REDUCTIVE DECHLORINATION OF PCBS AND OTHER CHLORINATED COMPOUNDS BY NOVEL ANAEROBIC BACTERIAL SPECIES

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Abstract
Bacteria that can reductively dechlorinate chlorinated compounds such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) have been receiving much attention in the field of environmental science and technology. In this study, the enrichment of bacteria that dechlorinate PCBs and PCDDs was performed by the sequentially transferred culture amended with 4,5,6,7-tetrachlorophthalide (fthalide) and single fatty acids from an uncontaminated paddy field. The enrichment cultures amended with lactate (KFL) or formate (KFF) maintained the activity to dechlorinate fthalide to 4-chlorophthalide in the sequential transfer but not the culture amended with acetate and butyrate. The only KFL-culture dechlorinated diverse chlorinated compounds including 2,3,4,5-tetrachlorobiphenyl, 2,3,4-trichlorobiphenyl, 1,2,3-trichlorodibenzo-*p*-dioxin. A further enrichment of KLF-culture provided an enrichment culture, KFC4A-culture, containing uniformly small short rods. Microbial community analysis based on 16S rRNA genes revealed the involvement of two phylogenetically distinct operational taxonomic units (OTUs) of *Dehalobacter* sp. (FTH1 and FTH2) with the dechlorination of chlorinated compounds. *Dehalobacter restrictus* TEA was their closest relative, and had 97.5% and 97.3% of 16SrRNA gene similarities for FTH1 and FTH2, respectively. These results demonstrate the capacity of uncontaminated paddy soil to dechlorinate PCBs and PCDDs, and the involvement of *Dehalobacter* sp.

Introduction
Reductive dechlorination of is one of promising bio-process to remediate the environments contaminated with polychlorinated biphenyls (PCBs) and dibenzo-*p*-dioxins (PCDDs). In this connection, bacteria dechlorinating PCBs and PCDDs have been enriched and isolated. The dechlorination of PCDDs by single pure microorganisms was reported for *Dehalococcoides* sp. CBDB1[1] and *Dehalococcoides ethenogenes* strain 195[2], which were initially shown to dechlorinate chlorobenzene[3] and chloroethene[4], respectively. Additionally, some reports have also suggested the possible association of other *Chloroflexi* with the dechlorination of PCBs and PCDDs[5, 6, 7, 8]. In this study, bacteria that dechlorinate PCBs and PCDDs were enriched from an uncontaminated paddy soil by the sequentially transferred cultures using a sediment-free medium supplemented with single fatty acids such as lactate, formate, butyrate, and acetate. The enriched culture was phylogenetically characterized based on the 16S rRNA gene by polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE), a 16S rRNA gene clone library.

Materials and Methods
Enrichment of dechlorinating bacteria A paddy soil sample was obtained from the plowed layer of a paddy field located at Yatomi-cho in Aichi Prefecture, Japan. The fresh paddy soil was sieved through a 5-mm sieve and water was added to adjust the water content to 65%. It was then stored in airtight plastic bags at 22°C for several weeks before use. Fifty grams-wet wt. of paddy soil were incubated with 100 ml of a mineral medium supplemented with 20mM of acetate, formate, butyrate, or lactate and spiked with 50 μM of fthalide at 30 °C for 2 weeks. The cultures exhibiting fthalide-dechlorinating activity were serially transferred to the new medium at 5% transfer rate. Further enrichment was performed by repeating of deep-agar culture and re-culture of the colonies and the serial diluted culture at the limit dilution rate.

Chemical analysis Identification and quantification of fthalide, PCBs, PCDDs, and their metabolites were performed using a gas chromatography (GC)–mass spectrometry (MS) system (Shimadzu, Kyoto, Japan) equipped with a DB-5MS column (J&W Scientific, Folsom, CA). The analysis of chloroethenes and chlorophenols was performed as described previously[9,10]. In the sample preparation for GC–MS, 1 mL samples of the cultures were extracted by mixing them with an equal volume of acetonitrile. The mixture was then separated by mixing it with 2 mL of ethyl acetate. The organic solvent phase was collected and dehydrated with

anhydrous sodium sulfate. The volume injected into the GC–MS was 2 μL. The gas in the headspace of the culture was analyzed in some cases by injecting 200 μL of headspace gas from the cultures. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}, with splitless injection. The injection and GC–MS interface temperatures were maintained at 280°C and 320°C, respectively. The column temperature was initially set at 140°C for 0.25 min, and then increased at a rate of 10°C min^{-1} to 320°C, where it was maintained for 3 min. The spiked chlorinated compounds and the dechlorinated products were identified by monitoring the mass spectra and retention times in full-scan mode, with quantification in selected ion mode. Mass spectra were obtained in the electron impact ionization mode at 70 eV. In the full-scan mode, the scanned mass range was *m/z* 50–400. In the selected ion mode, *m/z* 104, 139, 173, 207, and 243 were monitored to detect fthalide and its metabolites; *m/z* 154, 188, 222, 222, 256, and 290 to detect PCBs and their metabolites; and *m/z* 182, 217, 252, 287, and 322 to detect PCDDs and their metabolites.

Microbial community analysis Microbial community analysis was performed by PCR-DGGE, 16S rRNA gene clone library. DNA was extracted from the microcosm using the DNA extraction kit ISOIL (Nippon Gene Co., Tokyo, Japan), according to the manufacturer's instructions. PCR–DGGE and sequencing the major DGGE fragments were performed as described previously[8]. Bacterial 16S rRNA gene fragments encompassing nucleotides 341–537 and 27-1492 were amplified for PCR–DGGE and 16SrRNA gene clone library, respectively. DGGE was performed at 100 V for 16 h in 0.5 × TAE buffer at 60°C using a DGGE-gel with the detergent gradient at 40-60%. The clones of 16S rRNA gene fragments encompassing nucleotides 27-1492 were categorized as OTUs on the basis of their distinct RFLPs (restriction fragment length polymorphism) using *Hae*III, *Hha*I, and *Ssp*I. DGGE clones and representative clones for each OTU showing the unique RFLP pattern were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA) using the ABI 3100 DNA sequencer (Applied Biosystems).

Results and Discussion

Enrichment of fthalide-dechlorinating bacteria

All the cultures amended with single fatty acids dechlorinated fthalide. The uncontaminated paddy soil used in this study was also reported to dechlorinate pentachlorophnol to phenol[10]. The culture amended with formate or lactate (named as KFF-culture and KFL-culture, respectively) maintained the fthalide-dechlorination activity, whereas the transferred cultures with acetate or butyrate suddenly lost the capacity to dechlorinate fthalide after the second transfer of the culture using a 5% (v/v) inoculum (Fig.1). Although KFF-culture lost the dechlorination activity in 10th transferred culture when yeast extract was not amended, KFL-culture maintained the activity over more than 12 sequential transfers without yeast extract (data not shown).

Capability of KFL-culture and KFF-culture to dechlorinate chlorinated compounds

Both of KFL-culture and KFF-culture dechlorinated fthalide to 4-chlorophthalide and 2,4,6-trichlorophenol to 4-chlororphenol (Table 1). KLF-culture dechlorinated PCB, PCDD, and tetrachloroethene but not KFF-culture. KLF-culture dechlorinated 2,3,4,5-tetrachlorobiphenyl and 2,3,4-trichlorobiphenyl to 2- and 4-chlorobiphenyl.

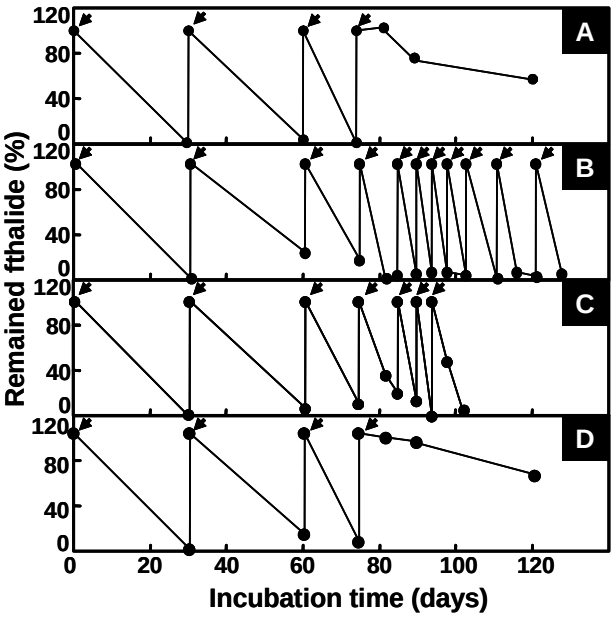

Fig.1 Fthalide-dechlorination activities of the sequentially transferred cultures amended with acetate (A), lactate (B), formate (C), and butyrate (D). Arrows indicate the timing of the transfer.

Table 1 The capability of the KFL-and KFF-cultures to dechlorinate chlorinated compounds.

chlorinated compounds	dechlorination		end products of dechlorination	
	KFL	KFF	KFL	KFF
2,3,4,5-tetrachlorophthalide (fthalide)	+	+	4-chlorophthalide	4-chlorophthalide
2,3,4-trichlorobiphenyl	+	–	2-MCB*, 4-MCB*	
2,3,4,5-tetrachlorobiphenyl	+	–	2-MCB*, 4-MCB*	
1,2,3-trichlorodibenzo-*p*-dioxin	+	–	2,3-DiCDD*, 2-MCDD*	
1,2,3,4-tetrachlorodibenzo-*p*-dioxin	–	–		
Tetrachloroethene	+	–	Trichloroethene	
2,4,6-trichlorophenol	+	+	4-chlorophenol	4-chlorophenol
Pentachlorophenol	–	–		

*2-MCB, 2-chlorobiphenyl; 4-MCB, 4- chlorobiphenyl; 2,3-DiCDD, 2,3-dihclorodibenzo-*p*-dioxin; 2-MCDD, 2-chlorodibenzo-*p*-dioxin.

The dechlorination of dichlorobiphenyl to monochlorobiphenyl has never been observed in the any dechlorinating isolates such as *Dehalococcoides* sp. strain 195[2] and other bacteria of the phylum *Chloroflexi*[5,6]. KLF-culture also dechlorinated 1,2,3-trichlorodibenzo-*p*-dioxin to 2,3-dihclorodibenzo-*p*-dioxin and trace amount of 2-chlorodibenzo-*p*-dioxin. The dechlorination pathway was identical to that observed in the culture of *Dehalococcoides* sp. CBDB1[1].

Further enrichment of fthalide-dechlorinating bacteria from KLF-culture

A further enriched culture of fthalide-dechlorinating bacteria was obtained by repeating agar shake cultivation and serial dilution cultivation of KLF-culture. Four out of 20 colonies exhibited the activity to dechlorinate fthalide, which were obtained from agar culture of 10^5-diluted KLF-culture amended with lactate after the one month of incubation at 30°C. The colonies in the agar-cultures at higher dilution rate did not dechlorinate fthalide. One of the four cultures was selected and further enriched by the serial diluted culture using liquid medium amended with 80% H_2, 20% CO_2, and acetate instead of lactate. A culture containing uniformly small short rods was obtained at 10^{-7} dilution rate and named as KFC4A-culture.

Microbial community analysis of KFC4A-culture

PCR-DGGE of KLF-culture and KFC4A-culture revealed the existences of two DGGE-bands in the both enrichment cultures (Fig. 2). The two DGGE-bands, disappeared in KLF-culture when KLF-culture was incubated without fthalide, suggested the involvement of the two bacteria corresponded to the two DGGE-bands with fthalide-dechlorination. In addition, the non-fthalide culture did not dechlorinated PCB and PCDD, indicated the capability of the two bacteria to dechlorinate not only fthalide but also PCB and PCDD (data not shown). Two operation taxonomic units (OTUs), OTU1 and OTU2 obtained in 16SrRNA clone library of the KLF-culture were corresponded to the DGGE bands A and B, and had 97.5% and 97.3% of 16S rRNA gene similarity with *Dehalobacter restrictus* TEA[11] as the closest isolate, respectively. The mutual similarity of OTU1 and OTU2 was 99.0%. OTU1 and OTU2 were named *Dehalobacter* sp. FTH1 and FTH2, respectively, to distinguish easily the bacteria that dechlorinate fthalide in this study. *Dehalobacter* sp. has been isolated as bacteria dechlorinating chloroethene[11,12] and chloroethane[13], but there are no reports of the capacities to dechlorinate PCBs and PCDDs. *Dehalobacter* species were recently detected in sediment cultures that dechlorinated 2,3,4,5-tetrachlorobiphenyl, predominantly with *Dehalococcoides* sp.[14] In conclusion, this study demonstrated the dechlorination of diverse chlorinated compounds such as PCBs and PCDDs by *Dehalobacter* sp. as a single dechlorinator which obtained from uncontaminated paddy soil and the important role of these bacteria in the dechlorination of PCBs and PCDDs in the environment as well as *Dehalococcoides* sp.

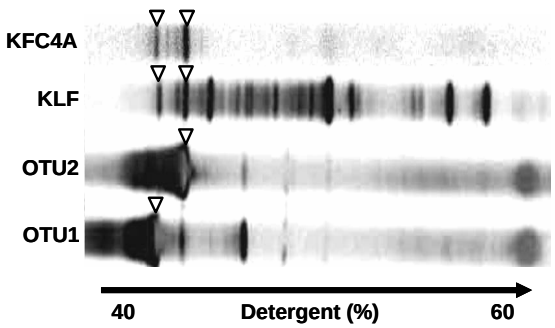

Fig.2 PCR-DGGE profiles of KLF-culture and the further enriched KF4A-cultures.

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