinated even though the quantity added was greater than that withstood by other CB-dechlorinating organisms.

In summary, bacterium DF-1 is a PCB dechlorinating bacterium that is also capable of catalyzing the dechlorination of CBs. A connection between PCB and CB dechlorination was previously reported with a microbial community in sediment (*44*), but this is the first time that an individual organism capable of dechlorinating both types of compound has been identified. This confirms the link between the dechlorination of the two different classes of compounds that are also pervasive environmental pollutants. The specificity of PCB dechlorination demonstrated by bacterium DF-1, namely of double flanked chlorines, extends to CB dechlorination. However, dechlorinating activity was specific for polychlorinated benzenes and biphenyls; hydroxylated chloroaromatic compounds including the polychlorinated hydroquinone metabolites of fungi were not dechlorinated. The dechlorination of penta-CB and hexa-CB by DF-1 is distinct in that no other organism has been identified that is capable of these reactions. We have also determined that an enrichment culture containing bacterium *o*-17 dechlorinates penta-CB to 1,3,5-CB (data not shown). In addition, PCR-DGGE showed the same DGGE pattern (not shown) as reported for this culture when bacterium *o*-17 was dechlorinating 2,3,5,6-PCB (*29*). These findings begin to explain some of the more common dechlorinations of CBs observed with freshwater and estuarine sediments and extends our understanding of the potential role of bacterium DF-1 in the detoxification of these and related pollutants during the global cycling of chlorinated organic compounds.

Acknowledgments

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