INDIGENOUS MIXED SOIL BACTERIA

IN PRESENCE OF COMPATIBLE PLANTS

ARE MORE EFFICIENT IN PCB DEGRADATION

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SUMMARY

This paper presents data on comparative studies of unknown mixed cultures of indigenous microorganisms versus single cultures isolated from PCB-contaminated soil samples containing varying concentrations of PCBs (polychlorinated biphenyls). Remediation of PCBs was studied using isolated indigenous mixed cultures, by indigenous single colony cultures, in presence or absence of the leguminous alfalfa (*Medicago sativa*) plant. The congener 2´, 3, 4-PCB was used as known standard to spike the mixture of clean soil and vermiculite. Analytical methods such as HPLC (high performance liquid chromatography) and GC (gas chromatography) were used for PCB degradation quantification. The plant experiments were carried out in a controlled growth chamber environment with a 16-hr fluorescent and incandescent light period which mimicked day light. Results obtained suggest that higher the PCB concentration in soil, the more efficient the indigenous soil bacteria particularly in presence of plants. Mixed indigenous cultures were 2–3 times more efficient than single cultures. When compared to known PCB co-metabolizing bacteria such as *Comamonas testosteroni* and *Rhodococcus* sp., indigenous bacteria showed not only higher amount of PCB degradation, but also significantly better growth as evidenced by the CFU (colony forming units) counts. When the alfalfa plant was present, the indigenous mixed cultures were even more effective than known and unknown single cultures. These findings suggest that a combination of endemic microorganisms utilized with alfalfa plant is a promising approach for bioremediation of PCB-contaminated soils.

KEYWORDS:
PCB, degradation, indigenous mixed culture, alfalfa plants.

INTRODUCTION

PCBs can be degraded by naturally occurring unknown microbes in the soil anaerobically as well as by known cultures aerobically [1]. Bioremediation treatment of such contaminants has been done by a technology that converts them to carbon dioxide, water, and inorganic ions. The technique is a more natural method relative to the conventional clean-up measures, because it depends on the degradation of contaminants by the microorganisms in the soil. The principle is based on using the contaminant as a carbon source for selection of a microbial population degrading the toxic materials. The growth of these microorganisms was affected by nutrients, oxygen supply and moisture content, which can be controlled [2]. When compared to other methods of remediation of contaminated sites, bioremediation has been proposed as a relatively low-cost mitigation strategy.

There is also a risk of the microorganisms competing with each other for nutrients. If this occurs, one species (and an important part of the overall process) may be lost. It has been suggested that to overcome this problem, an effective inoculum should contain a low density of a specific PCB metabolizer and a higher density of chlorobenzoate degraders [3]. Although the role of sediment in the enhancement of PCB dehalogenation is not known, it appears to be necessary for significant degradation. It may also be necessary to provide carbon or another growth factor that is needed by the microbial community or it may provide support for immobilized cells [4].

In the past, a number of strategies to isolate PCB degraders with enhanced ability to degrade more chlorinated congeners have been evaluated [5]. In 1991, *Pseudomonas putida* P111 was isolated and displayed the greatest range of chlorobenzoate utilization [5]. Chlorobenzoate degraders were shown to be frequently absent in soils while PCB-co-metabolizing strains were routinely isolated from contaminated soils by biphenyls [6]. Later, studies revealed that the presence of genes of *ortho*-chlorobenzoate 1, 2 dioxygenase (OCBD) on transmissible plasmids pPB11 was responsible for *Pseudomonas putida*'s broad degradation

activities [7]. The same type of dioxygenase was found and cloned from plasmid pBAH1 in the strain *Pseudomonas cepacia* 2CBS [8]. The frequency and diversity of chlorobenzoate degraders on PCB-contaminated sites and their impact on PCB degradation have not yet been fully investigated, although molecular evidence in such soils has been performed for classical biphenyl degraders carrying genes for the *bph* gene operon [6]. They have shown that soils which were not exposed to chloro-organics did not harbor chloroaromatic degraders.

Studies with plants have indicated that stimulation of microbial activity in the rhizosphere of plants can also stimulate biodegradation of various toxic organic compounds [9, 10]. This general "rhizosphere effect" is well-known in terrestrial systems. The rhizosphere soil has been described as the zone of soil under the direct influence of plant roots and usually extends a few millimeters from the root surface and is a dynamic environment for microorganisms [11]. The rhizosphere microbial community is comprised of microorganisms with different types of metabolism and adaptive responses to various environmental conditions. The production of mucilaginous material and the exudation of a variety of soluble organic compounds by the plant root play an important part in root colonization and maintenance of microbial growth in rhizosphere. Thus, microbial activity is generally higher in the rhizosphere due to readily biodegradable substrates exuded from the plant [9].

In this study two major questions were addressed:

a)   Is the pool of genes from several microbial genomes in the soil more effective when compared to single genome ?, and

b)   can the plant alone be effective in degrading PCBs or are plant–microbe interactions required for degradation to occur ?

In case of PCBs, plant compounds have been shown to induce the co-metabolic degradation of PCBs [10, 12, 13]. Dutta and Toure [14] have shown accelerated PCB degradation using the alfalfa plant and microbe combinations. The purpose of this study was to isolate PCB-degrading microbes from contaminated soils and measure their PCB degrading effectiveness in the presence or absence of plants.

MATERIALS AND METHODS

Soil samples and cultures

PCB-contaminated and non-contaminated soil samples were obtained from Army Waterways Experimental Station, Vicksburg, MS in an attempt to isolate PCB-degrading microbes from the soil. Table 1 summarizes a list of soil samples used in this study. Wild type cultures of *Comamonas testosteroni* and *Rhodococcus* sp. and genetically altered *C. testosteroni* (VP44) were donated by Professor James Tiedje of Michigan State Univ., East Lansing, MI.

Isolation of PCB-degrading unidentified microbes from soil and cultures

Enrichment cultures were prepared with approximately 1 g of the soil suspended in 100 ml of a phosphate-buffered mineral (PAS) medium (15) containing 200 mg of biphenyl (Aldrich) per L as sole organic carbon source. Experimental samples and controls were incubated on a shaker (200 rpm) in the dark at 30 $^{\circ}$C up to 14 days. Then, 0.1% of the culture was transferred to a homologous medium. Enriched cultures were diluted in PAS medium. Then, PAS with 1.5 % purified agar and 200 mg of biphenyl per liter was spread onto the agar surface. Isolated bacterial colonies were picked and analyzed as described by Master and Mohn (16).

Chlorine release studies

Isolates were grown on PAS with 1.5% purified agar plates, containing different concentrations (10, 20, 30, 50 and 100 ppm) of 2´, 3, 4-PCB. Dechlorinations in both media were determined colorimetrically by a method used by Bergman and Sanik (17) with sodium chloride (NaCl) as a standard. Cell densities were quantified by measuring the optical density at 600 nm.

Preparation of cultures and extraction of PCB using HPLC analysis

Microbial single vs. mixed cultures were grown for one day in LB broth, 500 µl from the growth medium was added to 25 ml Luria-Bertani (LB) broth medium with a concentration of 50 ppm of 2′, 3, 4-PCB. PCB was extracted with an equal volume of hexane in Teflon-lined screw-capped shaking vials (24 h), and stored overnight at 8 $^{\circ}$C. Then, as much as possible of the hexane-phase was separated from the culture. To the hexane phase 2 g of Na_2SO_4 were added in order to bind the remaining water. The hexane phase was stored at -20 $^{\circ}$C. The extract was evaporated down to approximately 1 mL, then 5 mL of hexane was added and the sample was again evaporated overnight under the chemical fume. The next day 500 µl of methanol and 1 µl of HCH (hexachlorocyclohexane) were added. The extract was passed through a 0.2 µ filter to ensure purity. A constant mobile-phase composition of 90:10 (v/v) acetonitrile:water was used. The mobile phase flow rate for both elution systems was 1.0 ml min^{-1} and detection was by UV absorbance at 214 nm.

Preparation of soil for the plant experiments

Equal amounts of vermiculite and clean-sieved soil were autoclaved at 121 $^{\circ}$C for 90 min. Then, the soil was allowed to cool overnight and stored at -20 $^{\circ}$C for one week. These steps were performed four times in order to restore the soil back to the normal chemical composition which had been changed due to the high temperature during autoclaving process.

Plant Experiments

Plant studies were done in Leonard jars filled with 300 ml of autoclaved and sieved clean soil before adding

about the same amount of vermiculite. The alfalfa seeds were surface-sterilized with hydrogen peroxide and rinsed in sterile water. Seeds of alfalfa were inoculated with 10 ml of one day old-cultures isolated from 100 ppm PCB-contaminated soil containing unknown microbes. When the first shoots of the plant appeared, 100 ppm of pure 2′, 3, 4-PCB dissolved in methanol were added around the root. Soil samples (1g) were picked from each treatment up to four weeks and extracts were estimated using HPLC analysis. Experiments were carried out in a controlled growth chamber set at 7:00AM temperature 18 °C with ½ light; at 10:00AM temperature 22 °C with all of the light on; at 3:00PM temperature 18 °C and ½ light; at 7:00PM temperature 15 °C with no light, and humidity equal zero).

Rhizosphere enhancement studies

Plant root plays an important part in root colonization and maintenance of microbial growth in the rhizosphere. The microbes were isolated from plant roots containing 100 ppm of 2′, 3 4-PCB and incubated in LB broth containing 50 ppm and 100 ppm 2′, 3 4-PCB. Then colony forming units (CFUs) were determined by plating serial dilutions on LB agar plates. CFUs were determined in four replicas.

RESULTS

Estimation of PCB concentrations in different soil samples used in this study

Table 1 summarizes concentrations of PCBs in different soil samples using GC/MS analysis. The control soil (uncontaminated soil) sample showed traces of PCB, Sample 1 P_{3-1} (number assigned by the U.S. Army Waterways Experimental station) possessed the least amount of PCBs; P_{3-2} showed more; P_{3-3} had the highest concentrations and the clean vermiculite sample had no PCB.

Growth of cultures obtained from different soil samples

Isolated colonies from 100 ppm 2′, 3 4-PCB were grown in LB broth for 24 hours and followed by plating on Luria-Bertani (LB) agar plates. Table 1 summarizes CFUs from these soil samples. Results indicate that the addition of 100 ppm PCB caused an increase in CFUs. In the presence of alfalfa plants, CFUs were higher with higher initial PCB concentration (Table 1). The CFU numbers in the presence of alfalfa plant were increased to a large extent (Table 1) which was not observed in absence of plants. This result supports the fact that the root exudates may serve as nutrient sources for the microorganisms.

Growth of unknown cultures from P_{3-2} plots at 50 ppm and 100 ppm PCB

Table 2 summarizes comparative studies where PCB concentration varied in the liquid Luria broth medium. Data show that CFU counts at 10^{-13} dilutions were consistently higher when PCB concentrations were increased. In

the presence of alfalfa plants, the CFU counts were even higher. Lesser numbers of CFUs indicated depletion of carbon sources with increasing growth of cultures after two weeks.

TABLE 1
Results of CFUs at 10^{-6} serial dilution from isolated cultures spiked with 100 ppm 2′, 3, 4-PCB in Luria-Bertani (LB) broth.

Soil Sample	PCB concentration (ppm)	Number of colonies (10^{-6} dilution), no plants	Number of colonies (10^{-6} dilution), presence of plants
C_3	< 0.05	15×10^6	166×10^6
P_{3-1}	48	20×10^6	175×10^6
P_{3-2}	57	46×10^6	189×10^6
P_{3-3}	77	38×10^6	200×10^6
Control	None	None	None

PCB concentrations (ppm) were estimated using the 2′, 3, 4 congener as standard in GC/MS analysis (mean of 3 determinations, standard deviation < 4%).

TABLE 2 - CFU counts of indigenous cultures grown for one day in LB broth containing 50 and 100ppm of PCB.

Growth in PCB concentration	without alfalfa plant at 10^{-13} dilution	with alfalfa plants at 10^{-13} dilution
One day		
50 ppm	9×10^{13}	28×10^{13}
100 ppm	14×10^{13}	49×10^{13}
One week		
50 ppm	12×10^{13}	38×10^{13}
100 ppm	18×10^{13}	52×10^{13}
Two weeks		
50 ppm	3×10^{13}	19×10^{13}
100 ppm	11×10^{13}	31×10^{13}
Three weeks		
50 ppm	1×10^{13}	5×10^{13}
100 ppm	3×10^{13}	8×10^{13}

Mean of 4 determinations; standard deviation < 4%.

Chlorine (Cl^-) release assays using mixed cultures indigenous to soil samples

Figure 1 shows Cl^- release by cultures isolated from different soil samples in PAS medium at different weeks. Results indicate that cultures isolated from 30 ppm-spiked media were most efficient in dechlorination after 14 days. There was no change in dechlorination of the control treatments. These observations suggest that the higher the PCB concentrations in indigenous cultures, the more they can adapt to yield enhanced dechlorination.

PCB Dechlorination: single vs. mixed indigenous cultures

Figure 2 summarizes numerous experiments done to perform comparative studies on dechlorination vs. growth of indigenous microorganisms using both single and mixed cultures in LB broth medium. Studies done with P_{3-3} contaminated soil spiked with 50 ppm showed that the consis-

tent increased Cl⁻ release at 460 nm was associated with cell growth (600 nm), particularly with mixed cultures. Several additional studies were done and all confirmed that higher PCB-containing cultures were always associated with increased Cl⁻ release. These studies using optical density for measurement, however, were not able to distinguish differences in performances between single vs. mixed cultures. Hence, more sensitive dechlorination tests were performed using HPLC analysis (Fig 3). The mixed cultures showed complete degradation at 50 ppm 2′, 3, 4-PCB after 14 days, while complete degradation by single cultures occurred after 28 days, and no degradation occurred in the autoclaved control.
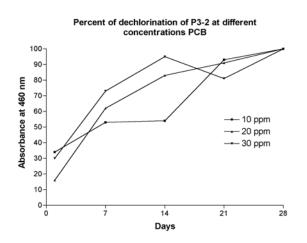

FIGURE 1- Summary of several tests (%) at different ppm levels (plot of P₃₋₂ soil sample). The chlorine release was expressed in percentages, 100% being complete dechlorination within 28 days. After 14 days the highest percentage of chlorine release was observed by microbes isolated from the 30 ppm-spiked soil sample.
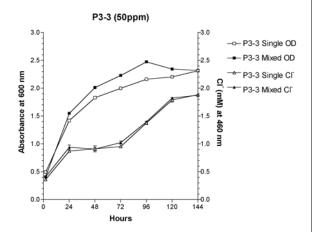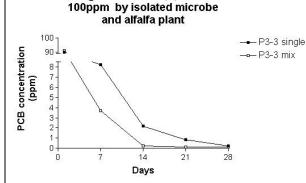

FIGURE 2 - Summary of chlorine release assays at 460 nm isolated from P₃₋₃ (50 ppm) plot as single vs. mixed indigenous culture with unknown microbes. The absorbance at 600 nm was to monitor culture growth.

FIGURE 3 - Degradation of PCBs (Summary of several HPLC analyses; the mixed cultures degraded PCB within 14 days and single cultures within 28 days; mixed cultures were isolated from soil sample P₃₋₃ spiked with 100 ppm 2′, 3, 4-PCB in the presence of alfalfa plants.

2′, 3, 4-PCB depletion by the wild types *Comamonas testosteroni* and *Rhodococcus* sp. and genetically altered *C. testosteroni* (VP44)

Comamonas testosteroni depleted approximately 58% of the PCB within the first week of cultivation whereas only 50% was depleted by *Rhodococcus* sp. during the same period (Table 3). Furthermore, *Comamonas testosteroni* completely depleted the pollutant within four weeks compared to 80 % depletion for *Rhodococcus* sp. The abiotic control showed less than 5% due to abiotic factors, indicating a good correction factor.

TABLE 3 - Composite data showing 2′, 3, 4 PCB depletion (ppm) by wild-type *Comamonas testosteroni*, *Rhodococcus* sp. and genetically modified *Comamonas testosteroni* with/without alfalfa plants.

Culture	presence of plants	PCB remaining (ppm)			
		Day 0	Day 7	Day 14	Day 28
Wild type *Comamonas testosteroni*	plant	101	5	4	0
	no plant	100	42	22	0
Genetically altered *C. testosteroni* VP44	plant	99	5	0	0
	no plant	99	7	5	0
Rhodococcus sp.	plant	99	45	30	8
	no plant	99	50	40	21
Indigenous mixed culture from PCB contaminated soil	plant	100	2	0	0
	no plant	50	23	10	0
alfalfa plants only sterile soil)	Plant	100	98	95	88
Control (autoclaved sterile soil only)	no plant	100	99	98	98

Mean of 3 HPLC determinations (ppm); standard deviation < 4%.

Genetically altered *Comamonas testosteroni* achieved 95 % depletion two weeks earlier than the wild type (Table 3). The CFU count was also significantly higher than that of the wild type (data not shown), confirming that these genes not only allow a faster and higher PCB degra-

dation but also bypass toxic steps that adversely affect the life of the bacteria. Comparative studies of 2′, 3, 4-PCB depletion in presence or absence of the wild type and genetically altered *Comamonas testosteroni* with/without addition of plants are summarized in Table 3 indicating significant differences between wild type and genetically modified *Comamonas testosteroni* tested.

DISCUSSION AND CONCLUSION

This paper presents data on indigenous bacterial cultures in contaminated soils. What is their primary source of carbon for bacterial growth? What are the interaction(s) of indigenous microbes with the habitats of specific soil? These basic fundamental questions were addressed in this study.

It was clear that indigenous cultures used PCB as the primary source of carbon for their growth. Three PCB-contaminated (P_{3-1}, P_{3-2}, P_{3-3}) and one non-contaminated (C_3) soil sample along with the autoclaved soil were tested for bacterial growth by means of CFUs. Data summarized in Tables 1 and 2 show that CFUs per gram of soil increased with increasing PCB concentrations in the soil samples. In the presence of alfalfa plants, CFUs increased 3- to 10-fold. CFU counts of indigenous cultures isolated from 100 ppm-spiked soil samples were significantly higher than those from 50 ppm. The presence of alfalfa plants also increased CFU values several-fold when cultures were grown in liquid medium, Luria Broth. The inability to grow is an indication of the high toxicity of 50 ppm and 100 ppm 2′, 3, 4-PCB in liquid medium (Table 2). It was inferred that indigenous cultures possess genes which are able to degrade PCBs and could help utilize the carbon from PCBs more intensive when cells were in direct contact with liquid growth culture. Decreasing CFUs after two weeks of growth (Table 2) confirmed these observations.

Numerous experiments conducted during this study show that dechlorination was always associated with increased cell growth. The isolates from mixed and single cultures were exposed to various concentrations of 2′, 3, 4-PCB (10, 20, 30, 50 and 100 ppm; data not shown). Chlorine concentration in the medium was determined colorometrically by a method of Bergman and Sanik [17] using sodium chloride (NaCl) as standard; cell densities were quantified by measuring the optical density at 600 nm. When these techniques were applied, growth and chlorine release were measured from 2 to 144 hours (Fig. 2). During this period, a high rate of growth and stochiometric amount of chlorine released was observed in all cultures incubated, single versus mixed cultures, except for sterilized (killed cells) or non-inoculum control cultures.

When the experiment was done with 100 ppm 2′, 3, 4-PCB, the unidentified microbes (single versus mixed) were capable of growing in this high concentration of PCB. As expected, these studies confirm that mixed cultures with their pool of PCB-degrading genes initiating multi-genic degradation pathways were impressively more effective than single culture genomes. When the data of the probable pathways are known and all pool genes responsible for PCB degradation are identified, the significance of these studies may be proved by reconstruction of PCB-degrading genes using Domain swapping and gene shuffling [18].

The alfalfa plant experiments indicated that the stimulation of microbial activity in the rhizosphere of the plants could also stimulate biodegradation of 2′, 3, 4-PCB. The unidentified mixed microbes isolated from rhizosphere were significantly faster (14 days) degrading to those containing a single colony (28 days). In the first two weeks single colony cultures seem to be slowly dechlorinated; however, all of the isolates (single versus mixed) in the presence of the alfalfa plant were able to degrade 2', 3, 4-PCB completely in half of the time (i.e. 14 days instead of 28 days; Fig. 3 and Table 3).

In conclusion, we have presented data showing that: (a) microbial cultures isolated from soils with higher PCB concentrations were more effective in its degradation; (b) mixed cultures were more efficient PCB degraders than single cultures based on both colorimetric and HPLC analyses; (c) mixed cultures in the presence of the alfalfa plant showed more colony forming units (CFUs) indicating more growth and degradation; (d) indigenous mixed cultures isolated from PCB-contaminated soil use PCBs as a sole carbon source for growth; (f) the well-known PCB degraders *C. testosteroni* and *Rhodococcus* sp. used in this study, also showed significantly enhanced remediation in the presence of alfalfa plant, but were less effective when compared to mixed indigenous cultures.

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