BIODEGRADATION OF 2,3-DICHLOROBIPHENYL AND 3,4-DICHLROBIPHENYL BY NOVEL PCB-UTILIZING BACTERIA

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Introduction

Although reductive dechlorination is sometimes very effective at removing chlorines from chlorinated hydrocarbons, toxicity problems still remain in many cases. For complete detoxification and degradation (mineralization) of halogenated compounds, subsequent oxidative reactions are required. Biologically mediated degradation of chlorinated compounds has been studied extensively, because several microorganisms can use lightly chlorinated compounds as a carbon or energy substrate or transform them cometabolically under aerobic conditions. There are still many limitations in the microbial degradation of PCBs. Most of all, PCBs are usually not suitable for a microorganism as a carbon and energy substrate, and only monochlorobiphenyls have been previously shown to support growth of natural isolates. The experiments were based on the hypothesis that, under suitable conditions, bacteria might evolve with novel abilities to degrade PCBs and related compounds. Using a mineral medium amended with Aroclor 1242, mixtures of congeners, or single congeners, bacterial isolates with novel abilities to degrade 2,3-dichlrobiphenyl, 3,4-dichlorobiphenyl and other chlorinated compounds were obtained. The isolates were able to grow on the dichlorobiphenyls as a single carbon source and the phylogenic studies of the isolates were made by analyzing 16 rRNA genes.

Materials and Methods

In order to isolate novel strains or consortia capable of aerobic degradation, the contaminated sludge from the Winston Thomas Wastewater Treatment plant in Indiana, USA was used as an enrichment inoculum. The tertiary lagoon sludge has been contaminated with PCBs (primarily Aroclor 1242) for over 20 years, at concentrations ranging from several hundred to several thousand mg/kg dry sludge. A mixture of 5 selected PCB congeners (2,3-CB, 2,4,4'-CB, 2,4,6-CB, 2,2',5,5'-CB, and 2,2',4,4',5,5'-CB; 20 PPM each) or Aroclor 1242 (100 ppm, w/v) was used as possible enriching substrate with or without the presence of a cometabolic substrate, biphenyl (0.1%, w/v). Enrichments were performed in 160-ml serum bottles containing a liquid volume of 40 ml, and crimp-sealed with Teflon-coated stoppers. Transfers (10% volume) were made every 4 to 6 weeks into fresh MS medium containing the 5 congeners. The bottle headspace was purged with air once a week to maintain aerobic conditions.

After 10 transfers, cultures were spread on MS medium solidified with 1.6 % noble agar and the medium surface was sprayed with biphenyl dissolved in a diethyl ether carrier. To isolate bacteria that also grow on chlorobenzoate, colonies appearing on these biphenyl plates were transferred to solid MS medium containing 2 mM mono- or dichlorobenzoates including 2-CBA, 3-CBA, 4-CBA, 2,3-CBA, and 3,4-CBA . In addition, single colonies growing on the biphenyl plates were transferred to liquid MS medium supplemented with a single PCB congener. By repeating the procedure above, we have isolated several PCB-degraders, including SK-1 (2,3-CB) and SK-2 (3,4-CB).

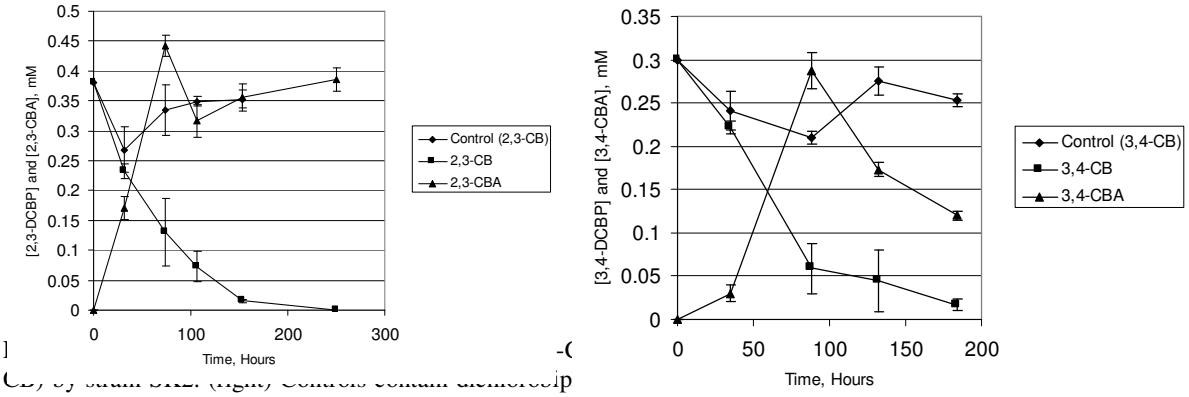
All experiments were conducted in Balch tubes containing 6 ml of MS medium and 21 ml of headspace with a crimp-sealed Teflon-coated butyl rubber stopper to prevent volatilization or sorption of PCBs. The volume of the initial oxygen content was sufficient for total oxidation of the added PCB. PCB congeners, biphenyl and Aroclor 1242 were added in 5 or 10 µl aliquots from stock solutions created by dissolving the carbon source in heptamethylnonane (HMN). HMN is a multi-branched nondegradable carrier, which was not able to support microbial growth of the isolates. When low-solubility substrates (PCBs) were supplied in an HMN carrier, an

Acridine Orange counting method was used.

Bacterial DNA preparation and PCR amplification and sequencing of 16S rRNA genes were carried out as described previously[1]. The resultant 16S rRNA gene sequence of strain was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred using the Fitch–Margoliash, maximum likelihood, maximum-parsimony and neighbour-joining methods[2]. The tree topologies obtained were evaluated by bootstrap analyses of the neighbor-joining method based on 1000 resamplings. The alignment and phylogenetic analysis were carried out using the jPHYDIT program (available at http://chunlab.snu.ac.kr/jphydit).

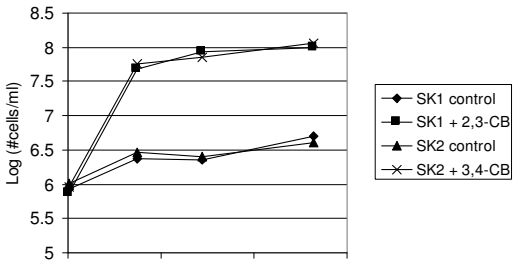
Results and Discussion

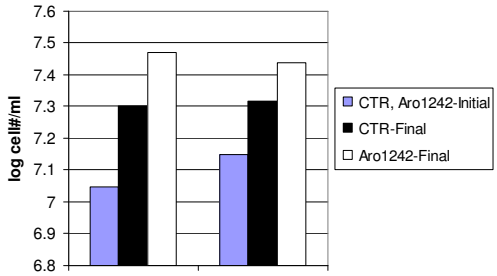
Four bacterial isolates that grow on a single PCB congener were isolated and they were temporarily named SK-1 and SK-2. Even though a non-chlorinated aromatic ring of a PCB can theoretically support microbial growth of a biphenyl-utilizer, only monochlorobiphenyls have been found to be growth substrates for naturally-occurring biphenyl-degraders. For successful PCB bioremediation, especially anaerobic-to-aerobic sequential biodegradation, a number of less chlorinated PCBs containing 2-4 chlorine substituents should be oxidatively degraded. Moreover, it is very desirable to discover microbial strains capable of using them as carbon and energy sources. SK-1 and SK-2 were found to be capable of using a 2,3-dichlorobiphenyl (2,3-CB) and 3,4-dichlorobiphenyl (3,4-CB), respectively, as a sole carbon and energy source. In experiments with 2,3-CB and SK-1, we observed almost total disappearance of 2,3-CB (0.38 mM, initial concentration) within 10 days (Figure 1). During the growth on 2,3-CB, SK-1 produced a nearly stoichiometric amount of 2,3-CBA. The rates of chlorobenzoate production were also parallel to the disappearance of the dichlorobiphenyls, but no distinct increase of chloride was observed in either case. Similarly, strain SK-2 also degraded 0.3 mM 3,4-CB during an incubation period of 200 hours (Figure 1). We also observed the stoichiometric production of 3,4-CBA from 3,4-CB. In contrast to SK-1 with 2,3-CB, the amount of 3,4-CBA decreased after 95 hours.



] -(CB) by strain SK2. (right) Controls contain dichlorobip and error bars represent a standard deviation.

During the degradation of dichlorobiphenyls, cell numbers of SK-1 and SK-2 had increased significantly. Figure 2 illustrates bacterial growth of SK-1 and SK-2 on 2,3-CB and 3,4-CB, respectively. During growth, cell numbers increased 100 fold over a 150-hour incubation. The pattern of these two growth curves was very similar. These two isolates did not need cometabolic substrates for microbial growth during degradation of PCBs. The results suggest that these novel strains contain enzymes capable of initial attack on the dichlorobiphenyl ring, and utilization of unchlorinated ring-cleavage products, i.e., unchlorinated 5-C fragments. To determine the ability of SK-1 and SK-2 to degrade a PCB mixture, 250 ppm (w/v) of Aroclor 1242 was incubated with benzoate-grown SK-1 and SK-2. The chromatograms of Aroclor 1242 illustrated several lightly chlorinated congeners were depleted during the incubation. During the incubation period, a small increase of cell numbers was also observed (Figure 2).





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numbers of SK-1 and SK-2 incubated with 200ppm Aroclor 1242.The initial concentration of the dichlorobiphenyls was 500 ppm (w/v). The controls were inoculated with cultures but lack the CBs. The cell numbers of the two isolates increased by 1.5 orders of magnitude in the presence of the CBs used as a growth substrate. Final cell numbers were enumerated after 12 days of incubation. (right)

Figure 3 illustrates a proposed degradation pathway of 2,3-CB. Considering a stoichiometrical production of 2,3-CBA and cell growth using the unchlorinated portion, it is assumed that SK-1 has a *bph*-like gene expressing proteins that can metabolize 2,3-CB in a conventional way. SK-2 seems to have a very similar metabolism. SK-1 and SK-2 are the first naturally occurring microorganisms that grow on dichlorobiphenyls chlorinated on one ring.

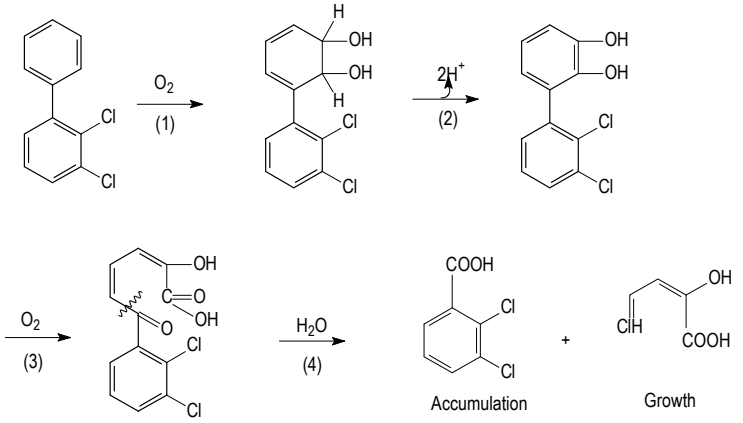

Figure 3 A proposed pathway of 2,3-CB degrdation by strain SK-1. This diagram was based on the conventional *bph* enzyme actions and the four enzymes are present: (1) biphenyl dioxygenase, (2) diol dehydrogenase, (3) catachol dioxygenase, and (4) hydroase. The biodegradation of 3,4-CB by SK-2 will contain two chlorines at the 3 and 4 position.

SK-1 was also a motile, oxidase-positive and catalase-negative, Gram-negative rod. Results of API E tests suggested that SK-1 has a good likelihood of being *Alcaligenes* or *Pseudomonas* species. The results of fatty acid characterization suggested strains of *Burkholderia solanacearum* (probability 0.265) and *B. piketti* (0.202). SK-1 was able to grow on a variety of organic compounds, including benzoate, biphenyl, all monochlorinated biphenyls, chloroacetate and phenol. SK-1 failed to grow naphthalene, 2,3,4-trichlorobenzene and all chlorobenzoates. Strain SK-2 was a motile, Gram-negative rod, which was positive in citrate-utilization, oxidase, catalase, and nitrate reduction. SK-2 was able to grown on biphenyl, benzoate, all monochlorobiphenyls, and 3,4-dichlorobiphenyl. SK-2 failed to grow on all chlorobenzoates, chloroacetate, phenol, 2,3,4-

trichlorobenzene or naphthalene. The results of fatty acid characterization also suggested that SK-2 is closely related to two *Alcaligenes* species: *A. pechaudii* (probability 0.471) and *A. xylosoxydans* (probability 0.427).

These two strains were also genetically identified. A nearly complete 16S rRNA gene sequence was obtained for strain from SK-1 and SK-2, and it was used for an initial BLAST search against the GenBank database. The search result clearly indicated that strain SK-1 belonged to *Proteobacteria, Betaproteobacteria, Burkholderiales* or *Burkholderiaceae*, and SK-2 belonged to *Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae,* or *Cupriavidus.* (Figure 4)

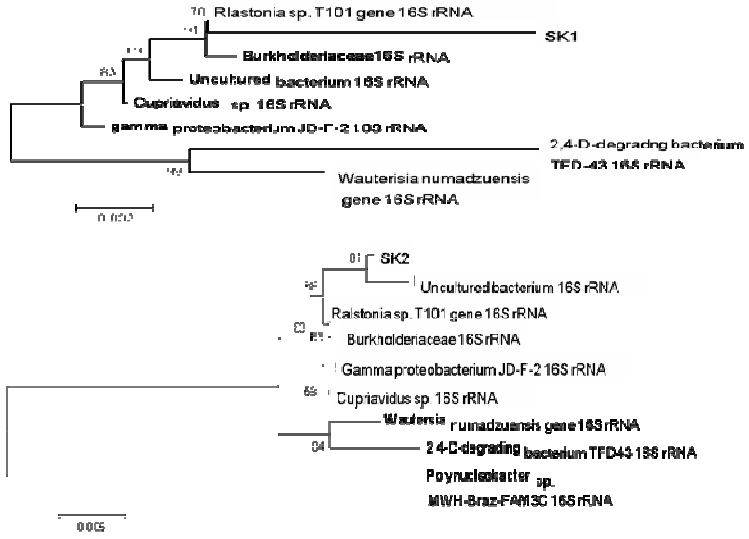

Figure 4 Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between the PCB-degrading bacteria (SK-1 and SK-2) and other strains. Percentages at nodes are levels of bootstrap support based on neighbor-joining analyses of 1000 resampled datasets

Acknowledgements
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References

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