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ORIGINAL PAPER

Use of a glass bead-containing liquid medium for efficient production of a soil-free culture with polychlorinated biphenyl-dechlorination activity

Daisuke Suzuki · Daisuke Baba · Velayudhan Satheeja Santhi · Robinson David Jebakumar Solomon · Arata Katayama

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Abstract We established a soil-free culture capable of dechlorinating polychlorinated biphenyls (PCBs) in Kanechlor-300 and Kanechlor-400 by establishing a PCB-dechlorinating soil culture in liquid medium containing 0.5 mm glass beads. PCB-dechlorination activity in liquid cultures with glass beads appeared to depend on the size of the glass beads, and soil-free cultures with 0.05-, 1.0- or 2.0 mm glass beads did not dechlorinate PCBs. Soil-free culture without glass beads also failed to dechlorinate PCBs. The soil-free culture containing 0.5 mm glass beads dechlorinated 42.6 ± 12.0 mol% in total PCBs. This soil-free culture was more effective than soil culture for dechlorinating PCBs ranging from dichlorinated PCBs to tetrachlorinated PCBs. Clone analysis of the 16S rRNA gene sequences showed that one of the predominant groups of microorganisms in the soil-free culture comprised heat-tolerant and spore-forming bacteria from the phylum Firmicutes. Heat treatment (100 °C, 10 min) did not destroy the PCB-dechlorination activity of the soil-free culture with glass beads. These results suggest that unknown species of the phylum Firmicutes were involved in PCB dechlorination in the soil-free culture. In this study, we succeeded in using a liquid medium containing glass beads as an inorganic soil substitute and showed that such a medium enhances PCB-dechlorination activity. Our study provides valuable information for developing PCB-bioremediation techniques using dechlorinating bacteria in anoxic contaminated soils and sediments.

Keywords Polychlorinated biphenyls · Dechlorination · Glass beads · 16S rRNA gene

D. Suzuki (✉) · D. Baba · A. Katayama
EcoTopia Science Institute, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
e-mail: dais@esi.nagoya-u.ac.jp

V. Satheeja Santhi · R. D. Jebakumar Solomon
Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India

Introduction

Polychlorinated biphenyls (PCBs) have 209 possible congeners and isomers containing chlorine atoms between one and ten. PCBs were in worldwide use for industrial and commercial purposes such as dielectric, heat transfer, hydraulic fluids, plasticizers and fire retardants until the 1970s (Pieper and Seeger 2008; Erickson and Kaley 2011). These PCB applications resulted in release into the environment of PCBs. Because PCBs are hydrophobic and chemically stable, they often accumulate in anaerobic environments such as sediments and estuaries (Wiegel and Wu 2000). PCBs can also bioaccumulate the fatty tissue of mammal animals and fish though the food chain (Sage 1993; German and Zakonnov 2003; Brázová et al. 2012) and some PCB congeners and isomers are known to be endocrine disrupters (Borja et al. 2005).

Commercial PCB mixtures such as Aroclor and a single PCB congener and isomer are converted from highly toxic to less toxic forms by the anaerobic dechlorination activities of microorganisms (Quensen et al. 1990; Wu et al. 1998; Bedard 2008). Anaerobic bioremediation has therefore attracted attention as a potential inexpensive technology for detoxifying PCBs in anaerobic contaminated sites. Most of PCB-dechlorinating cultures contain sediments or soils, which were needed to sustain PCB-dechlorination activities in cultures (Quensen et al. 1990; Williams 1994;

Bedard and Quensen 1995; Bedard and May 1996; Van Dort et al. 1997; Wu et al. 1997). PCBs have been known to adsorb to organic components in soils (Voice and Weber1983; Lee and Batchelor 2004). PCB-dechlorinating microorganisms may need attachment to the interface provided by the sediment or soil particles to where PCBs absorb. It is difficult to produce PCB-dechlorinating cultures that do not contain sediments or soils and there are few reports of such cultures (Bedard et al. 2006; Cutter et al. 1998; Wu et al. 2000). Sediments or soils should be excluded from PCB-dechlorinating cultures with sediments and soils in order to isolate or characterize PCB-dechlorinating microorganisms.

Some *Dehalococcoides* spp. from the phylum Chloroflexi and *Dehalobacter* sp. from the phylum Firmicutes, have been reported to have the ability to dechlorinate PCB congeners and isomers (Cutter et al. 2001; Wu et al. 2002; Fennell et al. 2004; Fagervold et al. 2005; May et al. 2008; Adrian et al. 2009; Yoshida et al. 2009b), but the factors that influence anaerobic dechlorination of PCBs are not well understood. Elucidating these factors would require sediment- or soil-free cultures in a defined synthetic medium. Methods for excluding sediments or soils from PCB-dechlorinating cultures need to be developed to elucidate the factors that influence PCB-dechlorination activity.

In this study, we used a liquid medium containing glass beads as an inorganic alternative to solid soil in order to exclude soil from our previously reported PCB-dechlorinating soil culture (Baba et al. 2007). To establish PCB-dechlorinating soil-free cultures, we incubated our culture in liquid media containing glass beads of different sizes. The soil-free culture containing 0.5 mm glass beads exhibited significantly greater PCB-dechlorination activity than the original soil culture. We investigated the effects of some electron donors and inhibitors on the PCB-dechlorination activity of the soil-free culture and estimated the compositions of the microbial communities by respiratory quinone analysis and clone analysis of 16S rRNA gene sequences.

Materials and methods

PCBs

A PCB mixture (technical formulations of Kanechlor [KC]-300 and KC-400 [1:1 w/w]; Kanegafuchi Chemical Industry, Osaka, Japan) was used for this investigation. Trichlorinated (39.9 % w/w) and tetrachlorinated (44.4 % w/w) biphenyls were the major constituents of the mixture, with the remainder comprising more than 50 different PCB congeners possessing 2–7 chlorine atoms. The PCB mixture was diluted in acetone to yield a stock solution of 1 g/l.

Inorganic soil substitute

Glass beads were used as an inorganic soil substitute. Each set of glass beads had uniform particle size (D_{50}). Glass beads with diameters of 0.05, 0.5, and 2.0 mm (Potters-Ballotini Co. Ltd., Ibaraki, Japan) were used in this study. The glass beads were washed with 10 % (v/v) nitric acid solution, rinsed thoroughly with distilled water, and dried before use.

PCB-dechlorinating soil-free cultures with glass beads

A liquid medium comprising the following components was used as a basal medium in this study (g/l): 0.5 K_2HPO_4, 0.5 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7\ H_2O$, 0.02 $FeSO_4 \cdot 7\ H_2O$, 0.5 yeast extract, 10 sodium lactate, 8 sodium acetate, and 0.001 sodium resazurin, adjusted to pH 7.0 with NaOH. This medium was designated AL medium glass beads of different size (4 g each) were placed in individual 100 ml glass vials and were autoclaved at 121 °C for 30 min. The PCB stock solution (1 g/l PCB mixture in acetone) was added at 100 µl/vial (100 µg of PCB mixture/vial), followed by the addition of 15 ml of the sterilized AL medium under anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). The vial was then filled with 100 % nitrogen and sealed with a Teflon-coated butyl rubber stopper.

We used the PCB-dechlorinating paddy soil culture (PS culture) from our previous study (Baba et al. 2007) as the inocula. The PS culture was inoculated into glass bead-containing medium and glass bead-free AL medium as a soil suspension at a ratio of 5 % (v/v), and the media were incubated. Unless otherwise noted, all cultures were statically incubated at 30 °C for 56 days in the dark under anaerobic conditions. All experiments were performed in triplicate or more. After incubation, each culture was transferred to fresh glass bead-containing or glass bead-free AL medium at a ratio of 5 % (v/v). For example, the culture containing 0.5 mm glass beads was transferred to fresh AL medium containing 0.5 mm glass beads, and the culture without glass beads was transferred to fresh AL medium without glass beads. Each soil-free culture was established after 2 such transfers. The soil-free cultures containing 0.05, 0.5, and 2.0-mm glass beads were designated the GB0.05, GB0.5, and GB2.0 cultures, respectively, and the soil-free glass bead-free culture was designated the L culture. Each soil-free culture was maintained using incubation and transfer procedures.

To evaluate the PCB dechlorination properties of each glass bead culture and the L culture, sterile control cultures of each culture were prepared by autoclaving each culture at 121 °C for 30 min, adding 0.1 % (w/v) paraformaldehyde, and then incubating under the same conditions as those used

for the other cultures. The PCB-dechlorination activity of each glass bead culture and L culture was determined by comparing the total residual PCBs in each glass bead culture and the L culture with that in the PS culture, which was also maintained as described previously (Baba et al. 2007).

To determine the maximum PCB-dechlorination activity of the GB0.5 culture, 15 ml of AL medium alone or 15 ml of AL medium containing 1,000 μg of PCB mixture was re-fed to the GB0.5 culture. After incubation, the PCB residues in both cultures were measured and compared with that in the sterile control to evaluate the PCB-dechlorination activity of the re-fed cultures.

AL medium without acetate/lactate and sulfate-free medium (SF medium) were used to examine the effects of various factors on the PCB-dechlorination activity of the GB0.5 culture after more than 15 passages. The composition of the SF medium was as follows (g/l): 1 NaCl, 0.5 KCl, 0.5 g NH_4Cl, 0.1 $CaCl_2 \cdot 2\ H_2O$, 0.1 $MgCl_2 \cdot 6\ H_2O$, 0.1 yeast extract, 0.001 sodium resazurin, 1 ml of trace element solution SL-10 (Widdel et al. 1983), 1 ml of Se/W solution (Widdel et al. 1983), 1 ml of 0.02 mM FeS solution (Ehrenreich and Widdel 1994), and 100 ml of 200 mM MOPS solution, adjusted to pH 7 with KOH. The GB0.5 culture was incubated under the conditions described below, and the PCB-dechlorination activity of the GB0.5 culture under each set of conditions was evaluated and compared with that of the PS culture in AL medium. To examine the effects of different carbon and energy sources on PCB-dechlorination activity, the GB0.5 culture was grown individually in the presence of 20 mM acetate, 20 mM formate, 20 mM lactate, 20 mM pyruvate, 5 % H_2/20 mM acetate, and 20 mM acetate/20 mM lactate. To examine the effects of inhibitors, the GB0.5 culture was grown individually in the presence of 2 mM sodium molybdate, an inhibitor of sulfate-reducing bacteria (Taylor and Oremland 1979), and 2 mM 2-bromoethanesulfonic acid, a methanogen inhibitor (Gunsalus et al. 1978). To examine the effect of heat treatment, the GB0.5 culture was incubated for 10 min at 100 °C and then continuously at 30 °C for 56 days. The dechlorination activity of the GB0.5 culture using a sole PCB congener as an electron acceptor was tested by growing the culture in the presence of PCB #61 (2,3,4,5-tetrachlorinated biphenyl). The effects of the inhibitors, heat treatment, and PCB #61 were tested on the GB0.5 culture grown in the presence of acetate/lactate (20 mM each), which functioned as electron donors.

Measurement of PCB residues

After incubation, the culture samples were extracted with a chloroform and methanol mixture (2:1). The PCBs partitioned into the organic solvent phase, which was further extracted with *n*-hexane and then cleaned up using Sep-Pak Plus Silica cartridges (Waters, Milford, MA, USA) and a deactivated Florisil column with *n*-hexane as the eluent. The cleaned-up PCB fractions were analysed with a gas chromatograph (GC-2014; Shimadzu, Kyoto, Japan) equipped with an ^{63}Ni electron-capture detector and an HT8 capillary column (inner diameter, 0.22 mm; length, 50 m; film thickness, 0.25 μm; SEG, Austin, TX, USA). Data collection and analysis were performed using a Shimadzu Class GC Station (Shimadzu), and the amounts of the individual PCB congeners present were calculated as weight per incubation sample. The detection limit was assessed in a culture spiked with 100 μg of the KC-300/400 mixture and was determined to be 35.7 ng for each individual PCB congener. Congeners with IUPAC #56 and #60 were excluded from PCB detection because their peaks in the chromatogram overlapped those of interfering materials extracted from the cultures. Two indicator congeners, #153 and #180, were also excluded from the congeners measured by this procedure because their concentrations in the KC-spiked culture were below the limit of detection. The sum of the PCB congener amounts measured as described above corresponded to 83.0 % (w/w) of the technical formulations of KC-300 and KC-400 reported by Kim et al. (2004). The total recovery of the PCB mixture by chloroform–methanol extraction of the soil-free cultures spiked with 100 μg of the PCB mixture was 94.2 ± 3.5 % (w/w), and the recovery of each congener and isomer was at least 91.3 %. The measurement procedure for the PCB residues has been described in more detail previously (Baba et al. 2007).

The PCB-dechlorination activity of each glass bead culture and the L culture was evaluated as the percentages of total PCBs and individual PCB congeners remaining in the experimental cultures relative to those in each sterile control glass bead culture and control L culture after incubation. The average total amount of residual PCBs in the sterile control cultures after incubation was 89.6 ± 0.5 % (w/w). The statistical significance of the degree of total PCB dechlorination was evaluated by analysis of variance (F-test), followed by Scheffe's multiple comparison test. The statistical significance of the dechlorination activity for each PCB congener and isomer was examined by the Aspin–Welch test. The statistical analyses were performed using the Ekuseru-Toukei 2004 program (Social Survey Research Information, Tokyo, Japan) according to the developer's instruction manual.

The dechlorination rate was calculated as the percent reduction in total chlorine atoms. Dechlorination efficiency was also expressed as the total mole amount of chlorine atoms (nmol-Cl) removed, as calculated from the molar decrease in PCBs in the PCB-spiked culture, and the total mole amount of electron donors (mmol-e-donors), i.e., acetate and lactate, consumed during the incubation.

Respiratory quinone analysis

Respiratory quinones were obtained from the GB0.5 culture in the same chloroform–methanol extraction step used to obtain the PCBs. The respiratory quinones in the extract were fractionated with Sep-Pak Plus Silica cartridges (Waters), the menaquinone fraction was eluted with *n*-hexane containing 2 % diethyl ether, and the ubiquinone fraction was eluted with *n*-hexane containing 10 % diethyl ether; both fractions were dried under a nitrogen stream. The quinone fractions were dissolved in 100 µl of acetone and analysed with a high-performance liquid chromatography system equipped with a photodiode array detector and 2 Zorbax ODS columns connected in series (inner diameter, 4.6 mm; length, 250 mm; DuPont Co., Wilmington, DE, USA). The mobile phase (1.0 ml/min) was a 9:2 mixture of methanol and isopropyl ether. The analytical procedure used to detect respiratory quinones has been described in detail previously (Katayama and Fujie 2000).

Measurement of chloride

For determining the chloride content in the GB0.5 culture, we prepared the samples by filtering the culture through a membrane filter with 0.45 µm pore size and diluting with pure water. The chloride content was determined using an ion chromatograph (Metrohm Compact IC761; Metrohm Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions.

DNA extraction and cloning and restriction fragment length polymorphism analysis

DNA was extracted from the GB0.5 culture after more than 15 passages in AL medium containing 0.5 mm glass beads. Genomic DNA was extracted from 2 ml of the culture using an ISOIL DNA Extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The extracted DNA was used as a PCR (Polymerase chain reaction) template with the universal primers 27f and 1492r for bacterial 16S rRNA genes (Lane 1991) and TaKaRa Ex *Taq*® DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan). PCR was performed with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles comprising denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 90 s, and a final elongation step at 72 °C for 7 min. A single band corresponding to a PCR product of approximately 1,500 bp was detected by agarose gel electrophoresis. The PCR product was excised from the gel and purified with a Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) according to the manufacturer's protocol. A TOPO TA Cloning Kit (Invitrogen) was used to clone the PCR product into *Escherichia coli* TOP10F' competent cells (Invitrogen) according to the manufacturer's protocol. Vector-harboring clones were selected on Luria–Bertani (LB) plates supplemented with 50 µg ampicillin/ml, 40 µg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml, and 100 µg isopropyl-β-D-1-thiogalactopyranoside/ml. White *E. coli* colonies ($n = 101$) were each inoculated into 7 ml of LB liquid medium, and the single-colony *E. coli* cultures were incubated at 30 °C. Plasmid DNA was isolated from the *E. coli* cultures by centrifuging 2 ml of each culture to collect the cells, followed by an alkaline-sodium dodecyl sulfate lysis procedure. Agarose gel electrophoresis revealed that plasmids were isolated from each culture (data not shown). Each clonal plasmid was used as a PCR template as described above. Agarose gel electrophoresis revealed that each clonal plasmid produced a PCR product of approximately 1,500 bp. All the clones were then subjected to restriction fragment length polymorphism (RFLP) analysis as follows: 2 µl of each PCR product was incubated for 2 h at 37 °C with *Hha*I and *Hae*III (TaKaRa Bio Inc.) in a 20 µl (total volume) reaction containing buffer and sterile water. Following the incubation, the digested PCR products were separated by agarose gel electrophoresis. The RFLP patterns were analyzsed visually under UV light after staining with ethidium bromide.

Sequencing and phylogenetic analyses

Cloned 16S rRNA genes representative of all of the unique RFLP patterns were sequenced from each end using primers 27f and 1492r. Chimeric 16S rRNA gene sequences were checked using the Pintail chimera detection program (Ashelford et al. 2005). Several inaccurate sequences were excluded from phylogenetic analysis. Multiple alignments of the sequences with reference sequences in GenBank/EMBL/DDBJ were performed with the BLAST program (Altschul et al. 1997). A phylogenetic tree using the aligned partial 16S rRNA gene sequences of the bacterial clones from the GB0.5 culture and the type strains of the closely related members was constructed by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL W program (Thompson et al. 1994). All gaps and unidentified base positions in the alignments were excluded before assembly.

Nucleotide sequence accession numbers

The nucleotide sequences of the cloned 16S rRNA genes have been deposited in the DDBJ database under the accession numbers AB678351–AB678376.

Results

Establishment of PCB-dechlorinating soil-free culture with glass beads

Table 1 shows the total PCB residues in the cultures after two passages of the PS culture in media containing glass

Table 1 Effect of the GB particle size on the total residual PCBs in second-passage cultures derived from the PS culture

Condition	PS	L	GB2.0	GB0.5	GB0.05
Material/particle size	Soil	None	GB/2.0	GB/0.5	GB/0.05
Residual amount (wt%)[a]	86.0 ± 2.6 %	89.2 ± 8.3 %	77.3 ± 11.6 %	72.9 ± 6.8 %*	92.5 ± 2.0 %

The conditions were defined in Materials and Methods

The experiments were performed in triplicate or greater

Abbreviations: PS paddy soil, *L* liquid, *GB* glass beads

* $P < 5$ %

[a] The significance of the decrease in the total residual weight % relative to that of the PS culture was determined by analysis of variance (F-test) followed by Scheffe's multiple comparison test

beads of different sizes. The amount of total PCB residues was significantly lower in GB0.5 culture than in the PS culture. The difference (in wt%) between the amounts of PCB residues in the original PS culture and the GB0.5 culture was approximately 13 %. Although the total PCB residues in the GB2.0 culture appeared to have reduced, the reduction, which we attributed to the wide fluctuations among the replicates, was not significant. The amount of the total PCB residues in the L culture or in the GB0.05 culture was higher than that in the PS culture. On the basis of these results, we selected the GB0.5 culture as the soil-free culture for the further investigation. The time courses of total PCB residue depletion and chloride formation in the GB0.5 culture were monitored after several transfers of the GB0.5 culture (Fig. 1). The decrease of PCBs was observed until 56 days, but not later. The increase of chloride was also observed until day 56. These results showed that chlorine was released from PCBs in the GB0.5 culture. The above results indicated that the PCB-dechlorinating soil-free culture was best established from a PS culture to which 0.5 mm glass beads had been added.

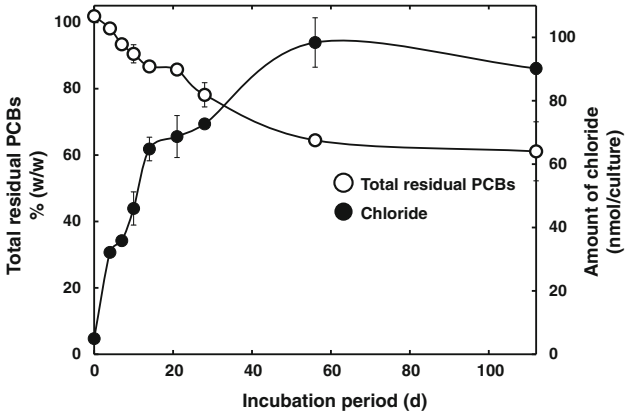

Fig. 1 Changes in the total amount of PCB residues (% w/w) and in the amount of chloride (nmol/culture) in the GB0.5 culture over 112 days of the incubation

PCB dechlorination activity and maximum dechlorination activity of the soil-free culture with glass beads

The PCB-dechlorination activity of the GB0.5 culture was successfully maintained over 3 years and was further enhanced by more than 15 passages at 5 % (v/v) ratios, with an incubation interval of 56 days. Table 2 lists the residual concentration (mol%), relative to that in the control culture, of each PCB congener and isomer in the GB0.5 culture after incubation for 56 days during culture maintenance by serial transfer. In comparison with the control, the GB0.5 culture dechlorinated most of the PCB congeners and isomers in the KC-300/400 PCB mixture. The total PCB residues in the GB0.5 culture decreased to 58.4 ± 12.0 mol% of that of the control (significant at $P < 0.01$). The average dechlorination was also calculated as 39.0 ± 10.7 mol% on the basis of the residual PCBs (significant at $P < 0.01$).

Table 2 also shows significant differences between the residual mol% of several PCB congeners and isomers in the KC-300/400 mixture of the GB0.5 and PS cultures (Baba and Katayama 2007). The GB0.5 culture significantly dechlorinated all the di- to tetrachlorinated PCB congeners and isomers in the KC-300/400 mixture except PCBs #6 (2,3-dichlorobiphenyl), #18 (2,2',5-trichlorobihenyl), #52/69 (2,2',5,5'-tetrachlorobiphenyl/2,3',4,6-tetrachlorobiphenyl). The lack of significant GB0.5 culture dechlorination of PCB congeners and isomers #6, #18, and #52/#69 was attributed to wide fluctuation in the residual percentages of these forms during the transfers. The GB0.5 culture significantly dechlorinated PCB #103 (2,2',4,5',6-pentachlorobiphenyl) but did not dechlorinate any of the other penta- or hexachlorinated PCB congeners and isomers in the KC-300/400 mixture.

When AL medium was re-fed into a GB0.5 subculture, the amount of total PCB residues in the culture was 23.1 ± 5.8 % (w/w) (25.7 ± 6.2 mol%) that in the sterile control. Approximately 75 μg of the added 100 μg total PCB mixture had been dechlorinated. Most of the PCB

Table 2 Average residual amounts of individual PCB congeners and isomers (mol%) in the GB0.5 culture and significant differences from those of the PS culture

IUPAC number	Cl-substituted positions	mol% In initial PCB mixture	Residual amount (mol%)	±SD	Significant difference[a]
6	2-3	0.6	104.7	±112.9	
8	2-4	4.5	38.8	±15.9	***
16	23-2	0.3	63.1	±18.8	**
20/33/45	23-3/34-2/236-2	7.9	57.6	±15.9	***
17	24-2	2.8	50.0	±14.5	***
28/53	24-4/25-26	10.1	51.2	±16.0	***
18	25-2	7.7	70.3	±40.4	
26/50	25-3/246-2	1.3	55.9	±14.6	***
31	25-4	9.6	58.8	±16.6	***
32	26-4	2.0	55.2	±23.8	***
44	23-25	6.1	71.3	±19.2	*
46	23-26	3.2	72.2	±16.8	*
49/43	24-25/235-2	4.2	64.8	±22.1	**
66	24-34	5.8	70.5	±11.0	**
52/69	25-25/246-3	6.6	79.0	±22.7	
70	25-34	7.0	69.1	±8.6	***
72/64	25-35/236-4	3.4	65.7	±18.3	**
41	234-2	0.8	69.9	±23.4	*
74/95	245-4	3.9	73.8	±13.9	*
110	236-34	2.6	83.9	±6.6	
105	234-34	1.2	78.8	±17.3	
97/117/86	245-23/2356-4/2345-2	1.1	75.2	±18.7	
101/150	245-25/236-246	3.1	76.1	±12.0	
118	245-34	1.9	76.3	±9.7	
103/71	246-25/26-34	1.2	69.2	±16.0	**
138	234-245	0.3	83.6	±16.2	
136	236-236	0.2	70.5	±26.8	
149/77	236-245/34-34	0.2	79.7	±14.3	
176	2346-236	0.2	76.7	±12.0	
Total		100.0	58.4	±12.0	***

The residual amounts are expressed as mol% of the sterile control. The values are the averages after 56 days of incubation for each of 15 serial transfers during the maintenance of the anaerobic PCB-dechlorinating culture

SD standard deviation

The symbols *, **, and *** denote significant differences at less than 5, 1, and 0.1 % probability, respectively

[a] The significance of the difference in the residual mol% of each PCB congener and isomer in the KC-300/400 mixture between the GB0.5 culture and the PS culture (Baba and Katayama 2007) was determined by the Aspin-Welch test

congeners and isomers in the KC-300/400 PCB mixture were dechlorinated during the incubation (Fig. 2), and biphenyl or less-chlorinated biphenyl as dechlorination products were not observed. These results showed that most of the PCB congeners and isomers in the GB0.5 culture were dechlorinated by re-feeding of the AL medium. In addition, when AL medium containing 10 times the amount of added KC-300/400 PCB mixture (1,000 μg) was re-fed to the GB0.5 culture, the culture dechlorinated approximately 60 μg of the total PCB mixture during the 56 days incubation. This means that the GB0.5 culture could dechlorinate approximately 60–75 μg of the KC-300/400 PCB mixture in 56 days, regardless of the amount of PCB added.

Effects of electron donors, inhibitors, and a single electron acceptor on PCB-dechlorination activity of GB0.5 cultures.

Table 3 lists the effects of various electron donors, inhibitors, and a single electron acceptor on the PCB-dechlorination activity of GB0.5 cultures grown in 2 different media. To examine the effects of these factors, AL medium containing modified carbon and energy sources and SF medium were used (see Materials and Methods). When AL medium was used as the basal medium, the total residual percentage of PCBs in the GB0.5 culture containing 20 mM acetate was significantly reduced by more than 10 % relative to that of the PS culture. The total residual percentage of PCBs in the GB0.5 culture containing 5 % H_2 and 20 mM acetate was not significantly different from that of the PS culture. When SF medium was used, the GB0.5 culture did not significantly dechlorinate PCBs relative to the PS culture under any tested condition. Neither heat treatment (100 °C, 10 min) nor inhibitors (molybdate and 2-bromoethanesulfonic acid) affected the

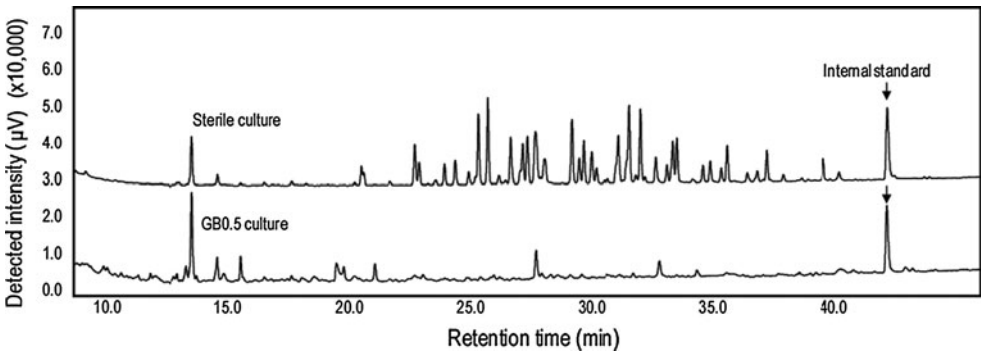

Fig. 2 Comparison of chromatograms obtained of PCBs from the GB0.5 culture and the sterile control

PCB-dechlorination activity of the GB0.5 culture. The GB0.5 culture did not significantly dechlorinate the electron acceptor PCB #61.

Microbial composition of GB0.5 culture based on respiratory quinone analysis

After the 56 days incubation period, the microbial composition of the GB0.5 culture was assessed by respiratory quinone analysis. We detected 2.65 μmol menaquinone-$7H_2$ in the GB0.5 culture, while the levels of any other quinones present in the culture were below the limit of detection (0.02 nmol).

Composition of the bacterial population in the GB0.5 culture

We amplified and cloned 101 partially complete bacterial 16S rRNA genes from genomic DNA extracted from the GB0.5 culture. The clones were classified into 36 groups by RFLP analysis. A representative clone from each group was sequenced. Ten sequenced clones were inaccurate or were chimeric sequences and were therefore excluded from the subsequent phylogenetic analysis. The phylogenetic affiliations of the sequenced clones were estimated. The phylogenetic analysis of the bacterial population of the GB0.5 culture showed that the 16S rRNA gene sequences fell into 4 different phyla: Chloroflexi, Proteobacteria, Firmicutes, and Bacteroidetes (Fig. 3). The sequence data from the remaining 26 clones from the GB0.5 culture showed that the most abundant bacteria in the culture were members of the class Deltaproteobacteria of the phylum Proteobacteria (10 clones, 38.5 %) and members of the phylum Firmicutes (10 clones, 38.5 %), followed by members of the Bacteroidetes (5 clones, 19.2 %) and Chloroflexi phyla (1 clone, 3.8 %). The closest relative of 9 of the clones affiliated with the class Deltaproteobacteria was *Desulfovibrio* sp. JG5, which exhibited approximately 98–99 % sequence similarity, while the closest relative of the remaining Deltaproteobacteria clone was *Desulfovibrio vulgaris* strain "Miyazaki F", which exhibited approximately 98 % similarity. The closest relative of 9 of the clones affiliated with Firmicutes was *Sporoanaerobacter acetigenes*, which exhibited approximately 87–98 % similarity. The remaining clone affiliated with the Firmicutes was closely related to *Clostridium methylpentosum*, exhibiting approximately 85 % similarity. The closest relatives of the clones affiliated with the phylum Bacteroidetes were *Dysgonomonas mossii* (approximately 99 % similarity), *Bacteroides* sp. W7 (approximately 95–97 % similarity), and *Parabacteroides* sp. Lind7H (approximately 93–94 % similarity). The closest relative of 1 clone affiliated with Chloroflexi was Bacterium K-4b6, exhibiting approximately 90 % similarity. The clone was distantly related to known *Dehalococcoides* spp. (less than 80 % similarity), which are dechlorinators in the phylum Chloroflexi.

Table 3 Effects of electron donors, bacterial inhibitors, and a single electron acceptor on GB0.5 cultures grown in 2 different media

	AL medium	SF medium
Electron donor		
Acetate (20)	++	−
Formate (20)	+	−
Lactate (20)	+	−
Pyruvate (20)	+	−
5 % H_2 + acetate (5)	−	−
Acetate (20)/Lactate (20)	++	−
Inhibitor[a]		
Heat treatment	++	+
Molybdate (2)	++	+
BES (2)	++	+
Electron acceptor[a]		
#61 (2,3,4,5-TeCB) (50 μM)	−	−

Abbreviations: BES 2-bromoethanesulfonic acid, *TeCB* tetrachlorinated biphenyl

Symbols correspond to the activity compared with that of the PS culture with AL medium; -: <1 %, +: <5 %, ++: >10 % increase. The numbers in parentheses show the concentrations in mM of each substrate added to the medium. The components of the AL medium and SF medium were described in Materials and Methods

[a] The effects of the inhibitors and a single electron acceptor were assessed in the GB0.5 culture amended with acetate/lactate as electron donors

Discussion

We attempted to establish a PCB-dechlorinating soil-free culture from a soil culture (PS culture) that originated from a gley paddy soil with no contamination history and had been reported previously (Baba et al. 2007). In the current study, liquid medium containing glass beads as an inorganic alternative to soil was used to exclude the soil from the PS culture in order to obtain a soil-free PCB-dechlorinating culture. Only the GB0.5 culture, comprising liquid medium containing 0.5 mm glass beads, exhibited significant PCB-dechlorination activity, whereas the glass bead-free L culture did not dechlorinate the PCBs added to the medium. The PCB-dechlorination activity of the GB0.5 culture differed somewhat from that of the PS culture. The former had greater PCB-dechlorination activity for most of the tri- and tetrachlorinated PCB congeners and isomers in the KC-300/400 mixture than the latter (Table 2). In a previous study (Baba and Katayama 2007), we reported another anaerobic PCB-dechlorinating culture obtained from the same PS culture from which the GB0.5 culture originated. This was a soil culture established by adding burnt soil (BS) originally obtained from uncontaminated paddy soil to the PS culture. The BS culture exhibited increased PCB-dechlorination activity relative to the PS culture (Baba and Katayama 2007). Compared with the BS culture, the GB0.5 culture exhibited strong dechlorination activity against trichlorinated PCB congeners and isomers in KC-300/400, but weak activity against tetrachlorinated PCB congeners and isomers. The dechlorination activity of the GB0.5 culture against the trichlorinated PCB congeners and isomers in KC-300/400 was stronger than that of both the PS and BS soil cultures. Therefore, a PCB-dechlorinating soil-free culture was obtained from a PCB-dechlorinating soil culture by using liquid medium containing glass beads and enhanced the dechlorination of some PCB congeners and isomers in KC-300/400.

Adding glass beads to liquid medium as a soil substitute effectively enhanced anaerobic microbial PCB dechlorination. Nonpolar organic compounds such as PCBs have been known to adsorb to organic components in soils (Voice and Weber 1983; Lee and Batchelor 2004). As glass beads are an inorganic material, it is likely that PCBs sorption are weaker to their surfaces. The enhancement of PCB-dechlorination activity in the GB0.5 culture may have been due to increased efficiency of PCB acquisition by dechlorinators in liquid medium with glass beads. However, in the current study, PCB-dechlorination activity in liquid cultures with glass beads appeared to depend on the size of the glass beads; only the GB0.5 culture dechlorinated PCBs. Therefore, enhancement of the PCB-dechlorination activity in the GB0.5 culture may not depend on increased efficiency of PCB acquisition by the dechlorinators. A medium containing glass beads also contains interspaces between the beads; these interspaces do not exist in glass bead-free medium. Dechlorination would be restricted to within these interspaces. In the current study, the glass bead-free L culture exhibited no PCB-dechlorination activity. Dechlorinators would require the interspaces formed between glass beads or soil particles for PCB dechlorination to take place. The interspaces formed between the 0.5 mm glass beads would have provided optimal space for PCB dechlorination.

The known sediment-free culture and "*Dehalococcoides*" sp. strain CBDB1 could dechlorinate highly chlorinated PCB congeners and isomers but not less-chlorinated PCB congeners and isomers (Bedard et al. 2006; Adrian et al. 2009). Our GB0.5 culture exhibited high dechlorination activity for the tri- or tetrachlorinated PCB congeners and isomers in the KC-300/400 mixture. The addition of glass beads to the medium would enhance the efficiency of the PCB-dechlorination activity of dechlorinators against less-chlorinated PCB congeners and isomers.

Many studies have reported anaerobic microbial dechlorination of simple PCB congeners by pure strains and sediment/soil-free cultures (Cutter et al. 2001; Wu et al. 2000, 2002; Fennell et al. 2004; Fagervold et al. 2005; Natarjan et al. 1998; May et al. 2006; Yan et al. 2006; Yoshida et al. 2009b). The GB0.5 culture exhibited high dechlorination activity against various PCB congeners and isomers in the KC-300/400 mixture but did not dechlorinate PCB #61, which had been added as a single PCB congener. PCB congeners and isomers such as PCB #61 that are chlorinated on a single phenyl ring were absent from the KC-300/400 mixture. The GB0.5 culture may exhibit dechlorination activity for PCB congeners and isomers chlorinated on both phenyl rings, and therefore only dechlorinate such PCB mixtures.

The 16S rRNA gene sequence-based clone analysis showed the composition of the bacterial population of the GB0.5 culture. The major groups were bacteria belonging to the class Deltaproteobacteria in the phylum Proteobacteria and to the phylum Firmicutes, while bacteria in the Bacteroidetes and Chloroflexi phyla formed minor groups. In addition, respiratory quinone analysis showed that the GB0.5 culture contained menaquinone-7H_2. Bacteria of the phylum Firmicutes and the class Deltaproteobacteria of the phylum Proteobacteria contain this quinone (Katayama and Fujie 2000). All 16S rRNA gene sequences of the bacterial clones belonging to the class Deltaproteobacteria in the GB0.5 culture were closely related to those of *Desulfovibrio* species, which are sulfate-reducing bacteria. *Desulfovibrio* species contain menaquinone-6 or menaquinone-6H_2 (Collins and Widdel 1986). The menaquinone-7H_2 in the GB0.5 culture would have been derived from bacterial species from the phylum Firmicutes.

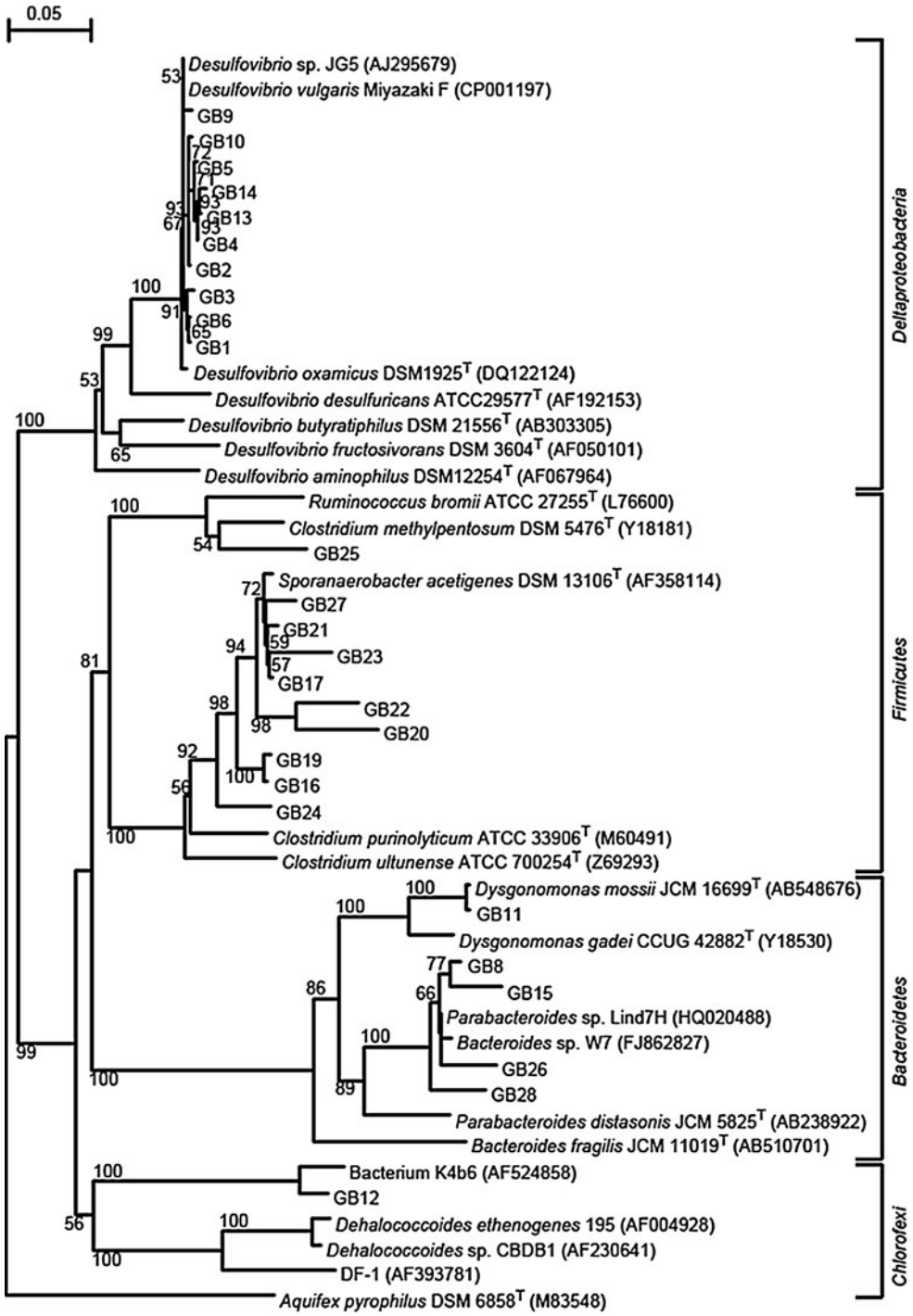

Fig. 3 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacterial clones from the GB0.5 culture and their closest relatives. *Bootstrap values* (expressed as percentages of 1,000 replications) above 50 % are shown at the branch nodes. *Bar*, 5 % estimated difference in nucleotide sequence position. *Aquifex pyrophilus* DSM6858^T was used as the outgroup

Bacterial species from Firmicutes have been found in other sediment-free PCB-dechlorinating cultures (Bedard et al. 2006; Yan et al. 2006). Several species from Firmicutes are dechlorinators, including the *Desulfitobacterium* and *Dehalobacter* species (Holliger et al. 1998; Villemur et al. 2006; Yoshida et al. 2009a). *Desulfitobacterium dehalogenans* can dechlorinate hydroxylated PCBs (Utkin et al. 1994; Wiegel et al. 1999). A previous study reported that an enrichment culture containing *Dehalobacter* species could dechlorinate 2,3,4,5-PCB and 2,3,4-PCB (Yoshida et al. 2009b). All the 16S rRNA gene clones of Firmicutes in the GB0.5 culture were unrelated to species of the *Desulfitobacterium* or *Dehalobacter* genus. Heat treatment (100 °C, 10 min) did not destroy the PCB-dechlorination activity of the GB0.5 culture. Bacterial species from Firmicutes could therefore play a role in PCB dechlorination by the GB0.5 culture because most of these species form heat-tolerant spores. This result suggests that unknown

species of the phylum Firmicutes were involved in PCB dechlorination by the GB0.5 culture.

Sulfate-reducing bacteria such as the *Desulfovibrio* species reduce sulfate to sulfide and oxidize lactate to acetate (Kuever et al. 2005). *Desulfovibrio* species in the GB0.5 culture would have oxidized lactate to acetate and reduced sulfate to sulfide. *Desulfovibrio* species have been found in both sediment-containing and sediment-free PCB-dechlorinating cultures (Cutter et al. 2001; Wu et al. 2002). The anaerobic ultramicrobacterium strain DF-1 has been reported to require coculture with *Desulfovibrio* species or with a *Desulfovibrio* species cell extract for growth on hydrogen and PCBs in mineral medium (May et al. 2008). Neither the addition of molybdate nor culture in SF medium inhibited the PCB-dechlorination activity of the GB0.5 culture. Our findings suggest that the *Desulfovibrio* species in the GB0.5 culture did not dechlorinate PCBs and were not involved in PCB dechlorination by other microorganisms in the GB0.5 culture.

Bacterial species of the phylum Bacteroidetes were a minor group in the GB0.5 culture. No known species of Bacteroidetes is a dechlorinator. Species from Bacteroidetes in the GB0.5 culture would have fermented lactate or yeast extract.

A single 16S rRNA gene clone in the GB0.5 culture belonged to the phylum Chloroflexi. The clone was distantly related to members of the genus *Dehalococcoides*, which are dechlorinators and belong to Chloroflexi (less than 85 % 16S rRNA gene sequence similarity). A 16S rRNA gene clone distantly related to members of *Dehalococcoides* has been detected in a recently reported PCB-dechlorinating marine sediment culture (Zanaroli et al. 2010). This clone was closely related to the sequence of the uncultured Chloroflexi bacterium clone SF1 that had been identified as a PCB dechlorinator in a sediment culture (Fagervold et al. 2005) that dechlorinated PCB congener #91. The 16S rRNA gene sequence analysis showed that 2 bacterial species that dechlorinated several single PCB congeners and isomers, a dechlorinator in an *o*-17 culture (Cutter et al. 2001) and the anaerobic ultramicrobacterium strain DF-1 (May et al. 2008) of the phylum Chloroflexi, were also distantly related to members of the genus *Dehalococcoides*. The bacterial clone from the phylum Chloroflexi in the GB0.5 culture may be an unknown PCB dechlorinator that is distantly related to members of the genus *Dehalococcoides*.

Conclusions

In this study, we succeeded in establishing a PCB-dechlorinating soil-free culture from a PCB-dechlorinating soil culture by using a liquid medium containing glass beads as an inorganic soil substitute. In addition, this soil-free culture exhibited enhanced PCB-dechlorination activity. Our study provides valuable information for developing PCB bioremediation techniques involving dechlorinators.

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