Enrichment and Isolation of Novel Anaerobic Microorganisms Capable of degrading various kinds of POPs

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Abstract
From the flooded field soils in Japan, and PCB contaminated river sediments in US, 17 anaerobic microorganisms, which decreased the residual content of various kinds of POPs such as hexachlorobenzene, Endrin, Aldrin, Dieldrin, Lindane, decafluorobiphenyl, and decachlorobiphenyl, were isolated. As halogenated reaction intermediates were not ordinary produced in most reactions and biphenyl or mono-chlorobiphenyl was sometimes produced from hexachlorobenzene, highly-chlorinated chemicals seemed to be dehalogenated sequentially by these anaerobic microorganisms without accumulation of halogenated intermediates. As the isolated microorganisms had novel morphological and physiological characters, which had clearly distinguished these microorganisms from any of the known microorganisms, they were supposed to be novel microorganisms, which had not been isolated until this time. As these microorganisms were indigenous to soil or sediments and seemed to have novel effective dechlorination mechanism, they seemed to be useful for bioremediation of various kinds of POPs accumulated in soils and sediments.

Introduction

Because of extremely high resistance to oxidative degradation of aerobic microorganisms, highly-chlorinated chemicals, such as PCBs, Dioxins, HCB, and Drins (POPs: Persistent Organic Pollutants), have extremely high environmental persistency. As the dechlorinated POPs became less toxic and more easily degradable by aerobic bacteria, the reductive dechlorination was supposed to be an effective bioremediation method for POPs. Dehalorespiration is an efficient dechlorination mechanism in which halogenated compounds can be used as the final electron acceptor[1]. Several bacterial groups, e.g., sulfur reducing bacterial group such as *Desulfitobacterium* sp.[2], Phylum Chloroflexi such as *Dehalococcoides* sp.[3], reported to have anaerobic dehalorespiration, which dechlorinated reductively tetrachloroethene[2)] or chlorobenzenes[4]or chlorophenols or PCBs or Dioxins[5]. We had isolated novel anaerobic microorganisms, which could degrade various kinds of POPs by anaerobic dehalorespiration[6,7]. Hear we represent isolation, substrate specificity, and mechanism of degradation of the novel anaerobic microorganisms.

Materials and Methods

1) Culture medium[8]

All media in these experiments included modified basal medium composed the following components in grams per liter: NaCl, 8.4; KCl, 0.27; Na_2HPO_4, 0.6; NH_4Cl, 0.5; cysteine-HCl・H_2O,0.25; resazurin, 0.001. To the solution, 1% (vol/vol) trace element solution (nitrotriacetic acid, 1.5; $MgSO_4$, 3.0; $MnSO_4$,0.5; NaCl, 1.0; $FeSO_4$, 0.1; $CaCl_2$, 0.1; $CoCl_2$, 0.1; $ZnSO_4$, 0.1; $CuSO_4$, 0.01; $AlK(SO_4)_2$, 0.01; HBO_3, 0.01; Na_2MoO_4, 0.01) was added (Berkaw et al 1996). The medium was boiled under oxygen-free N_2 to remove O_2 completely. After addition of Na_2S・$9H_2O$(0.25 g) and $NaHCO_3$(2.53 g), the pH of the medium was adjusted to 7.3 to 7.5 by flushing N_2-CO_2 (95:5).

The medium was dispensed into N_2 flushed serum bottles (160 ml serum bottles), and was sealed with teflon-lined butyl stoppers secured by aluminum crimp collars. The bottles were sterilized by autoclave tree times (120℃, 1 hr). To the sterile medium, 2% (vol/vol) anaerobic sterile Wolin vitamin solution[9] (mg/L; biotin, 2; folic acid, 2; pyridoxine hydrochloride, 5; riboflavin, 5; thiamine, 5; nicotinic acid, 5; pantothenic acid, 5; vitamin B12, 0.1; p-aminobenzoic acid,5; thioctic acid, 5), electron donor; sodium acetate or formate or lactate (20 mM), and salt solution; $MgCl_2$・$6H_2O$(3.95) and $CaCl_2$・$2H_2O$(0.05), were added from sterile anaerobic stock solution (x50).

2) Samples, enrichments, and isolation[6,7]

Paddy field soil, Andosol at Nishigoshi, Kumamoto, had stored in reservoir tank under flooded condition with PCB 500ppm. After incubation for 26 months at 28℃ in dark condition, hexachlorobenzene （HCB） was applied to a final concentration of 50 ppm. After100 days of the incubation, 50 g of soil was sampled and the degradation intermediates were analyzed by using GC-MS.

Freshly sampled paddy field soil, Andosol at Koshi, Kumamoto (PCB nor the other poly-chlorinated aromatic compounds had not been applied; PA), and the above mentioned PCB applied field soil (B), were inoculated into duplicated bottles (1 and 2) with the anaerobic media (20% vol/vol), Hexachlorobenzene (200 ppm), and sodium acetate, formate, and lactate (2.5 mM) on Oct. 12, 2000, and incubated at 42℃with vigorous reciprocal shaking (250 rpm). On Nov.14 (1st), and Dec.2, 2000 (2nd), 10% transfer was made using the same anaerobic medium, respectively.

On Jan.18,2001(3rd transfer), 10% and 1% transfers were made using the anaerobic media adding 2-bromoethansuflonate (BES; specific inhibitor of methanogenic bacteria)[10], or pre-incubating 80℃ 20 min to observe the effect of selective incubation on residual HCB content (%) after 53 days of anaerobic incubation.

On Mar.5, 2001(4th transfer), 5% and 1% duplicated transfers were made using the anaerobic media containing sodium acetate (2.5 mM), sodium formate (2.5 mM), or sodium lactate (2.5 mM) to observe the effect of electron acceptor on the residual HCB content (%) after 11 days of anaerobic incubation.

Serial dilution transfers, from 10^{-1} to 10^{-8} dilutions, were made from 4th transfer solutions showing the lowest residual HCB content (%). After 2 weeks anaerobic incubation, anaerobic microorganisms were isolated from these enrichments showing the residual HCB content by using 2 kinds of isolation method, 1) by using anaerobic agar plate methods which consisted of 10 mL of the anaerobic medium solidified with 1.5% agarose and supplemented with pentachlorobenzene and acetate or formate to final concentration of 250µM and 25 mM respectively, 2) by using anaerobic agar plate methods which consisted of 10 mL of the anaerobic medium solidified with 1.5% agarose and acetate or formate to final concentration 25 mM respectively, and Hexachlorobenzene (2-5 mg) was placed on a lid of the plate.

By the same method, enrichment was stared from Hudson river sediment, Hot spot 28 (January 24, 2001)[10,11], Red Ceder Rive sediment and Little Missouri river sediment (April 9, 2001). By the similar method, 8 strains were isolated from the enrichment cultures.

3) Degradation of various POPs

Degradation activities of various kinds of halogenated compounds, HCB (200ppm), Endrin (100ppm), Aldrin (100ppm), Dieldrin (100ppm), Heptachlor (100ppm), γ BHC(100ppm), PCP(100ppm), PCNB (100ppm), decafluoro- biphenyl (100ppm), and decachlorobiphenyl (100ppm), were evaluated using the isolated microorganisms.

After anaerobic incubation of isolates and the halogenated compounds at 42℃with vigorous reciprocal shaking (250 rpm), incubation medium (1 mL) was sampled from the bottle. Pentachlorobenzene or tetrachlorobenzen (10ppm) was added to the sample, which was used as an internal standard to calculate a recovery ratio. The residual halogenated compound in the sample were extracted by shaking with 1 mL ether, and their amount was analyzed using a gas chromatograph equipped with ECD detector (Shimadzu GC-14B) and a Hewlett-Packard Ultrabond 1 capillary column (25 m by 0.2 mm〔inner diameter; SE-54 equivalent). GC-MS analyses were performed on a Perkin Elmer GCMS Q-910 instrument equipped with SUPELCO SPB™1 column (length: 30m, diameter:0.32 mm).

Results and Discussion

1) Enrichment and Isolation

One hundred days left under water-lodged condition, trace amount of tetrachlorobenzenes (1,2,3,4-TCB, 1,2,4,5-TCB) was produced from HCB in the upland field soil, which had been applied with PCB(500ppm) and left under water-lodged condition for 26 months . As 1,2,3,4-TCB and 1,2,4,5-TCB were also observed after 1 month anaerobic incubation with sodium acetate, sodium formate, and sodium lactate as electron donors of the paddy field soil, the reaction was supposed to be dechlorination by the anaerobic microorganisms. After 56 days of third enrichment culture from paddy field soil, one of the 1% transfer bottles using acetate as an electron donor had the activity to decrease residual HCB content. After 56 days from PCB treated soil, both of the 1% transfer bottles using acetate as an electron donor, both of the 5% and 1% transfer

bottles using formate as an electron donor, and 5% transfer bottles using lactate as an electron donor had the same activity. On the all the anaerobic agar plates applied with the highest dilution series showing the lowest residual HCB content, crystallized colonies, which were distinguishable from any of the other microorganisms were formed within 3 days of anaerobic incubation under N_2(Fig.1). As isolated eleven microorganisms formed the same colony and all the re-isolated microorganisms had the same activity, which lowered the residual HCB content, the isolated microorganisms were found to decrease residual HCB content.

Table 1. Residual content of various drins

	% of un-inoculated control			
Strain No.	Heptachlor	Endrin	Aldrin	Dieldrin
1.Hud 3-2	91.2	0.142	0.033	0.386
2.Hud 3-1	93.8	0.153	0.012	0.231
3.LM1-1	96.3	0.160	0.064	0.247
4.LM2-2	85.7	0.103	0.049	0.282
5.RR1-1	98.4	0.136	0.018	0.406
6.RR1-12	90.8	0.123	0.099	0.288
7.RR1-3	93.4	0.114	0.012	0.282
8.RR2-2	81.7	0.113	0.009	0.269

Fig.1. Isolated anaerobic microorganism (strain KW2). One month after anaerobic incubation.

2) Substrate specificity

Isolated microorganisms also decreased the residual content of several various Drin Insecticides, such as Endrin, Aldrin, and Dieldrin from 0.01% to 0.4% in 33 days, while they did not decreased the residual content of Heptachlor, which was remained from 81.7% to 98.4% in 44 days (Table 1). They also decreased the residual content of γ BHC (from 3.7% to 30.0%), decafluorobiphenyl (from 0.40% to 1.03%), and decachlorobiphenyl (from 23.80 to 57.54%), but did not decrease the residual content of PCP(from 46.4% to 102.4%), and PCNB (from 33.5% to 61.8%).

3) Degradation mechanisms

Degradation intermediate, such as chlorinated benzenes, have not been formed in the most of the culture medium. GC-MS analysis of culture medium of the isolate (LM2-2) indicated that 3-chlorobipenyl was produced from HCB. In the culture medium of the isolate (Hud2), 3-chlorobipenyl and biphenyl was produced from HCB. These results suggested that HCB was sequentially dechlorinated, and that biphenyl or monochlorobiphenyl was supposed to be formed by the coupling reaction with benzene, which had been produced by sequential dechlorination of HCB, and mono or dichlorobenzene, which had been produced by sequential dechlorination of HCB.

The isolated microorganisms had novel morphological and physiological characters, which had clearly distinguished these microorganisms from any of the known microorganisms; 1) they formed rigid crystallized colonies, 2) they had high affinity to the organic solvents, 3) they had high affinity to the a glass bottle, 4) they were not stained by any of the known staining solutions (gram stains solution etc.), 5) cell wall were too hard to extract chromosomal DNA by any of the known DNA extraction method such as lysozyme/SDS, freeze-thaw, and beed beading. No amplification product was also observed by PCR using universal primer for 16S rDNA nor 18S rDNA from purified chromosomal DNA fractions.

Only by using french press, small amount of chromosomal DNA could be extracted and the 18S rDNA was amplified by PCR by using the extract. Their taxonomical analysis is under progress.

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