Detection of a Bacterial Group within the Phylum Chloroflexi and Reductive-Dehalogenase-Homologous Genes in Pentachlorobenzene-Dechlorinating Estuarine Sediment from the Arakawa River, Japan

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We enriched a pentachlorobenzene (pentaCB)-dechlorinating microbial consortium from an estuarine-sediment sample obtained from the mouth of the Arakawa River. The sediment was incubated together with a mixture of four electron donors and pentaCB, and after five months of incubation, the microbial community structure was analyzed. Both DGGE and clone library analyses showed that the most expansive phylogenetic group within the consortium was affiliated with the phylum Chloroflexi, which includes Dehalococcoides-like bacteria. PCR using a degenerate primer set targeting conserved regions in reductive-dehalogenase-homologous (rdh) genes from Dehalococcoides species revealed that DNA fragments (approximately 1.5–1.7 kb) of rdh genes were amplified from genomic DNA of the consortium. The deduced amino acid sequences of the rdh genes shared several characteristics of reductive dehalogenases. The mixed culture could be maintained by transferring small inocula into fresh synthetic mineral medium containing either acetate or pyruvate, which supported the dechlorination of pentaCB by acting as an electron donor.

Key words: Persistent organic pollutants, Pentachlorobenzene, Chloroflexi, Reductive dehalogenation, Dehalococcoides

Persistent organic pollutants (POPs) is the common name of a group of pollutants that have toxic properties, bioaccumulate, and resist degradation. POPs are semi-volatile and transported across international boundaries through air, water, and migratory species. As a result, POPs are deposited far from their place of release, accumulating in terrestrial and aquatic ecosystems. A key environmental concern is that some POPs have been found in polar regions at surprisingly high levels. To raise awareness of the need for global action on POPs, the Stockholm Convention on Persistent Organic Pollutants was held in May 2001. The global treaty included several measures, such as the prohibition and elimination of the production, use, import, and export of POPs, and a call for reduction of their release. Currently, 12 types of POPs, i.e., aldrin, chlordane, dieldrin, endrin, heptachlor, hexachlorobenzene (hexaCB), mirex, toxaphene, polychlorinated biphenyls (PCB), polychlorinated dibenzop-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), are specifically listed in the treaty.

HexaCB, one of the POPs listed, was used as a pesticide,
fungicide, and seed protectant, and as a consequence has been detected all over the world in air, water, sediments, and biota. The compound persists in the environment for many decades under natural conditions and shows hepatocarcinogenic activity\(^\text{10,20}\). According to an environmental monitoring report published by the Japanese Ministry of the Environment (2004) (<http://www.env.go.jp/chemi/kurohon/http2004/index.html>), hexaCB has been found to accumulate in aquatic environments (both water and sediments) in Japan. Until now, the maximum acceptable concentration of hexaCB has not been determined, but protection of the environment from its polluting effects is regarded in Japan as a matter deserving urgent attention.

The reductive dechlorination of highly chlorinated benzenes by microbes has been studied over the last two decades\(^\text{1,4,6,7,11,16,28,29}\). Some of these reports describe that hexaCB is first dechlorinated to pentachlorobenzene (pentaCB) and this reaction is accompanied by a negative change in Gibbs free energy\(^\text{1,16}\). Using hydrogen as the electron donor, the change in free energy under natural conditions (\(\Delta G^\circ\)) is \(-174.1\) kJ/mol for hexaCB\(^\text{1}\)).

Considering that reductive dechlorination of hexaCB releases per dechlorination step \(4-10\) kJ/mol more than dechlorination of pentaCB\(^\text{1}\), it is thought to be more difficult to dechlorinate pentaCB than hexaCB in terms of energy. In addition, pentaCB also shows hepatocarcinogenic activity\(^\text{10}\) and persists in the environment\(^\text{29}\). Hence, in this study, we used pentaCB as the model compound in order to abide by the Stockholm Convention regarding the use of POPs (hexaCB).

So far, pure cultures of only two strains, *Dehalococcoides* sp. CBDB1\(^\text{1,13,16}\) and *D. ethenogens* 195\(^\text{6,18}\), both of which dechlorinate hexaCB and pentaCB, have been obtained. These strains also reductively dechlorinate diverse chlorinated aromatic pollutants, such as PCBs and PCDD/Fs. Recently, the complete genome sequences of *Dehalococcoides* sp. CBDB1\(^\text{17}\) and *D. ethenogens* 195\(^\text{23}\) were determined (1,395,502 bp in strain CBDB1 and 1,469,720 bp in strain 195) and found to contain, respectively, 32 and 17 reductive-dehalogenase-homologous (rdh) genes\(^\text{17,23}\). Nevertheless, reductive dehalogenase genes for the highly chlorinated benzenes have not yet been identified. The aim of the present study is to obtain an enrichment culture that can respire highly chlorinated benzenes for the future isolation of a pure culture other than *Dehalococcoides* species.

### Materials and Methods

#### Sampling and culture conditions

In 2004, 820–1500 pg of hexaCB (g dry weight\(^{-1}\)) was detected in Arakawa River sediment (<http://www.env.go.jp/chemi/kurohon/http2004/index.html>). The sediment sample used in this study was collected from the mouth of the Arakawa River (35\(^\circ\)38'80" N, 139\(^\circ\)50'71" E) on 10 September 2003, using a core sampler (45 mm in diameter). A sample of river water was taken from the same point. The sediment and the river water were put into plastic bags and bottles, respectively, kept in a cooler box, and brought back to the laboratory within several hours. The basal medium used in these initial experiments consisted of river water in which a mixture of four electron donors, acetate, pyruvate, benzoate, and fumarate (final concentration of 5 mM each), and resazurin (0.001 g L\(^{-1}\)) had been dissolved. In an anaerobic glove box, approximately 1 g of the sediment sample and 30 ml of sterile basal medium were dispensed into 100-ml glass bottles. NaS (final concentration, 1 mM), as a reducing agent, and then pentaCB, dissolved in dimethylformamide (DMF) (final concentration, 500 \(\mu\)M), were added to each bottle. The bottles were sealed with Teflon-coated butyl-rubber septa and aluminum caps, and the headspace was exchanged with N\(_2\)-CO\(_2\)-H\(_2\) (3:1:1, vol/vol) using a deoxygenated-gas pressure injector (Sanshin Kogyo, Yokohama, Japan). The sample bottles were incubated at 30\(^\circ\)C in the dark.

#### GC-MS analysis

Fluoranthene was added to each sample at a final concentration of 0.2 mM as an internal standard, after which the samples were extracted with 30 ml of either n-hexane or ethylacetate. A JMS Automass 150 GC-MS system (JEOL, Tokyo, Japan) fitted with a fused silica chromatically bonded capillary column DB-5 (0.25 mm I.D. by 15 m, 0.25 \(\mu\)m film thickness; J&W Scientific Inc., Folsom, CA, USA) was used to determine the amount of pentaCB and to identify less chlorinated metabolites. Each sample was injected into the column and the temperature was increased by 16\(^\circ\)C min\(^{-1}\) to 280\(^\circ\)C. The head pressure of the helium carrier gas was 65 kPa. The rate of pentaCB depletion was calculated by setting the peak area for the molecular ion of pentaCB (m/z=250) at 0 months (or weeks) incubation at 100%. Chlorinated metabolites were identified by comparing the mass spectra and retention times of the metabolites with those of authentic samples.
DNA extraction

DNA was extracted from sediment samples according to the method of Zhou et al. (1996) with some modifications. Briefly, 2 ml of the sediment slurry was centrifuged and the pellet was suspended in 900 μl of DNA extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM sodium phosphate buffer, 1.5 M NaCl, and 1% CTAB, pH 8.0). Next, 10 μl of 10 μg proteinase K/μl was added to the suspension, which was then incubated for 30 min at 37°C. One-hundred microliters of 20% SDS was added and the mixture was further incubated for 2 h at 65°C. After three cycles of freezing (~80°C for 30 min) and thawing (65°C for 10 min), the suspension was extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). Isopropyl alcohol (0.7 volumes) was added to the recovered aqueous solution, the mixture was gently mixed, and DNA was precipitated by centrifugation. The DNA-containing precipitate was washed with 70% (vol/vol) ethanol and then treated with Tris-EDTA buffer containing RNase.

PCR-DGGE

The V3 region of the bacterial 16S rRNA gene was PCR-amplified using primer set 341F-GC (5’-CCTACGGGAGGCAGCAG-3’, with a GC clamp attached to the 5’-end) and 534R (5’-ATTACCGCGGCTGCTGG-3’). The PCR conditions reported by Muyzer et al. (1993) were followed. First, the samples were incubated for 10 min at 94°C, followed by two cycles of 1 min at 94°C, one cycle of 1 min at 65°C, and one cycle of 2 min at 72°C (i). Second, the annealing temperature was subsequently decreased by 1°C every second cycle until it reached 55°C, at which point 20 additional cycles were performed (ii). Third, one cycle of 10 min at 72°C was carried out (iii). The PCR products were applied to 8% (wt/vol) polyacrylamide gels containing 30–70% denaturing gradient of formamide and urea and electrophoresed at 60°C for 6 h in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at a constant voltage of 130 V with a DCode System (Bio-Rad, Hercules, CA, USA). The gels were stained with ethidium bromide (0.5 μg ml−1) and visualized with a UV transilluminator. Excised DNA fragments were amplified using the PCR conditions described above, purified using a QIAEX II gel extraction kit (Qiagen) according to the manufacturer’s instruction. Purified DNA fragments were cloned into pT7 Blue T-Vector (Novagen), which was used to transform Escherichia coli DH5α according to published procedures. Plasmid DNA was isolated and purified with the Quantum Prep plasmid miniprep kit (Bio-Rad) according to the manufacturer’s instructions.

Sequencing and phylogenetic analysis

Cloned 16S rRNA genes and rdh genes were sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or a 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences obtained were analyzed with DNASIS-Mac software (version 3.7; Hitachi Software Engineering, Tokyo, Japan). Homology searches were carried out with the BLASTN or BLASTX program, and multiple sequence alignment with CLUSTALW. Phylogenetic trees were constructed by the neighbor-joining method, and the topology of the trees was evaluated by bootstrapping with 1000 resamplings. The nucleotide sequences were deposited at the DDBJ, EMBL, and GenBank databases under accession numbers AB253368 to AB253381 and AB251615 to AB251618.

Composition of the basal salt medium and culture conditions

Several synthetic media were tested for their ability to stably maintain the pentaCB-dechlorinating activity of an inoculum obtained from the Arakawa estuary sediment. A basal medium containing 10 g NaCl, 0.2 g KH2PO4, 0.27 g NH4Cl, 4.5 g MgCl2·6H2O, 0.52 g KCl, 0.15 g CaCl2·2H2O, 0.25 g cysteine·HCl·H2O, 2.5 g NaHCO3, 0.001 g resazurin, 0.5 ml vitamin mixture, and 1 ml mineral mixture (L−1) was selected. The vitamin mixture consisted of 4 mg p-aminobenzoate, 1 mg biotin, 10 mg nicotinic acid, 5 mg pantothenic acid, 15 mg pyridoxine, 10 mg thiamine, and 10 mg cobalamin (100 μl−1), and the mineral mixture of 1.5 g FeCl3, 70 mg ZnCl2, 100 mg MnCl2·4H2O, 6 mg H2BO3, 190 mg CoCl2·6H2O, 2 mg CuCl2·2H2O, 24 mg NiCl2·6H2O, 36 mg NiMoO4·2H2O, and 10 ml HCl (7.7 M) (100 ml−1). Acetate, pyruvate, or benzoate was added to the basal medium as an electron donor at a final concentration of 5 mM. The inoculum (1% vol/vol) and 30 ml of sterile synthetic
basal medium were dispensed into 100-ml glass bottles in an anaerobic glove box, 0.8 mM Ti (III) citrate was added to each bottle as a reducing agent, and pentaCB was directly added to the cultures as crystals at a final concentration of 250 µM. The bottles were sealed with Teflon-coated butyl-rubber septa and aluminum caps, the headspace was exchanged with N₂-CO₂-H₂ (3:1:1, vol/vol) as described above, and the sample bottles were incubated at 30°C in the dark.

**Chemicals**

All chlorobenzenes (98–99% purities) used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of the highest purity (>98%) commercially available and were purchased from Merck (Whitehouse Station, NJ, USA), Sigma-Aldrich, Kanto Chemical (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

**Results and Discussion**

**Detection of dechlorinated metabolites of pentaCB**

We tried to enrich the pentaCB-dechlorinating mixed culture from Arakawa estuarine sediment by initially adding 500 µM of pentaCB to the sediment samples. To prevent the enrichment of dechlorinating bacteria growing well on specific synthetic media, Arakawa River water was used instead of the defined synthetic medium, e.g., for cultivation of *Dehalococcoides* strains. After 5 months of incubation, the amount of pentaCB in the sediment was approximately 10% of the initial value (data not shown). GC-MS analysis showed two less-chlorinated metabolites of pentaCB in the sample after 5 months of incubation, but not after 1 or 3 months of incubation. The mass spectrum of one metabolite exhibited fragment ions at m/z 216 (M⁺, 100), 179 (16), 143 (10), 108 (24), 84 (18), and 74 (36) at a retention time of 4.7 min (the relative intensities expressed as percentages are given in parentheses). This fragmentation pattern of the metabolite was identical to that of authentic 1,2,4,5-tetrachlorobenzene (tetracB). The mass spectrum of another metabolite showed fragment ions at m/z 180 (M⁺, 100), 145 (27), 109 (40), 84 (19), and 74 (48) at a retention time of 4.0 min. Its fragmentation pattern was identical to that of authentic 1,2,4-trichlorobenzene (triCB). These results suggested that the sediment sample may have dechlorinated pentaCB by a pathway generating the metabolites 1,2,4,5-tetracB and 1,2,4-triCB. However, a quantitative relationship of the amounts of pentaCB, 1,2,4,5-tetracB, and 1,2,4-triCB could not be established because only trace amounts of the latter two compounds were detected (data not shown). It also remains to be determined whether pentaCB was dechlorinated in the sediments to di- or monochlorobenzenes.

**PCR-DGGE and clone library analyses**

A PCR-DGGE analysis was carried out to investigate changes in the microbial population in the sediment before and after incubation with pentaCB (Fig. 1). The former and latter sediment samples were designated as AR0 (0 months of incubation) and AR5 (5 months of incubation), respectively. Two bands, A and B, in the DGGE gel (Fig. 1) were reproducibly detected in repeated experiments only when DNA extracted from AR5 was used as a template. Hence, these two bands were excised and sequenced. The nucleotide sequences of bands A and B exhibited 96.8 and 99.9% identity to that of an uncultured Chloroflexi bacterial isolate, ODP1176A6H_1_B (AY191351), and *Clostridium litorale* (X77845), respectively.

Furthermore, we investigated changes in the bacterial
populations in AR0 and AR5, especially those of the phylum Chloroflexi, by amplifying partial 16S rRNA gene fragments, which were used to construct two clone libraries. From these two libraries, 50 clones were randomly selected and sequenced (Table 1). No fundamental differences in phylogenetic composition between AR0 and AR5 were observed except for the phyla Chloroflexi, Firmicutes, and Deltaproteobacteria. In the AR0 library, 46% of the clones were affiliated with the phylum Firmicutes and 16% with Deltaproteobacteria. However, the proportion of these bacteria decreased in the clone library from AR5; instead, bacteria from the phylum Chloroflexi became increasingly common (26%) (Table 1).

To obtain a more detailed picture of the affiliation of the AR5 clones within the phylum Chloroflexi, we constructed a phylogenetic tree of the AR5 clones within the phylum Chloroflexi and their relatives, including the Dehalococcoides group (Fig. 2). None of the clones fell into the cluster within the known dehalorespiring Dehalococcoides strains, e.g., strains 195, CBDB1, and BAV1; however, five clones (CLA-4, 33, 36, 72, and 76) formed a cluster with an uncultured bacterium, DF-1, which has the ability to dechlorinate PCB and hexaCB\textsuperscript{76}. Formed with a degenerate primer set designated for amplifying putative reductive dehalogenase genes of Dehalococcoides species\textsuperscript{10} (Fig. 2). PCR was performed with a degenerate primer set designated for amplifying putative reductive dehalogenase genes of Dehalococcoides species\textsuperscript{10} (Fig. 2). Amplicons with the expected sizes (approximately 1.5–1.7 kb) were detected in AR5 but not in AR0 (Fig. 3). The AR5-derived amplicons were purified and clone libraries were constructed, from which four clones were randomly selected and sequenced. All four sequences contained a nearly complete rdhA region at the 5'-end (Fig. 3a). The sequenced rdhA regions were designated as rdh1, rdh3, rdh4, and rdh6. The deduced amino acid sequences of these four rdhA genes showed 30–50% identity with rdh genes from strains 195, CBDB1, and BAV1 (data not shown), and contained two iron-sulfur cluster (ISB) motifs in their C-terminal region (Fig. 4). Furthermore, the first ISB motif of all four RDHs possessed the conserved consensus sequence CXXCXXCPXXP (PceA in Fig. 4), whereas in RDH4 and RDH6 the first cysteine residue of the motif was lacking (Fig. 4b). A similar tendency was observed in PceA from Sulfurospirillum multivorans (PceASm in Fig. 4b), PceA from Desulfitobacterium sp. Y51 (PceADy), and CprA from Desulfitobacterium dehalogenans (CprAd) (Fig. 4b). Electron paramagnetic resonance (EPR) analysis of CprAd strongly suggested the presence of one 4Fe-4S cluster and one 3Fe-4S cluster, consistent with two ISB motifs CXXCXXCPXXP and CXXCXXCPXXP\textsuperscript{20}. Thus, the second ISB motif, CXXCXXCP, in both RDH4 and RDH6, may correspond to the 3Fe-4S motif was lacking (Fig. 4b). A similar tendency was observed in PceA from Sulfurospirillum multivorans (PceASm in Fig. 4b), PceA from Desulfitobacterium sp. Y51 (PceADy), and CprA from Desulfitobacterium dehalogenans (CprAd) (Fig. 4b). Electron paramagnetic resonance (EPR) analysis of CprAd strongly suggested the presence of one 4Fe-4S cluster and one 3Fe-4S cluster, consistent with two ISB motifs CXXCXXCPXXP and CXXCXXCPXXP\textsuperscript{20}. Thus, the second ISB motif, CXXCXXCP, in both RDH4 and RDH6, may correspond to the 3Fe-4S motif.
However, in RDH3, the first and third cysteine residues of the motif were lacking (Fig. 4b).

In this experiment, only four rdh clones were selected and analyzed; nonetheless, the results suggest that Dehalococcoides-like bacteria may also have rdh genes similar to those from Dehalococcoides species. Supposing that a Deh-
Fig. 3. (a) Reductive-dehalogenase-homologous \( (rdh) \) gene fragments amplified from the known dehalorespiring \textit{Dehalococcoides} strains with the primer pair RRF2-B1R. The conserved dehalogenase features are also shown: the Tat signal peptide RRDFMK, two 4Fe-4S clusters (ISB motifs) in the C-terminal portion, and a conserved motif WYEW within the \textit{Dehalococcoides} \( rdhB \) gene. Conserved amino acid residues in dehalogenases are labeled with an asterisk. (b) PCR amplification of the \( rdh \) gene fragments with the primer pair RRF2-B1R. DNA extracted from AR0 (before incubation; lane 1) or AR5 (after 5 months incubation; lane 2) was used as a template.

(a)

(b)

Fig. 4. Alignments of the C-terminal region of the deduced amino acid sequences of \( rdhA \) genes. Alignment of \( RdhA \)s with two ISB motifs corresponding to two 4Fe-4S clusters (a) and one 4Fe-4S and one 3Fe-4S cluster (b). Sequence abbreviations, species, and accession numbers are as follows: TceADe \textit{Dehalococcoides ethenogenes} 195, AF228507; VcrADv \textit{Dehalococcoides} sp. VS, AY322364; PceASm \textit{Sulfurospirillum multivorans}, AF022812; CprAdD \textit{Desulfitobacterium dehalogenans}, AF115542; PceADy, \textit{Desulfitobacterium} sp. Y51, AB070709. \( Rdh1 \), \( Rdh3 \), \( Rdh4 \), and \( Rdh6 \) (AB251615 to AB251618) are reductive dehalogenase homologues from this study.

(a)

(b)
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_4_ -like bacterium also possesses multiple _rdh_ genes, a number of _rdh_ genes can be isolated from the AR5 clone library. To date, no methods using _E. coli_ and other bacterial hosts to analyze the expression and function of the _Dehalococcoides rdh_ genes have been established. The development of such systems would facilitate analysis of the functions of various _Dehalococcoides rdh_ genes, including the reductive dehalogenase gene for pentaCB.

**Effect of electron donors on pentaCB dechlorination**

The DGGE and clone library analyses indicate that further enrichment of the mixed culture is necessary for identifying and isolating pentaCB-dechlorinating bacteria. However, repeated transfer of the sediment sample to sterile river water containing electron donors resulted in a loss of dechlorination activity. Therefore, several types of synthetic basal salt media were screened for their ability to maintain stable subcultivation. After 34 kinds of synthetic media, with different constituents at varying concentrations (NaCl: 1, 5, or 10 g (L$^{-1}$); MgCl$_2$·6H$_2$O: 0.42, 2.2, or 4.5 g (L$^{-1}$); reducing agents: 1 mM Na$_2$S or 0.8 mM Ti (III) citrate; pentaCB addition: as DMF solution or as crystal), had been tested (data not shown), the synthetic medium described in the Materials and Methods was chosen. Using this synthetic medium, we then set out to identify which of three electron donors (acetate, pyruvate, or benzoate) was most effective in mediating dechlorination of pentaCB in the microbial communities. Compared to the sterilized control sample, 5 mM acetate resulted in a significant decrease in the concentration of pentaCB after 2 months of incubation (Fig. 5a). Supplementation with pyruvate resulted in more than 80% pentaCB depletion within 2 months of incubation (Fig. 5b), whereas benzoate was ineffective (Fig. 5c). These results indicated that either acetate or pyruvate can be used as an electron donor in the pentaCB-dechlorinating consortium.

In conclusion, we obtained a microbial consortium that reductively dechlorinates about 90% of 250 µM pentaCB.

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