CONSTRUCTION AND DETECTION OF THE RECOMBINANT *RHODOCOCCUS* SP. STRAIN RHA1 FOR MINERALIZATION OF PCBs IN SOIL

By

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ABSTRACT

CONSTRUCTION AND DETECTION OF THE RECOMBINANT *RHODOCOCCUS* SP. STRAIN RHA1 FOR MINERALIZATION OF PCBs IN SOIL

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PCBs are still one of the most important environmental pollutants. We developed a strategy of combining the biphenyl and 4-chlorobenzoate (4-CBA) degradation pathways into the same microorganism for PCB degradation. The genes from *Arthrobacter globiformis* strain KZT1 responsible for transforming 4-CBA into 4-hydroxybenzoate were sequenced and their products expressed in *E. coli*. Three open reading frames, organized in an operon, were required for this activity: 4-CBA CoA-ligase (*fcbA*), 4-CBA dehalogenase (*fcbB*), and 4-CBA thioesterase (*fcbC*). These genes are cotranscribed into one polycistronic mRNA. The presence of the 3.3 kb fragment downstream of the operon repressed the expression of the *fcb* operon when 4-CBA was absent. Gene expression was restored to its normal level in response to 4-CBA addition, indicating that the *fcb* operon is regulated by a repressor. The Gram-positive bacterium *Rhodococcus* species, strain RHA1, which contains the biphenyl oxidation pathway, was

electroporated with a plasmid containing the 4-CBA operon. The recombinant strain grew on 4-CBA and 4-chlorobiphenyl (4-CB) as the only source of carbon, with stoichiometric release of chloride and a molar growth yield on 4-CB that suggested utilization of both biphenyl rings. Similar conversion rates were observed for wild type and recombinant strains for the eight most common congeners from the anaerobic dechlorination of Arochlor 1242 (pattern M), but the recombinant strain accumulated lower amounts of chlorinated *meta*-cleavage products and no 4-CBA. The recombinant cell population, when added to non-sterile 4-CB contaminated soil, increased to a density consistent with the 4-CB consumed and the *fcb* operon remained stable. A real time PCR assay using fluorescently labeled oligonucleotides (TaqMan probes) was developed to quantify *Rhodococcus* sp. RHA1(*fcb*) in soil. The TaqMan-16S rDNA probe detected RHA1(*fcb*) and phylogenetically related species while TaqMan-*fcb* probe was specific for the recombinant strain. The method had a 6-log dynamic range of detection (10^2 to 10^7) for both probes. In other microcosms, two recombinant strains: *Rhodococcus* sp. RHA1(*fcb*) and newly engineered *Burkholderia cepacia* LB400 containing the 2-chlorobenzoate (*ohb*) degradation operon, were added to Aroclor 1242-contaminated sediment that had undergone anaerobic dechlorination. Both recombinant strains increased their populations in the PCB contaminated sediment. The recombinant RHA1 cell number and *fcb* gene copies were quantified over the experimental period by real time PCR and results agreed well with plate counts of this strain. Inoculation at cell densities of 10^4 and 10^6 cells g^{-1} sediment resulted in equivalent PCB removals, 57% and 54%, respectively. The residual PCB congener profile after 30 days was the same for both high and low cell density inoculation.

DEDICATION

To Maeli

I will always feel very fortunate that I found you

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I am deeply in debt with all individuals who, in some way, have helped me through this small step:

Dr. James M. Tiedje, my academic advisor, for his guidance, patience, and encouragement during all this years.

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PREFACE

I conducted the work described in these chapters except as follows:

Chapter II: Dr. Tamara Tsoi worked on the protein labeling for expression profiles, Dr. J. Cole instructed me on bootstrapping the sequences, and Dr. S. Rech and I worked on plasmid constructions and β-galactosidase assays.

Chapter III: Dr. O. Maltseva and I jointly planned the resting cell experiments, Dr. J. Quensen run the PCB analysis from the extracts I provided.

Chapter IV: M. Aiello, during his rotation as a new graduate student, worked on optimizing the conditions for real time PCR, Dr. J. Urbance provided guidance in using the ARB program, and Joel Klappenbach helped me with the 16S rRNA gene copy determination.

Chapter V: The work described here was designed to integrate accumulated information so that we could bench test the complete anaerobic-aerobic PCB bioremediation scheme. I designed the experiment with suggestions from Dr. J. Quensen and Dr. O. Malteva. M. Aiello performed the real time PCR, J. Quensen conducted the PCB analysis and Dr. T. Tsoi provided the LB400 recombinant strain.

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CHAPTER I

GENERAL INTRODUCTION

Polychlorinated biphenyls (PCBs) became widespread in a variety of commercial applications as replacements for flammable petroleum products such as: lubricants, hydraulic fluids, capacitors, plasticizers, and electric transformers (Miller 1982). All these applications are due to the physico-chemical properties of PCBs such as stability, low flammability, high insulation capacity, low volatility, low water solubility, resistance to oxidation and good dielectric characteristics (Boyle et al. 1992).

PCBs refers to mixtures of 209 possible isomers of a chlorinated biphenyl molecule. Each one of those congeners has an assigned name (and IUPAC number) according to the number and position of chlorine atoms covalently bound to the two carbon rings that comprise the biphenyl molecule. Commercial PCB products are complex mixtures of the different congeners. PCB mixtures were sold in many countries under different names: Clophen (Germany), Phenoclor (Italy), Kaneclor (Japan), Pyralene (France), Solvol (Russia), and Aroclors (USA). Aroclors are described by a number following the Aroclor term, i.e. 1242, 1254, and 1260 (Abramowicz 1990). The first two digits correspond to the number of carbon atoms in the biphenyl molecule and the last two numbers refer to the percentage of chlorine by weight present in the mixture, i.e. 42, 54, and 60% of chlorine (Figure 1.1)

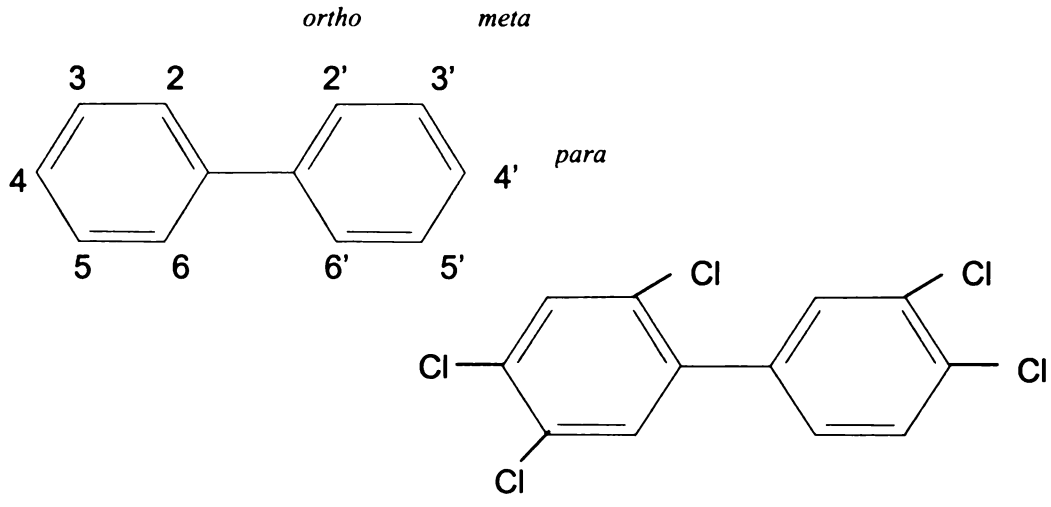

Figure 1.1. At left, the numbering scheme of PCBs and positional references, relative to the opposing phenyl ring. At right, a sample pentachlorobiphenyl known as 2,4,5,3',4'-pentachlorobiphenyl by the IUPAC nomenclature, or simply as congener number 118.

PCBs as environmental contaminants

PCBs have been a subject of environmental concern since 1966, when unexplained peaks appeared in chromatographic analysis of soil and water analyzed for DDT (Jensen 1972, Waid 1986). Although PCB production was later discontinued, PCBs have persisted and became ubiquitous environmental contaminants (Bedard and Quensen 1995).

PCBs are considered hazardous to human health, having potential estrogen-like activity and are known to cause liver damage (Sager 1991; Silberhorn et al 1990). In laboratory animals, PCBs elicit a variety of responses such as: dermal toxicity, total body weight loss, immunological supression, reproductive and developmental effects (Sager 1991, Bittman and Cecil 1970), neurotoxicity, and carcinogenicity. (Safe 1989, Safe 1994, Silberhorn et al. 1990).

The threat to health possessed by this widespread contaminant is compounded by their persistence and the difficulty of cleaning up PCBs. They are neither photochemically deactivated nor hydrolyzed, remaining among the most difficult hazardous waste cleanup problems. Incineration or land-filling have been used, but these procedures are expensive and not long term alternatives. Costs for incineration in US contaminated sites were estimated to a total $20 billion with prices around $800 per ton of soil while land-filling ranges about $400-500 per ton (Funk et al. 1993). In contrast, bioremediation offers a potentially inexpensive and effective way to treat PCB-contaminated soils.

PCBs biodegradation

The biodegradability and toxicity of individual congeners are largely governed by the number and position of the chlorines on the biphenyl nucleus (Abramowitz 1990, Boyle et al. 1992). Due to the high number of PCB congeners, efficient microbial degradation of PCBs requires either diverse catabolic activities from one microorganism or many microorganisms with different degradation capabilities. Because of this, a sequential anaerobic-aerobic biological treatment system has been proposed as the best available alternative for PCB degradation (Abramowitz 1990).

Anaerobic PCB degradation

Evidence of microbially mediated anaerobic reductive dechlorination of PCBs (a process in which a chlorine is replaced by hydrogen in the aromatic ring) came from altered profiles of PCB congener distribution in Hudson River sediments (Brown et al. 1984). Depletion of the tri- and higher chlorinated congeners with a corresponding

percentage increase in mono- and di-chlorobiphenyl isomers was later confirmed to be a microbially catalyzed process in laboratory studies (Quensen et al. 1988, Quensen et al. 1990, Ye et al. 1992). At least six different altered PCB congener profiles, known as dechlorination patterns, have been identified (Table 1.1). Each one of them is thought to be a result of different congener selectivities by dechlorinating microbial consortia. The six individual dechlorination profiles may occur separately, or in combination (Bedard and Quensen 1995). The specificity of the microbial dechlorination is thought to be determined by three major factors: 1) the composition of the microbial community, 2) the molecular structure of the congener, and 3) the environmental conditions.

Table 1.1. PCB congeners remaining after microbial dechlorination and their pattern nomenclature. Adapted from Bedard and Quensen (1995).

Dechlorination products	Dechlorination pattern						
	M	Q	H'	H	P	N	C[a]
2	+	+					+
2-2/2-6	+	+					+
2-3		+	+	+			
2-4	+		+				
23-25					+		
24-2	+		+				
24-3			+	+			
24-4	+					+	
25-2		+	+				
25-3			+	+			
26-2	+	+					+
26-3		+	+	+			+
24-4/25-4			+	+			
24-24			+	+		+	
24-25			+	+	+	+	
24-26						+	
25-25			+	+	+		
235-23					+		
235-24			+	+			
235-25			+	+	+		
236-24			+	+			
236-25			+	+			
246-24						+	
2356-24						+	

[a] Process C is believed to be a combination of processes M and Q.

Many *in situ* and laboratory experiments have confirmed the presence of PCB-dechlorinating organisms in a variety of sediments and their activity could be maintained when they are sequentially reinoculated into PCB contaminated soil or sediment matrices. But, enrichment of PCB dechlorinators through serial transfers has generally not led to increased activity and has not resulted in any PCB dechlorinating pure cultures. (Morris et al. 1992, Tiedje et al. 1993). Kin and Rhee (1997), however, did show an increase of two orders of magnitude in the number of dechlorinating organisms by the most-probable-number technique during the course of Aroclor 1248 dechlorination, but no isolation was attempted. Putative PCB dechorinating organisms have been identified on the basis of molecular monitoring of enrichment cultures and sequence analysis of the 16S rDNA gene amplified from members of the community (Holoman et al. 1998). During active dechlorination of PCBs, sequence similarities with the following groups were found: an undescribed species in the δ subgroup of the class *Proteobacteria*, members of the low G+C gram positive subgroup, the *Thermotogales* subgroup, and *Dehalococcoides ethenogenes*. With exception of the latter microorganism, all the other groups include sulfur-, sulfate-, and/or iron(III)-respiring bacterial species. These results agree with Ye's hypothesis that spore-forming dissimilatory sulfate-reducers are at least one of the physiological groups responsible for PCB dechlorination (Ye et al. 1999).

Aerobic PCB degradation

PCB degraders, their metabolic pathways and genetics

Aerobic biodegradability of PCBs was first confirmed with the isolation of two species of *Achromobacter* (later both were reclassified as *Rhodococcus globerulus*) from

biphenyl and 4-CB enrichments (Ahmed and Focht 1973). Since the final products were found to be different for the two isolates, the authors suggested different metabolic pathways of degradation. Biphenyl-utilizing bacteria represent a diversity of genera including: *Nocardia, Pseudomonas* (Baxter et al. 1975, Kimbara et al. 1988), *Alcaligenes* (Furukawa and Matsumara, 1976), *Acinetobacter* (Furukawa et al. 1978), *Burkholderia* (Bopp 1986), *Comamonas* (Pellizari et al. 1996) and *Rhodococcus* (Maeda et al. 1995). These PCB degraders usually can utilize biphenyl or similar aromatic compounds as the sole source of carbon and energy.

The biphenyl degradation gene cluster (*bph* operon) from several bacterial strains has been cloned and sequenced, allowing comparisons. Genetic analysis of a few of these microorganisms has revealed a striking similarity of *bph* gene sequences, but with variations in how the genes are organized in the operon (Furukawa. 1994).

The aerobic degradation pathway for PCBs involves a sequence of four enzymatic reactions (Furukawa et al. 1993): (1) molecular oxygen is introduced at the 2 and 3 positions of one of the two rings by a biphenyl dioxygenase; (2) the resulting dihydrodiol is dehydrogenated by a dihydrodiol dehydrogenase; (3) the 2,3-dihydroxybiphenyl formed is cleaved between the 1 and 2 positions by a 2,3-dihydroxybiphenyl dioxygenase, and (4) the compound produced from *meta* cleavage (a chlorinated derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) is hydrolyzed by a hydrolase, resulting in benzoic acid and 2-hydroxy-pent-2,4-dienoic acid (Figure 1.2).

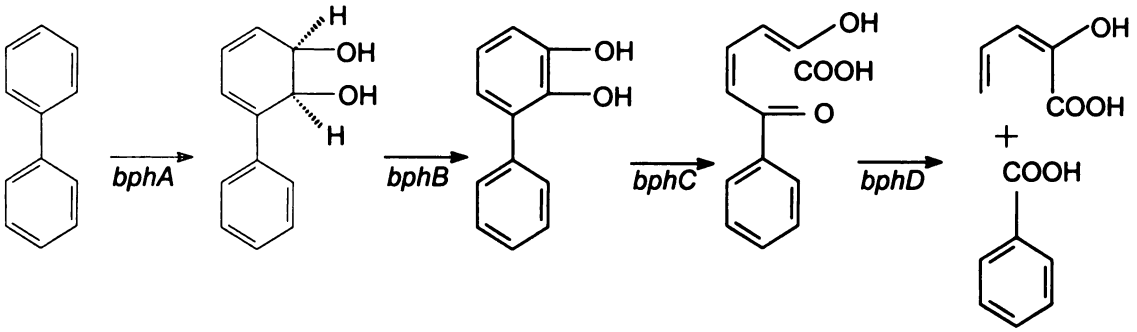

Figure 1.2: The upperpathway for degradation of biphenyl and chlorobiphenyls. The genes coding for each step are indicated below the arrow for each reaction.

The biphenyl locus of the PCB degrader *Burkholderia* sp. strain LB400 (Bopp 1986) has been extensively characterized (Mondello 1989; Erickson and Mondello 1992). At least ten different genes are involved in biphenyl breakdown. The 2,3-biphenyl dioxygenase is a multicomponent enzyme comprised of four subunits: a large subunit of terminal dioxygenase encoded by the *bphA1*, a small subunit of the terminal dioxygenase encoded by the gene *bphA2*, a ferredoxin encoded by *bphA3*, and the ferredoxin reductase encoded by the *bphA4*. The *bphB* gene encodes for a dehydrogenase, the *bphC* encodes for the 2,3-dihydroxybiphenyl-1,2-dioxygenase, and the *bphD* gene encodes for a hydrolase. These four genes and their respective encoded proteins belong to the "upper" pathway of the biphenyl degradation, leading to the formation of benzoate and 2-hydroxypenta-2,4-dienoate (pentadiene) (Erickson and Mondello 1992).

"Lower" pathways are required for the conversion of pentadienes and benzoates to Krebs cycle intermediates (Hofer et al. 1994). The enzymes required for the pentadiene pathway are encoded by the following genes: *bphK* encodes for a glutathione-S-transferase, *bphH* encodes for the 2-hydroxypenta-2,4-dienonate hydrase, *bphJ* encodes

for an acetaldehyde dehydrogenase, and *bphI* encodes for the 4-hydroxy-oxovalerate aldolase (Figure 1.3).

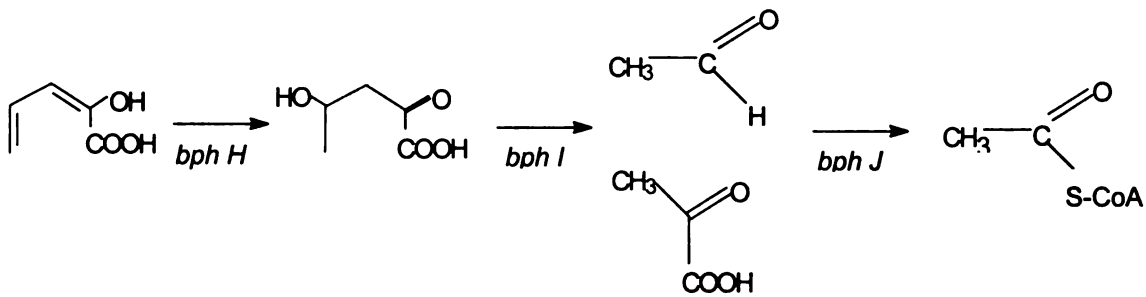

Figure 1.3: The lower pathway for degradation of biphenyl and chlorobiphenyls. The genes coding for each step are indicated below the arrow for each reaction. The gene *bphK* is not shown in this scheme.

Constructing a better PCB-degrading bacterium

Although many bacterial species possess the biphenyl (*bph)* pathway, none of the isolated bacteria are able to degrade a large range of PCB congeners nor are any of them able to completely mineralize any important Aroclor congener. The failure of organisms to grow on PCBs is due to either or both the lack of chlorobenzoate degradation capabilities and the production of toxic intermediates. In the later case a particular problem is the formation of acyl halides from 2- and 3-chlorocatechol produced from 2- and 3-chlorobenzoates. These acyl halides readily deactivate the biphenyl degrading oxygenase.

The production of toxic intermediates from chlorobenzoic acids can be avoided if chlorines are removed before ring cleavage (Stratford et al. 1996). Establishment of dehalogenation as the first step of degradation is attractive for development of novel

recombinant metabolic pathways. The need of a PCB-mineralizing bacterium able to express all genes required for catabolism of chlorobenzoic acids was first recognized by Furukawa and Chakrabarty (1982). However, only in recent years has the construction of recombinant catabolic pathways been considered viable (McCullar et al. 1994, Brazil et al. 1995). Brenner et al. (1994) suggest two different strategies for the construction of catabolic pathways: i) one based on the recruitment of isofunctional enzymes from various recognized pathways, and ii) another involving mutational alteration of the substrate specificity of existing key enzymes. Our approach for construction of a new metabolic pathway was based on the first strategy.

The aerobic metabolism of chlorobenzoic acids can be achieved through three degradation pathways (Peel and Wyndham 1999) known to date: i) a non-specific dioxygenase attack of the ring (Frantz and Chakrabarty 1987), ii) the 3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway, which forms a protocatechuate that will be further utilized (Nakatsu et al. 1995), and iii) the 4-chlorobenzoate (4-CBA) hydrolytic dehalogenation (*fcb*) pathway, which removes a *para*-positioned chlorine, yielding 4-hydroxybenzoate (4-HBA) (Figure 1.4) (Zaitsev et al. 1991). This third pathway is advantageous because dechlorination occurs first in the degradation process, avoiding formation of chlorocatechols and hence the toxic intermediate (Marks et al. 1984).

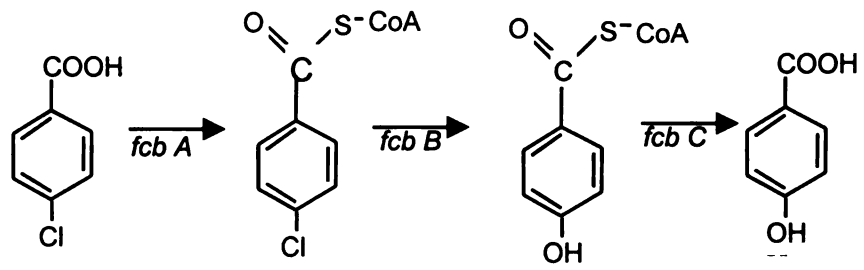

Figure 1.4: The 4-chlorobenzoate (*fcb*) degradation pathway from *Arthrobacter globiformis* strain KZT1. The genes coding for each step are indicated below the arrow for each reaction.

Rhodococcus sp. strain RHA1

The genus *Rhodococcus* includes a wide variety of morphological diversity with the ability of many strains to form hyphae, which can fragment into rods and cocci (Goodfellow 1989). This Gram-positive genus defines a group of actinomycetes that have: a) a peptidoglycan consisting of N-acetylglucosamine, *N*-glycolylmuramic acid, D- and L- alanine, and D-glutamic acid with *meso*-diaminopimelic acid; b) a cell wall with major amounts of arabinose and galactose; c) a phospholipid combination of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides, d) a fatty acid profile containing straight-chain, unsaturated and tuberculostearic acids and mycolic acids, and e) dedydrogenated menaquinones with eight or nine isoprene units. These chemical characteristics as well as hyphal fragmentation make them very resistant to stressful environmental conditions (Warhust and Fewson 1994).

The PCB degrader *Rhodococcus* sp. strain RHA1 was isolated by Fukuda's group (Masai et al. 1995) from a PCB-contaminated Japanese soil. This strain possesses

multiple aromatic catabolic enzymes, allowing transformation of up to 45 congeners from different Aroclor mixtures (Seto et al. 1996). In addition, this Gram-positive bacterium grows on other toxic compounds such as *o*-xylene, ethylbenzene, toluene, benzene, propylbenzene, and butylbenzene (Hatta et al. 1998) which can be co-contaminants at PCB sites. Hence, this strain seemed to be at least among the most promising for introduction of dehalogenase genes and to use in PCB bioremediation.

Major aims of this study

In this thesis, the 4-chlorobenzoate degradation (*fcb*) operon was combined with the broader PCB cooxidation capabilities and environmental endurance of the *Rhodococcus* sp. strain RHA1 to test whether the constructed strain had desired properties for PCB bioremediation. Specifically, I addressed the following points:

1. Investigate the regulation of the 4-chlorobenzoate (*fcb*) degradation operon so that the expression of this pathway could be understood;
2. Introduce the *fcb* operon into the PCB degrader *Rhodococcus* sp. strain RHA1 and test the *fcb* operon expression;
3. Investigate the aerobic degradation of defined anaerobic dechlorination PCB products by recombinant bacteria;
4. Test whether the recombinant bacteria grew and degraded PCBs in non-sterile soil microcosms;
5. Track and quantify the recombinant strain in soil by its molecular signature;
6. Test the effectiveness of the recombinant bacterium in removing PCBs from an Aroclor 1242 contaminated sediment microcosm.

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CHAPTER II

SEQUENCE ANALYSIS, EXPRESSION, AND REGULATION OF THE 4-CHLOROBENZOATE DEGRADATION (*fcb*) OPERON FROM *ARTHROBACTER GLOBIFORMIS* STRAIN KZT1.

ABSTRACT

The genes from *Arthrobacter globiformis* strain KZT1 responsible for transforming 4-chlorobenzoate (4-CBA) into 4-hydroxybenzoate (4-HBA) were sequenced and their products expressed in *E. coli*. Three open reading frames, organized in an operon, were required for this activity: 4-CBA CoA-ligase (*fcbA*), 4-CBA dehalogenase (*fcbB*), and 4-CBA thioesterase (*fcbC*). Mini cells showed that each gene was translated into a protein with the expected molecular weight. Reverse transcription-PCR with two sets of primers designed to amplify the regions spanning the *fcbA-fcbB* and *fcbB-fcbC* yielded amplification products of 2.1 and 0.4 kb, respectively, indicating that these genes are cotranscribed into one polycistronic mRNA. The presence of the 3.3 kb fragment downstream of operon repressed the expression of the *fcb* operon in *E. coli* when the specific substrate, 4-CBA, was absent. Gene expression was restored to its normal level in response to 4-CBA addition. This indicates that the *fcb* operon is regulated by a repressor.

INTRODUCTION

Chlorinated benzoic acids (CBAs) reach nature from use as industrial chemicals, herbicides or as intermediate metabolites from the degradation of some pesticides, polychlorinated biphenyls (PCBs) or other chloroaromatic compounds. The high solubility of CBAs in water and the lack of assessment of their toxicity and degradation has raised concerns (Ducrocq et al. 1999). CBAs have been regulated under the Toxic Substance Control Act (US EPA, 1976).

Many laboratories have reported the isolation of microorganisms capable of growing on CBAs as the only source of carbon (Fulthorpe et al. 1996, Marks et al. 1984, Peel and Wyndham 1999, Zaitsev et al. 1991). Three different CBA biodegradation pathways have been described (Peel and Wyndham 1999). The first biochemical pathway is encoded by the *clcABD* genes, causing an *ortho*-ring fission of chlorocatechols after initial attack by a non-specific dioxygenase (Frantz and Chakrabarty 1987). A second pathway, known as the *meta*-ring fission or 3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway, is encoded by the *cbaABC* genes with production of a dioxygenase, reductase, and dehydrogenase, respectively (Nakatsu et al. 1995). These enzymes degrade 3-CBA and 3,4-DCBA by formation of a protocatechuate. In both pathways, the removal of chlorines always occurs after ring cleavage and the metabolism of chlorobenzoates occurs via production of chlorocatechols (Marks et al. 1984). While catechols are readily utilized by soil bacteria or chemically polymerized into soil organic matter through quinone and free radical additions, metabolism of some chlorocatechols results in production of acylchlorides which inactivate dioxygenases (Focht 1995).

The third biochemical pathway avoids the production of toxic intermediates because halogens are removed before ring cleavage. Occurrence of dehalogenation as the first step of degradation is attractive for development of novel recombinant metabolic pathways because toxic intermediates could be avoided. Tsoi et al. (1991) were the first to report the isolation of plasmid-borne genes responsible for 4-CBA hydrolytic dehalogenation, yielding 4-hydroxybenzoate (4-HBA). The genes encoding for the enzymes responsible for dehalogenation of 4-CBA found in the *fcb* operon of a *Pseudomonas* strain have been cloned and partial expression investigated (Babbit et al 1992, Chang et al. 1992, Löffler et al. 1995, Schmitz et al. 1992, Tsoi et al. 1991).

In this paper, we report the nucleotide sequence of the *fcb* operon from *Arthrobacter globiformis* strain KZT1 as well as 3.3-kb of the downstream region which has not been investigated. We also report on the organization, expression, and regulation of this operon. To our knowledge this is the first study showing quantitative data for the expression of the operon in response to 4-CBA as well as 4-HBA.

MATERIALS AND METHODS

Plasmids, bacterial strains, bacteriophages, and growth conditions. The genotypes and origins of the bacterial strains, plasmids and bacteriophages used in this study are shown in Table 1. *Escherichia coli* strain JM109 was grown at 37°C while *Rhodococcus* sp. strain RHA1 was grown at 30°C. For plasmid manipulations and phage infections, *E. coli* was grown in Luria-Bertani (LB) medium. Ampicillin (100 mg/l), tetracycline (20 mg/l) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 mg/l) were added when required. For β-galactosidase assays, cells were grown in M63

medium containing trace amounts of thiamine, 10 mM of glucose and 0.1% casamino acids. 4-Chlorobenzoate (4-CBA) and 4-hydroxy-benzoate (4-HBA) were added at final concentrations of 1, 2, and 5 mM as indicated. Cultures (15 ml) in 100 ml flasks were shaken at 200 rpm during aerobic growth. The flasks containing the indicated medium were inoculated with overnight cultures grown under the same conditions, and the cells were allowed to grow to mid-exponential phase prior to harvesting for enzyme analysis.

Plasmid constructions. Cloning of different fragments from the plasmid pCA311 (Tsoi et al. 1991) into the high copy number plasmid pUC19, yielded plasmids pCH1, pXB3, pBP6, pXB11, and pKXS7 (Table 1). Cloning of the *Xho*I-*Eco*RI fragment into plasmids pBlueScript KS(+), and pBlueScript SK(+) gave plasmids pJT01 and pJT02 (Figure 2.1). The same fragment was cloned into the low copy number plasmid pSP329, resulting in the plasmid pGL10. Plasmid DNA was isolated and purified by using a WIZARD miniprep Kit (Promega, Madison, WI.). Restriction and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions. Chloride release was analyzed as previously described (Hrywna et al. 1999).

Minicell assay. *E. coli* strain χ925 transformants were assayed as previously described (Stocker et al. 1984). Induction of protein synthesis of the *fcb* operon was evaluated by $Na_2{}^{35}SO_4$ labeling. Cells were serially transferred three times in a two fold-diluted LB medium, harvested in early stationary phase, and washed in phosphate-buffered saline (pH 7.0). Cells were resuspended in PBS and starved for 6 h at room temperature. Induction was carried out with 100 μM 4-CBA and 0.4 μCi of ^{35}S at room temperature for 68 h. Incubated cells were harvested, washed with PBS, and resuspended

in H_2O. Total proteins (20 μg) from lysed cells (Lowry et al. 1951) were analysed in a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The Kodak X-OMAT Ar film (Eastman Kodak, Rochester, NY) was exposed for 5 days.

TABLE 2.1. Plasmids, bacterial strains, and phages used in this study.

Plasmid, strain, or phage	Genotype or phenotype	Reference or source
Plasmids		
pUC19	Amp^r α*lacZ*	Yanischi-Perron et al. 1985
pCA311	Same as pUC19 with the 7.6 Kb insert	Zaitsev et al. 1991
pCH1	Same as pUC19 with *Sau*3A insert	Zaitsev et al. 1991
pXB3	Same as pUC19 with *Sma*I-*Bal*31 insert	This work
pXB11	Same as pUC19 with *Xho*I -*Bal*31 insert	This work
pBP6	Same as pUC19 with *Pst*I-*Bam*HI insert	This work
pKXS7	Same as pUC19 with *Xho*I-*Eco*RI insert	This work
pBluescript II KS(+)	Amp^r α*lacZ* ColE1 origin	Stratagene
pBluescript II SK(+)	Amp^r α*lacZ* ColE1 origin	Stratagene
pJT01	Same as pBSKS(+) with *Xho*I-*Eco*RI insert	This work
pJT02	Same as pBSSK(+) with *Xho*I-*Eco*RI insert	This work
pSP329	*Tc^r*, IncP, *lacZ* α	Dr. V. Ksenzenko[a]
pGL10	Same as pSP329 with *Xho*I-*Eco*RI insert	This work
pRHD34	Same as pSP329, pRC1 replicon, *fcb*ABC	Rodrigues et al. 2000
pRS415	*lacZ lacY*$^+$ *lacA*$^+$ Amp^r	Simons et al. 1987
pSJ1	Same as pRS415 with P_{fcb}	This work
Strains		
Escherichia coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB) [F'(traD36)] proAB lacI ZΔM15*	Promega
JfcbP	Same as JM109 containing P_{fcb}-*lacZ* fusion	This work
χ925	*F*$^+$ *minA minB thr leu thi ara lacY gal malA xyl mtl tonA rpsL supE*	Stocker et al. 1984
Rhodococcus sp. RHA1	grows in biphenyl, *o*-xylene, ethylbenzene, toluene, benzene, propylbenzene, and butylbenzene	Seto et al. 1995
Phage		
λRS45	*lacZ lacY*$^+$ *lacA*$^+$	Simons et al. 1987
λSJ1	*φ(fcbA-lacZ)lacY*$^+$*lacA*$^+$ (operon fusion)	This work

[a] Institute of Biochemistry and Physiology of Microorganisms, Puschino, Russia.

Construction of *fcb-lacZ* fusions. Plasmid pCA311 served as the template to amplify a 422 basepair fragment carrying the promoter region of the *fcb* operon as well as the first 20 basepairs of *fcbA*. The foward primer JT0846 (5'-GGAATTCCGCGCCGAGACCTGTG-3') was designed to contain an *Eco*RI restriction site and the reverse primer JT0847 (5'-GGATCCGCGGCTGGCAAGTA-3') included a *Bam*HI site. The amplified fragment was purified using the Wizard PCR Purification Kit (Promega, Madison, WI) and ligated into the reporter plasmid pRS415 to obtain plasmid pSJ1. These operon fusions were transferred to the lambda vector λRS45 and introduced into the chromosome of *E. coli* strain JM109 as previously described (Simons et al. 1987). Lysogens that contained a single inserted phage were isolated and stocked for subsequent studies.

β-galactosidase assay. β-Galactosidase levels were determined by hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) (Rech et al. 1995). The protein concentration was estimated by assuming correspondence between *E. coli* optical density of 1.4 at 600 ηm and 150 μg of protein per ml (Miller et al. 1972). Units of β-galactosidase are expressed as ηmoles of ONPG hydrolyzed per min per mg of protein. An extinction coefficient for ONPG of 0.0045 mM^{-1} cm^{-1} was used. The β-galactosidase values were averaged from at least three independent experiments with two replicates each. Values did not vary more than 8%.

Nucleotide sequence of the 4-CBA degradation operon. Plasmids pCA311, pJT01, and pJT10 were submitted to *Exo*III deletions. Resulting fragments were ligated and submitted to the Michigan State University Sequencing Facility (East Lansing, MI) for automated fluorescent sequencing. Internal primers were used for filling gaps by

primer walking. Sequence alignment and editing were performed with the software Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI). Primers were designed by using the LASERGENE software package (DNASTAR Inc., Madison, WI). The completed sequence was submitted to similarity searches with the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology (NCBI) sequence database (National Institutes of Health, Bethesda, MD). Alignment was made by using the CLUSTALW program, version 1.7 (Thompson et al. 1994) at the European Bioinformatics Institute homepage. An unrooted Neighbor-Joining dendrogram was derived from the alignment and repeated on 100 bootstrap samples by using PHYLIP software program (Felsenstein 1993).

Nucleotide sequence accession number. The nucleotide sequence of the *fcb* DNA region has been deposited in GenBank.

RNA extraction and reverse transcription PCR (RT-PCR). Recombinant *Rhodococcus* cells were grown in K1 medium amended with 3 mM 4-CBA (Rodrigues et al. 2000). Cells were harvested by centrifuging 10 min at 1,500 *x g* and resuspended in TE buffer (pH 8.0) containing 3 mg/ml of lysozyme. Cell were lysed by two passages through a French press at 20,000 lb in^2. The Quiagen RNAeasy Kit (Quiagen, Valencia, CA) was used to purify the total RNA from lysate according to the manufacturer's instructions. DNA removal was obtained with two on-column treatments with addition of the RNAase - free DNAase I (Davis et al. 1999). RT-PCR was performed with the Pro-Star HF Single Tube RT-PCR System (Stratagene, LaJolla, CA) according to instructions, using a first strand synthesis at 43°C for 30 min. Primer pairs used during RT-PCR were synthesized at the Macromolecular Structure Facility, Michigan State

University, as follows: 1) region between *fcbA* and *fcbB*; foward JT-679 (5'-AACTGATCCGCCGAGACAACATCC-3') and reverse JT-688 (5'-CCCCCGCACACCGCATCAAG-3') and 2) between *fcbB* and *fcbC*, forward JT-1286 (5'-GAGGCGTATGAGTGGGGAGTGGTC-3' and reverse JT-1287 (5'-AGCGCTGGCGTAGGGTGTCTGTA-3'). Annealing temperatures during amplification for the two primer pairs were 55°C and 53°C, respectively. PCR products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Materials. ONPG, 4-CBA, 4-HBA, ampicillin, and tetracycline were purchased from Sigma Chemical Company (St. Louis, Mo). All others reagents used were of laboratory grade.

RESULTS

Mapping and sequence of the *fcb* operon. A number of deletional inserts of the plasmid pCA311 were constructed, introduced into *E. coli* cells, and analyzed for the conversion of 4-CBA to 4-HBA by chloride release. The plasmid pCH1 retained the dechlorination function. The whole DNA fragment containing 7,612 nucleotides (nt) was completely sequenced from the plasmid pCA311. The mol % G + C of the fragment was 61.17, which is consistent with the genomic nucleotide composition for the species *A. globiformis* (62.0-65.5 %) (Keddie et al. 1986). Translation of the DNA sequence in all six reading frames revealed 14 open reading frames (ORFs) in both orientations (Figure 2.1). The ORF1, ORF2, and ORF3 were identified as structural genes responsible for the dechlorination activity and named *fcbA*, *fcbB* and *fcbC*, respectively. Preceding the *fcbA*

gene, a putative ribosomal binding site consisting of –35 (TTGCGA) and –10 (TATGTT) regions spaced by 18 bp was found, followed by the Shine-Dalgarno sequence AAGGAG at the position -5 from the start codon. The *fcbA* gene shows a GTG start codon at nt 271 and ends at nt 1833, which could code for a polypeptide of 520 aminoacids (aa) with a molecular mass of 56,417 Da. A GenBank database search using the *fcbA* sequence revealed that it was identical to the 4-chlorobenzoate CoA-ligase from the *Arthrobacter* sp. strain SU (Schmitz et al. 1992). The second ORF, *fcbB*, starts at nt 1843 and ends at nt 2673, encoding for a 276 aa polypeptide of 29,897 Da. This ORF was found to be 99% similar to a 4-chlorobenzoate-CoA dehalogenase from strain SU (Schmitz et al. 1992). The final ORF required for dehalogenation (*fcbC*) starts at the nt position 2681 and ends at the position 2764, with a frameshift of two nt in the reading strand. This third ORF was 100% identical to a 4-chlorobenzoate thioesterase from the same organism described above. The ORF3 encodes for a 151 aa polypeptide of 16,393 Da.

Expression of the *fcb* genes. The newly constructed plasmids (Figure 2.1) containing different size fragments of the 7.6-kb *Arthrobacter globiformis* DNA were introduced into *E. coli* strain JM109 and assayed for dechlorination. Only pCH1- and pXB3-plasmid containing cells showed dechlorination activity. Induction of protein synthesis in minicells containing one of these plasmids was carried out to confirm activity. Minicells containing plasmids pCH1 (lane 2) or pXB3 (lane 3) showed polypeptides with molecular masses of approximately 57, 30, and 17 kDa (Figure 2.2). The pCA311-plasmid containing minicells (lane 4) expressed an additional polypeptide of approximately 50 kDa. The band correspondent to this polypeptide was also shown for minicells with the plasmid pKXS7 (lane 6). Polypeptides of approximately molecular

weight of 46 and 34 kDa appeared when minicells contained plasmid pXB11 (lane 5). These are believed to be truncated polypeptides as indicated by restriction analysis of this plasmid.

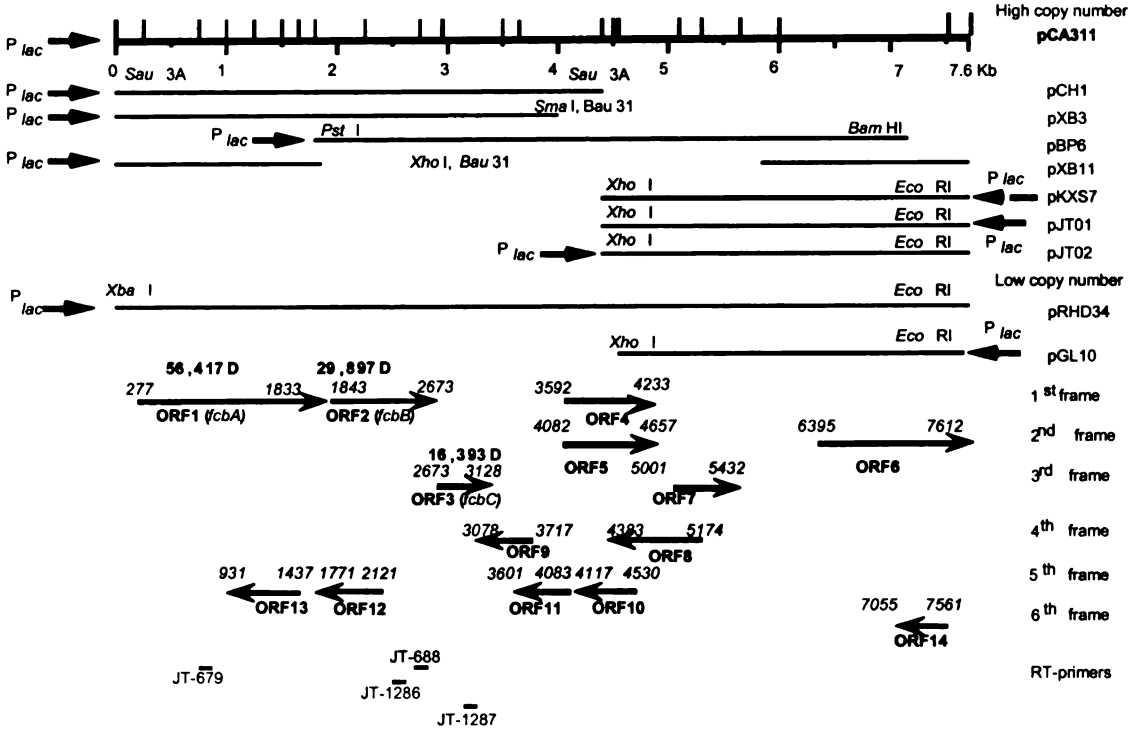

Figure 2.1. A) Physical and restriction enzyme map of the 7,612 bp DNA cloned fragment and deletional variants used for plasmid constructions. Bold arrows represent the direction of the *lac* promoter. B) The location and transcriptional direction of identified open reading frames are indicated by arrows, along with the calculated molecular masses of the corresponding *fcb* polypeptides. The positions of primers used for RT-PCR are also shown. Note that the primer identification number does not reflect the primer's position.

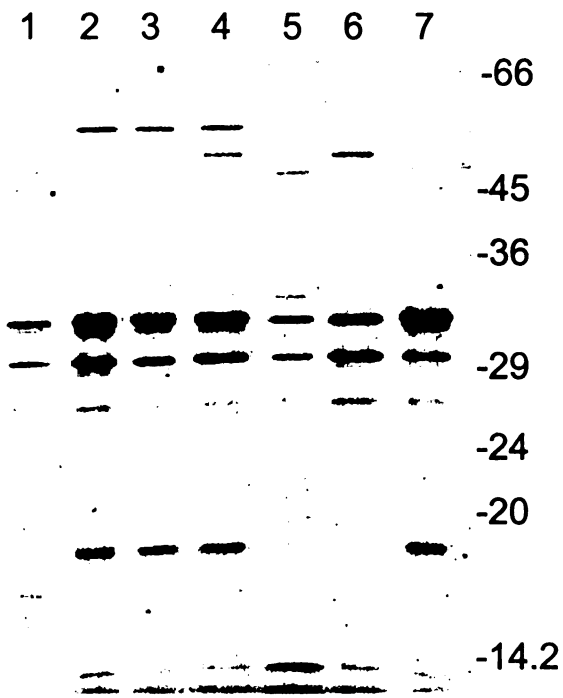

Figure 2.2. Autoradiogram shows ^{35}S-labeled proteins synthesized during induction with 4-CBA (100 μM). Lane 1 - pUC19 (control), lane 2 - pCH1, lane 3 - pXB3, lane 4 - pCA311, lane 5 - pXB11, lane 6 - pKXS7, and lane 7 - pBP6. Molecular mass standards (SDS-7, Sigma Co. St Louis, MO) were as follows: bovine serum albumin (66.2 kDa), hen egg white ovalalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Effect of 4-CBA and 4-HBA on *fcbA-lacZ* gene expression. To determine the expression of the *fcb* operon in response to 4-CBA as well as the first intermediate 4-HBA, a *fcbA-lacZ* operon fusion was inserted into the chromosome of the *E. coli* strain JM109. The resulting strain, JfcbP, was then grown in minimal medium with various concentrations of 4-CBA and 4-HBA (Figure 2.3) and the β-galactosidase activities were measured. Expression of the operon fusion occurred even when cells were grown in minimal medium without additions (Figure 2.3). Addition of increasing amounts of 4-CBA resulted in increased expression. This increase became significant when 2 mM and 5 mM 4-CBA were added to the medium. Addition of 1 mM 4-HBA repressed the expression of the fusion by about one-third. When the concentration of 4-HBA was doubled the repression was removed. In both cases the fusion showed reduced expression compared to the expression in the presence of corresponding amounts of 4-CBA.

Complementation of the *fcbA-lacZ* fusion with low copy number plasmids carrying the *fcb* operon and downstream region. To further study the expression of the *fcb* operon in the presence of potential regulators, we transformed JfcbP with two low copy number plasmids, pGL10 and pRHD34 which carry a 3.3-kb fragment downstream from the *fcb* operon or the entire sequenced region, respectively (Figure 2.3). Cells transformed with the parent plasmid only showed similar expression to the non-complemented strain (data not shown). In the absence of 4-CBA the expression from the putative *fcb* promoter was repressed in strains transformed with pRHD34 while repression in strains carrying pGL10 was minimal. Addition of increasing concentrations of 4-CBA resulted in increased expression. The expression was especially high in the

strains complemented with pGL10 and grown at 5 mM 4-CBA. The first intermediate of the pathway, 4-HBA had no effect on the expression of the *lacZ* fusion.

Complementation with high copy number plasmids. In the absence of 4-CBA strains carrying plasmids containing the full length fragment or the downstream region showed expression very similar to the uncomplemented parent strain. The expression in the strain carrying pCA311, i.e. the *fcb* operon plus the downstream region, also increased with the addition of increasing amounts of 4-CBA. Concentrations of 5 mM 4-CBA also seemed to be toxic to the cells, since *E. coli* growth was slower (Figure 2.4). Addition of plasmid pKXS7, which contains the same downstream region as plasmid pGL10, resulted in an increase in activity in the presence of 5 mM 4-CBA. This increase in expression was also observed with pGL10 as described above. Addition of 4-HBA did not result in an increase of activity, but rather a slight repression.

Transcriptional analysis of the *fcb* operon. Two sets of primers were specifically designed to test whether the genes identified as part of the 4-CBA operon were cotranscribed. The first primer set was positioned between ORF1, the 4-CBA-CoA ligase and ORF2, the 4-CBA-CoA dehalogenase and the second set overlap the ORF2 and ORF3, the 4-HBA-CoA thioesterase. The fragment sizes of the RT-PCR products were 2,106 and 476 bp, respectively, for the primer set one and two (Figure 2.5). The amplification product sizes are the same as predicted from the sequences. Control treatment without reverse transcriptase showed no amplification indicating a DNA free PCR reaction.

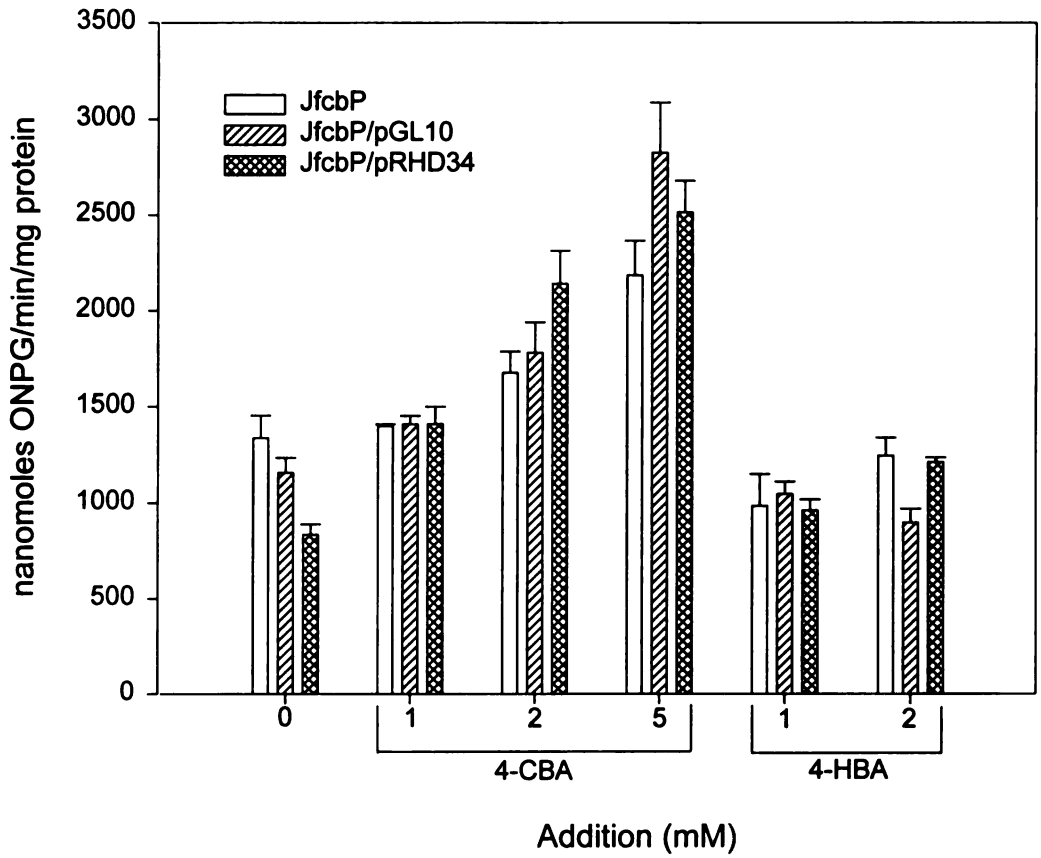

Figure 2.3. Effect of different concentrations of 4-CBA and 4-HBA on the expression of the *fcbA-lacZ* operon fusion with low copy number plasmids containing the 3.3 kb fragment downstream of the *fcb* operon.

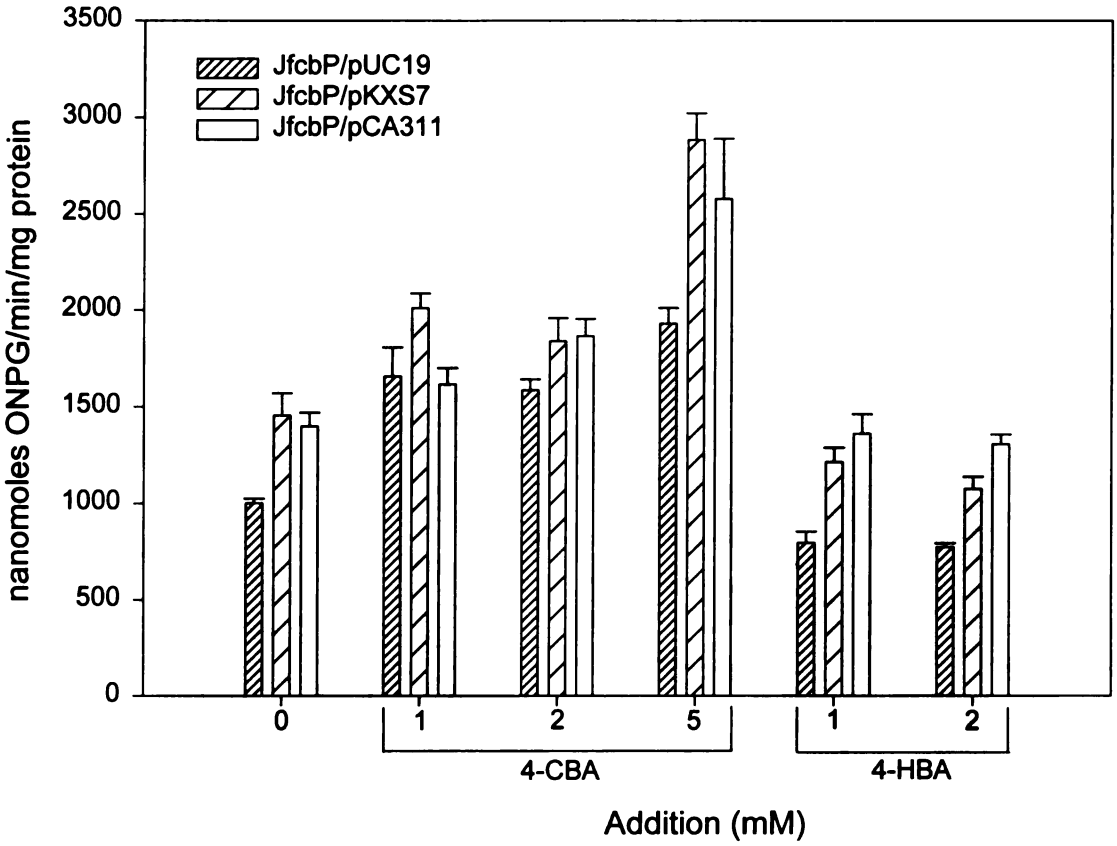

Figure 2.4. Effect of different concentrations of 4-CBA and 4-HBA on the expression of the *fcbA-lacZ* operon fusion with high copy number plasmids containing the 3.3 kb fragment downstream of the *fcb* operon.

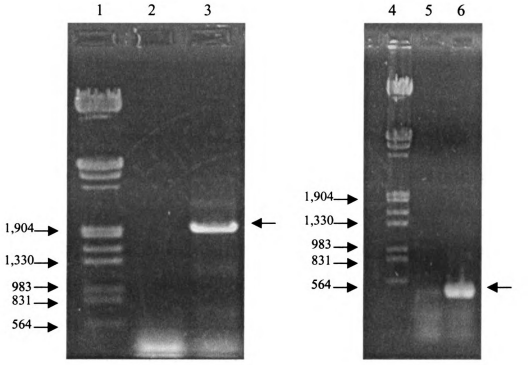

Figure 2.5. Ethidium bromide-stained agarose gel of RT-PCR products. Lanes 1 and 4 - Molecular marker λ restricted with *Eco* RI and *Hin*d III, lane 2 and 5 – control without reverse transcriptase, lane 3 – PCR product of expected size between ORF1 and ORF2 (2.0 kb), and lane 6 - PCR product of expected size between ORF2 and ORF3 (0.4 kb).

DISCUSSION

We have determined the nucleotide sequence and structural organization of the operon involved in the degradation of 4-chlorobenzoate by *Arthrobacter globiformis* strain KZT1. Sequence comparison show that the *fcbA*, *fcbB* and *fcbC* genes encode a 4-chlorobenzoate CoA-ligase, a dehalogenase, and a thioesterase, respectively. Expression of the *fcb* genes agrees (Figure 2.2) with protein sizes predicted by sequence analysis (Figure 2.1). While enzyme purification and kinetic studies have been done for the 4-CBA dehalogenase isolated *Pseudomonas* sp. strain CBS-3 (Chang et al. 1992, Löffler et al. 1995), the dehalogenase of *Arthrobacter* spp. has not been examined in detail. In addition no protein expression and operon regulation studies have been reported for the *fcb* genes isolated from any bacterial strain. The *fcb* operon in *Arthrobacter* strains is plasmid borne while the operon is chromosomal in *Pseudomonas*, which agrees with the pattern of sequence similarity shown in Figure 2.6 for FcbB. Gene regulation of chromosome- and plasmid- borne *fcb* operon from *Pseudomonas* and *Arthrobacter*, respectively, remains to be compared.

Sequence analysis revealed information about the evolutionary relationships of the dehalogenase gene, the promoter of the operon and the binding site of a potential regulator. The translated nucleotide sequence of the 4-chlorobenzoate dehalogenase (*fcbB*) gene showed significant sequence identity to the enoyl-CoA hydratase family. This group of enzymes is involved in fatty acid β-oxidation and catalyzes the hydratation of 2-*trans*-enoyl-CoA into 3-hydroxyacil-CoA (Minami-Ishii et al. 1989). The mechanism of action for the dehalogenase and hydratase is similar (Babbitt et al. 1992), i.e. H_2O activation for addition across a carbon-carbon bond in conjuction with a CoA-

thioester group. Babbitt at al. (1992) suggest a probable evolutionary relationship between them, as well as the *menBE* operon involved in the menaquinone biosynthesis (Driscoll et al. 1992). The sequence relationship among these enzymes is shown in Figure 2.6. The dehalogenase might have evolved from enzymes involved in fatty acid oxidation and menaquinone biosynthesis which are processes central to cell metabolism.

The promoter region controlling the *fcb* operon, assigned based on established σ^{70} *E. coli* consensus sequences, shared 50-60% of similarity with other promoter sequences previously identified for the operons encoding the chlorocatechol 1,2-dioxygenase (*clcABC*) (Frantz and Chakrabarty 1987) and *cis,cis*-muconate lactonizing enzyme (*cat*BC) (Rothmel et al. 1990) (alignment not shown). These promoter regions, as well as promoter regions for the naphthalene metabolism operon, are positively induced by the presence of the substrate (Schell 1993). Our studies showed that the expression of the *fcb-lacZ* fusions was induced by 4-CBA (Figures 2.3 and 2.4) providing additional evidence that this promoter belongs to the same group.

The sequence upstream of the –35 site resembles the same consensus sequence observed for the LysR family of transcriptional activators (72 – CTGA – N_7 – TCAG – 58), suggesting that the *fcb* operon would be under the same type of regulation. Since the three catalytic genes are aligned in the same direction, the result of the RT-PCR suggests that the *fcb* operon is transcribed as a polycistronic mRNA, indicating that these genes are controlled by the same promoter region. However, none of the open reading frames downstream from the *fcb* operon showed any similarity to transcriptional regulators. We constructed operon fusions in order to determine the general pattern of *fcb* gene expression and to determine if any of the downstream ORFs had a role in regulation of

gene expression. *E. coli* was used because the operon fusion could be inserted into the chromosome in single copy and mini cell studies had established that the genes could be expressed in this bacterium.

Initial studies showed that the promoter was constitutively expressed at a measurable level. The expression of the fusion increased slightly with the addition of increased amounts of the substrate while it remained low if 4-HBA was added. Therefore the operon is expressed in response to a specific substrate. The constitutive expression indicated the action of a repressor, which was not present in *E. coli*.

Several open reading frames located downstream from the *fcb* operon could not be identified as regulators based on database searches. In order to determine if any of the ORFs were involved in the expression of the operon, low and high copy number vectors carrying part of or the entire downstream region (Figure 2.1) were introduced into the fusion strains. Vectors pGL10 and pKXS7, which carry most of the downstream 3.3-kb of the sequenced fragment did not change expression of the operon significantly compared to the uncomplemented strains. However, when the full-length fragment was introduced on a low copy number vector the expression was significantly reduced in the absence of 4-CBA. The repression was reversed when the substrate was added. This observation indicates that the *fcb* operon seems to be regulated by a repressor, which is inactivated in the presence of 4-CBA. The homologous multicopy vector did not have the same effect. This could be due to a dilution of the repressor because each vector carries a copy of the operon and a potential binding site for a repressor.

Since the vectors carrying the shorter region of the fragment did not have an effect on expression, the region between the *fcbC* gene and the *Xho*I site at 4331

basepairs must be involved in regulation. This region contains several open reading frames. In order to determine the role of these open reading frames, future experiments will include the subcloning of this fragment into a low copy number vector. The effects of the resulting construct on gene expression will then be examined using the strains constructed in this study. This procedure would also eliminate interference of the functional genes.

The *fcb* operon *in Arthrobacter globiformis* is composed of three genes, which are transcribed as a single unit and produced three proteins with the expected molecular weight. We were successful in constructing reporter fusions which indicate that expression is induced in response to the substrate, 4 CBA, and is regulated by a repressor. Further research will focus on the identification of the regulator and its mechanism of action. These studies will not only allow us to understand the mechanisms of repression, but also will eventually contribute to improve conditions for bioremediation of chlorinated aromatics by combining both biphenyl and 4-chlorobenzoate degradation pathways into the same PCB-degrading bacterium.

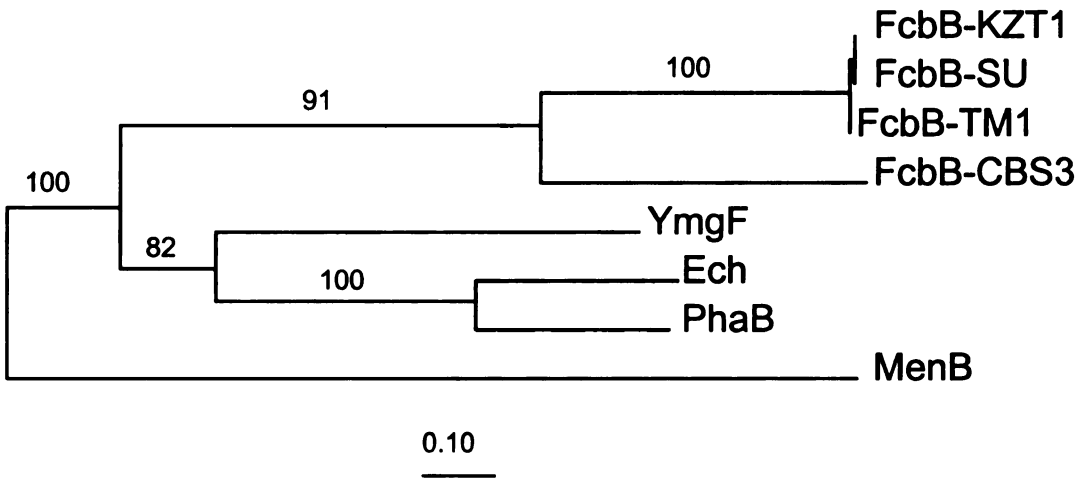

Figure 2.6. Unrooted Neighbor-Joining dendrogram showing evolutionary distance of 4-chlorobenzoate dehalogenases and hydratases. The horizontal axes are scaled in terms of expected number of aminoacid substitutions per site. Numbers at the internal nodes indicate the number of times out of 100 bootstrap samples that the cluster defined by the node was monophyletic. In descending order of overall sequence similarity, proteins were the FcbB from *Arthrobacter globiformis* strain KZT1, the FcbB from *Arthrobacter* sp. strain SU (Genbank accession nos. in parenthesis. A48956), FcbB from *Arthrobacter* sp. strain TM1 (AF042490), FcbB from *Pseudomonas* sp. strain CBS3 (A42560), YngF (enoyl-CoA hydratase) from *Bacillus halodurans* strain C-125 (BAA75329), enoyl-CoA hydratase from *E. coli* (X97452), PhaB (enoyl-CoA hydratase II) from *Pseudomonas putida* (AF029714), MenB (naphthoate synthetase) from *Bacillus subtilis* (F69656).

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CHAPTER III

DEVELOPMENT OF A *RHODOCOCCUS* RECOMBINANT STRAIN FOR DEGRADATION OF PRODUCTS FROM ANAEROBIC DECHLORINATION OF AROCLOR 1242

ABSTRACT

The Gram-positive bacterium *Rhodococcus* sp. strain RHA1, naturally containing the biphenyl pathway, was electroporated with a broad host range plasmid containing the 4-chlorobenzoate (4-CBA) degradation operon (*fcb*) isolated from *Arthrobacter globiformis* strain KZT1. The recombinant strain grew in medium containing 4-CBA and 4-chlorobiphenyl (4-CB) as the only source of carbon, with stoichiometric release of chloride and a molar growth yield on 4-CB that suggested utilization of both biphenyl rings. Similar conversion rates were observed for wild type and recombinant strains for the most common eight congeners from the anaerobic dechlorination of Arochlor 1242 (pattern M), but the recombinant strain accumulated lower amounts of chlorinated *meta*-cleavage products and no 4-CBA. Recombinant cells inoculated at 10^4 cells/g into non-sterile soil contaminated with 4-CB grew to $6 \cdot 10^5$ cells/g, a density consistent with the 4-CB consumed. 4-CB was removed only in the inoculated soil and the recombinant strain did not grow in the same soil but with no PCB. The *fcb* operon remained stable in the recombinant strain reisolated from soil after 60 days. This work provides proof of

concept that a *Rhodococcus* strain constructed to grow on a PCB would actually grow in non-sterile soil contaminated with the appropriate PCB substrate.

INTRODUCTION

Polychlorinated biphenyls (PCBs) were extensively used in industry owing to their physico-chemical properties such as stability, incombustibility, high insulation capacity, and low volatility. These same properties contribute to their long-term persistence in the environment where they bioaccumulate in higher trophic levels. Some PCBs are potentially hazardous to human health, have potential estrogen-like activity and can cause liver damage (Silberhorn et al. 1990). Because complete mineralization of commercially used PCB mixtures is not achieved by aerobic microorganisms, a sequential anaerobic-aerobic biological treatment system has been proposed (Abramowicz 1990). Reductive dechlorination of PCBs by anaerobic microorganisms (Bedard and Quensen 1995, Holoman et al. 1998, Quensen et al. 1988, Quensen et al. 1990, Wu et al. 1998, Ye et al. 1995) converts highly chlorinated congeners into less chlorinated biphenyls, primarily *ortho-* and *para-* substituted, which are then subject to aerobic attack. The aerobic stage of the PCB treatment scheme is limited by the inability of aerobic biphenyl degraders to grown on and completely metabolize the PCBs and to thrive in soil typical of contaminated sites. Most PCB degraders can not use the chlorobenzoates produced from the biphenyl pathway for growth and these products as well as the *meta*-cleavage products, chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDAs), can be inhibitory (Furukawa 1982, Seto et al. 1996, Stratford et al. 1996).

Several hybrid strains of PCB degraders have been constructed, usually by transferring biphenyl pathway genes to chlorobenzoate degraders (Havel and Reineke 1991, Hickey et al. 1992, Lajoie et al. 1993, McCullar et al. 1994, McKay et al. 1997), but also recently by the opposite transfer (Hrywna et al. 1999). All these constructions were done in Gram negative bacteria. Gram positive bacteria, especially *Rhodococcus* strains, offer a number of advantages for environmental use. These include higher growth yields on biphenyl, the presence of multiple PCB metabolic systems allowing co-oxidation of a wider range of PCB congeners (Masai et al. 1997, Seto et al. 1995), and more tolerance to environmental stresses such as drought or exposure to toxic compounds (Hatta et al. 1998, Warhust and Fewson 1994). Genetic engineering of catabolic pathways in *Rhodococcus*, however, is not well developed. In this study, we developed a broad host range vector suitable for transfering dehalogenase gene cassettes into *Rhodococcus* as well as Gram negative strains. Our objectives were to test whether the 4-chlorobenzoate degradation (*fcb*) operon (Tsoi et al. 1991) in this new cassette would allow *Rhodococcus* strain RHA1 to grow on 4-CB and 4-CBA, to determine the stability of this construct, and to test whether this strain inoculated at low densities into soil would grow on and degrade the targeted PCB.

MATERIAL AND METHODS

Bacterial strains and culture conditions. The strains used in this study were *Rhodococcus* sp. strain RHA1 (Masai et al. 1995) and *Escherichia coli* strain JM109 (Promega, Madison, WI). Cells were grown in Luria-Bertani (LB) broth, and when required, ampicilin (100 μg/mL for *E. coli*) or tetracycline (10 μg/mL for *E. coli* and 40

μg/mL for *Rhodococcus* sp.) were added. *Rhodococcus* transformants were grown on synthetic medium K1 (Zaitsev et. al. 1991) containing 2 mM 4-CBA or 3 mM biphenyl (nominal concentration). Strain RHA1 is unable to grown on or metabolize 4-CBA.

Construction of recombinant *Rhodococcus* strain. A Gram +/Gram – shuttle vector was developed by cloning the 2.7 Kb *Rhodococcus* replicon from pRC1 (Hashimoto et al. 1992) into *Pst*I-*Xba*I sites of a broad host range vector pSP329 (Tsoi et al. 1999), resulting in the plasmid pRT1. Cloning of the 4.4 Kb *Xba*I fragment from the plasmid pCH1 containing the structural *fcbABC* operon from *Arthrobacter globiformis* strain KZT1 (Tsoi et al. 1991) into the plasmid pRT1 under the control of P_{lac} promoter, yielding pRHD34. Plasmid DNA was isolated and purified by using a WIZARD miniprep kit (Promega, Madison, WI.). Restriction and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions.

The plasmid was transformed into *Rhodococcus* RHA1 by electroporation. Cells were grown in 10 mL LB medium at 30°C to OD_{600} of 0.6, harvested by centrifuging 10 min at 1500*g*, and washed three times with ice-cold 0.3 M sucrose. Cells were resuspended in 250 μL ice-cold 0.5 M sucrose; 100 μL of the cell suspension was mixed with 3 μg of plasmid DNA (1 μg/μL). The mixture was transferred to pre-chilled 0.1 cm cuvettes and subjected to electroporation with at 12 kV/cm, 800 Ω; and 25 μF (Bio-Rad Laboratories). LB medium (1 mL) containing sucrose (0.5 M) was added to the mixture and the suspension was shaken at 150 rpm and 30°C for 12 h. The suspension was plated onto LB plates containing tetracycline (40 μg/mL) and incubated at 30°C for 5 days. Colonies were screened for their ability to grow on K1 plates amended with 1.25 mM 4-

CBA. Individual colonies were transferred to liquid medium of the same composition with 4-CBA.

Stability of the *fcb* operon. Transformants with pRHD34 were grown in liquid medium with 2 mM 4-CBA and inoculated into 200 mL of non-selective medium containing 3 mM biphenyl. Cultures were grown at 30°C on a rotary shaker to final OD_{600} 1.0 (first cycle of growth). Cells were plated on LB medium when OD_{600} was 0.30, 0.67, 1.0. At the end of the first cycle, a 0.1% inoculum was transferred to fresh non-selective medium and plated when OD_{600} reached 1.0 (second cycle). The initial and final percentages of *fcb*-containing colonies were determined by picking 50 colonies from each sampling time and screening for *fcb* genes by PCR amplification. Template DNA for PCR was prepared by lysing whole cells in 100 μL NaOH 0.05N at 95°C for 15 min. Primers were specifically designed for *fcb*A (5' AACTGATCCGCCGAGACAACATCC 3' and 5'AGGCATTTTTCGAGACGCTTCA 3') and *fcb*B (5' GGTCCAGCGCGAAATCCAGTC 3' and 5' CCCCCGCACACCGCATCAAG 5') genes to yield PCR products of 598 and 599 bp, respectively. Amplifications were performed in a 20 μL reaction volume containing 10 pmol of primers, 200 μM each deoxynucleoside triphosphate, 400 ηg/mL of Bovine Serum Albumin, 1 X *Taq* Buffer, 1.5 U *Taq* DNA polymerase (Sigma, St Louis, MO), and 2 μL of DNA template. The PCR was initiated with a 3 min denaturation step at 94°C; followed by 30 cycles at denaturation temperature of 94°C for 1 min, primer annealing at 60°C for *fcb*A and 55°C for *fcb*B for 30 s, extension at 72°C for 2.1 min, and final extension for 5 min. Three μl aliquots of the PCR products were analyzed on 1% agarose gels.

Resting cell assay. Transformed *Rhodococcus* cells were grown in K1 liquid medium with repeated additions of 2 mM 4-CBA up to OD_{600} = 2.0. Substrate exhaustion was verified by HPLC. The total amount of substrate utilized was 10 mM. Cells were used to inoculate new liquid cultures with the same medium but using biphenyl as the only C source. When OD_{600} was 1.0, cells were harvested by centrifuging 10 min at 1500*g* and washed three times with 50 mM phosphate buffer (pH 7.0). Cells were concentrated and resuspended in the same buffer to OD_{600} = 2.0 and assayed in triplicate (Maltseva et al. 1999). Cells were assayed for metabolism of a mixture of eight PCB congeners that represent 70 % of the PCBs produced by pattern M dechlorination (Bedard and Quensen 1995). Chlorobenzoic acids were analyzed by high performance liquid chromatography (HPLC) in a 1050 Hewlett-Packard chromatograph equipped with a reverse-phase RP-18 column (Alltech) and a diode array detector. Aqueous solvent contained 1 ml of 85% *ortho*-phosphoric acid and 600 ml of methanol per liter. Formation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDAs) was analyzed by visible spectral scanning of the cell-free supernatant (Maltseva et al. 1999).

Growth assay. Transformants were inoculated into K1 synthetic medium containing 1 mM (nominal concentration) 4-chlorobiphenyl (4-CB). Cultures in triplicate were incubated in a rotary shaker at 30 °C. Growth was measured by an increase in optical density at 600 nm. Chloride release (Hrywna et al. 1999) and protein (Stoscheck 1990) were analyzed as previously described. For protein analysis, albumin was used as the standard and exposed to the same alkali conditions as the samples. Molar growth yields as cell dry weight were calculated from protein yields assuming the cells contain 60% protein.

Soil microcosms. Soil samples were obtained from a non-contaminated area adjacent to PCB contaminated soils at Picatinny Arsenal, New Jersey (28 miles northwest of New York City). The soil, a loamy sand (84.6 % sand, 12.1 % silt, and 3.4 % clay) with 3.5 % organic matter, pH 7.6, was passed through a 4-mm mesh sieve and stored at 4 °C until used. Total soil bacterial counts before inoculation were performed by staining with 5-(4,6-dichlorotriazine-2-yl) aminofluoroscein (DTAF) followed by epifluorescence microscopy (Bloem 1995). Naturally occurring rifampicin (Rif $^{+}$) mutants from the recombinant RHA1 were isolated from K1 medium amended with biphenyl (Smith and Tiedje 1980).

Rif-resistant recombinant cells grown on 4-CBA-containing medium were washed twice with 50 mM phosphate buffer (pH 7.0), resuspended in K1 medium, diluted, and added to 20 g of Picatinny soil previously amended with 100 ppm of 4-CB, giving a density of 10^4 cells/g of soil. Sterile treatment was obtained by autoclaving soil for 1 h during 3 consecutive days. A non-contaminated soil control was inoculated at the same cell density. Soil was brought to 30% moisture content with sterile K1 medium, mixed well, and incubated in triplicate at 30 °C. Cells were recovered from soil by vortexing 1 g of soil with phosphate buffer (9 mL) for 10 min. Appropriate dilutions were spread on LB plates containing rifampicin (50 μg/mL). Colony forming units (CFUs) were counted after one week of incubation. Fifty randomly selected colonies from each sampling time were used for PCR with the *fcb*B specific primers and plated on K1 medium containing 4-CBA or biphenyl as the only C source.

RESULTS

Construction and activity of the recombinant strain. The replication site from the broad-host range vector pRC1 was cloned into the plasmid pSP329, yielding the plasmid pRT1, which contains the *lac* promoter and also confers tetracycline resistance. The *fcb* operon responsible for hydrolytic dechlorination of 4-CBA (Tsoi et al. 1991) was cloned into the vector pRT1 under the regulation of the *lac* promoter yielding pRHD34. Transformation of the *Rhodococcus* strain RHA1 by electroporation with pRHD34 enabled cells to grow on LB plates containing tetracycline (40 μg/mL). Whole cell PCR amplification with *fcb*A and *fcb*B specific primers confirmed the presence of the two catabolic genes in the transformants (Figure 3.1). No PCR product was detected when the wild type RHA1 strain was used as a template, but PCR products were obtained with *E. coli* strain JM109 harboring the plasmid pCH1 used as positive control. The recombinant strain, RHA1(pRHD34), grew in medium containing 4-CBA as the sole carbon source, and released stoichiometric amounts of chloride (Figure 3.2). Complete disappearance of 4-CBA was confirmed by HPLC (data not shown). Controls of the wild type strain did not grow in medium containing 4-CBA.

The *fcb* operon in strain RHA1 appeared to be stable under non-selective conditions. All screened cells from the first cycle of growth contained both *fcb* genes and 49 out 50 colonies tested from the second cycle contained the genes. All colonies in which PCR amplification products were observed grew on plates containing 4-CBA.

Figure 3.1. Ethidium bromide-stained agarose gel of whole-cell PCR products after amplification of the *fcb*A (lanes 2 to 5) and *fcb*B (lanes 7 to 10) genes. The expected sizes are 598 and 599 bp, respectively. Numbers above lanes refer to (1 and 11) molecular marker, (2 and 7) recombinant strain RHA1, (3 and 8) wild type strain RHA1, (4 and 9) *E. coli* strain JM109 harboring plasmid pCH1 (positive control), and (5 and 10) no cells (negative control). Lane 6 was not loaded.

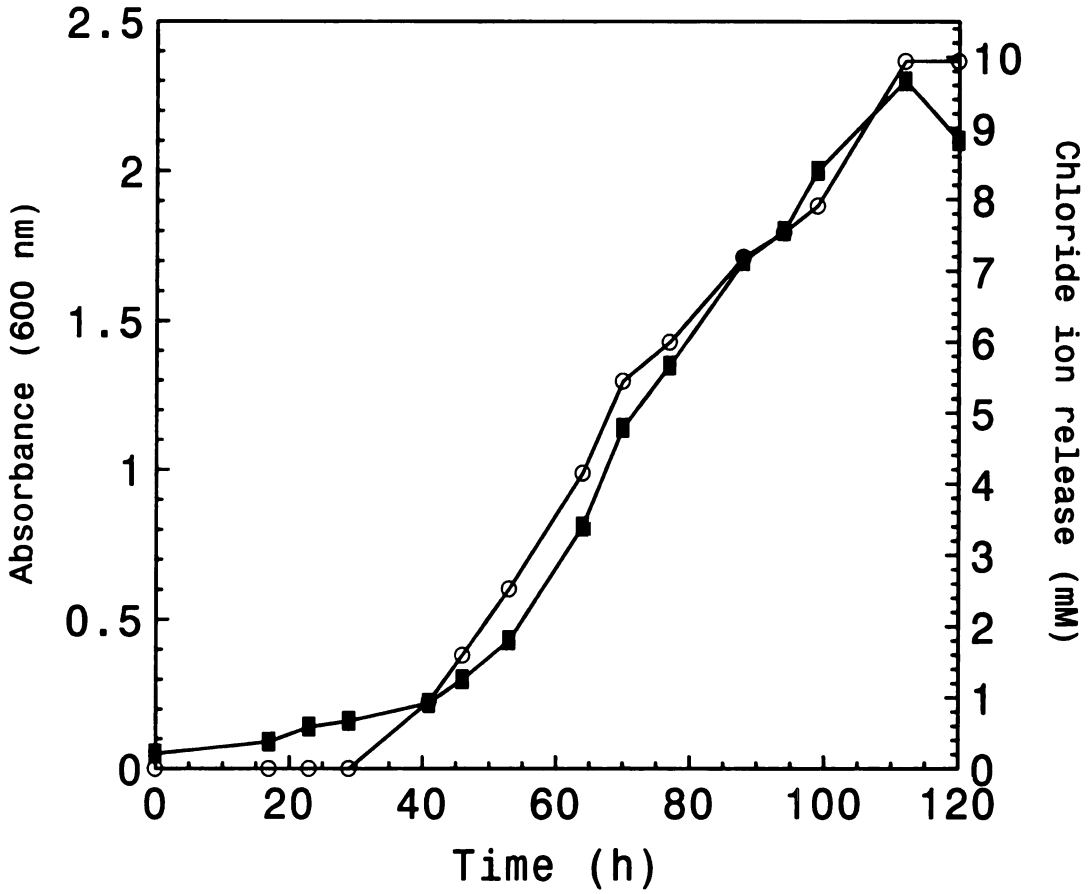

Figure 3.2. Growth of recombinant *Rhodococcus* sp. strain RHA1 containing the *fcb* operon in 4-CBA: (■) Optical density values at 600 nm, (○) chloride concentration. Data are mean values from duplicates.

Resting cells of the recombinant strain grown on biphenyl as the only C source, completely degraded of 2-, 4-, and 2,4-CB after 24 h of incubation (data not shown). No 4-CBA was detected, whereas accumulation of equimolar amounts of 2- and 2,4-CBA was observed. Wild-type RHA1 accumulated stoichiometric quantities of 4-CBA from 4-CB. Complete utilization of 4-CBA occurred in all treatments in which 4-CB was degraded, suggesting activity of the enzymes encoded by the *fcb* operon in the recombinant strain.

Transformation of PCB anaerobic dechlorination products. Because the wild type strain RHA1 could degrade the major products from pattern M anaerobic dechlorination with a theoretical recovery of 50% of *para*-chlorobenzoates (Maltseva et al. 1999), the eight PCB congeners that most accumulated in this pattern were used to test the activity of the recombinant strain. Similar degradation rates for mixture M congeners by the wild type RHA1 and its recombinant were obtained (Figure 3.3). Sixty percent of PCBs present in the mixture M were degraded by the wild-type strain with accumulation of 2-, 4- and 2,4-CBAs. In contrast, strain RHA1 containing the *fcb* genes accumulated only trace concentrations of 4-CBA. HOPDA concentrations were 38% less for the recombinant strain (data not shown). Final concentrations of 2- and 2,4-CBA were similar for both wild type and transformant strain, with no utilization of these metabolites in either case (Figure 3.3).

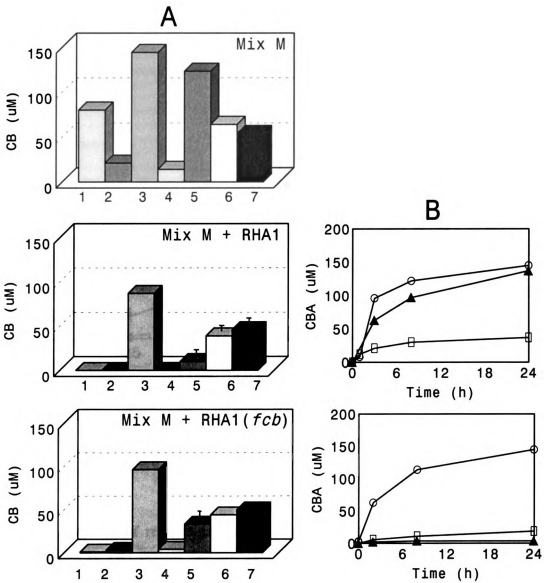

Figure 3.3. Degradation of PCB mix M (A) and accumulation of chlorobenzoates (B) by resting cells of wild-type and recombinant *Rhodococcus* sp. strain RHA1. A: Bars 1 through 7 in mix M represent congeners 2-CB, 4-CB, 2,2' and 2,6-CB, 2,4-CB, 2,4'-CB, 2,4,2'-CB, and 2,4,4'-CB, B: (○) 2-CBA, (▲) 4-CBA, and (□) 2,4-CBA. Data are mean values and standard error from triplicates. Standard errors for CBA's are smaller than symbols.

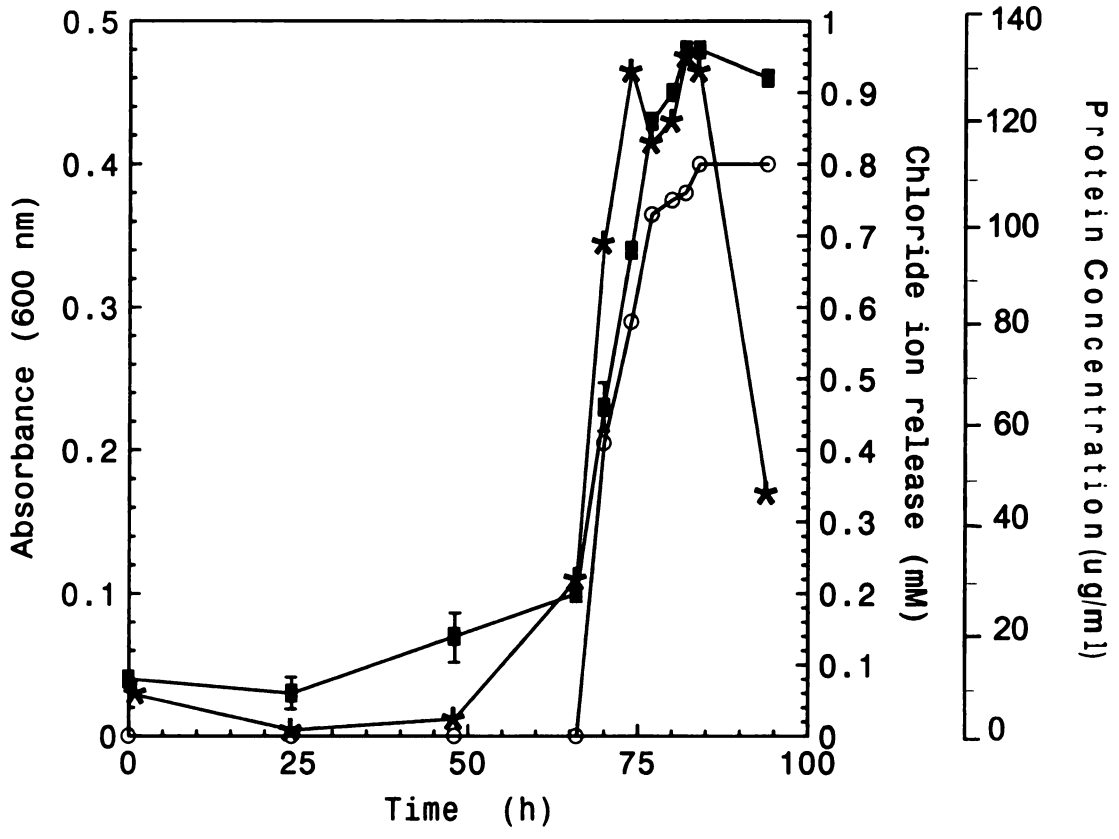

Figure 3.4. Growth of recombinant *Rhodococcus* sp. strain RHA1 containing the *fcb* operon on 4-CB: (■) optical density, (○) chloride ion concentration, and (*) protein concentration. Data are mean values and standard error from triplicates for absorbance values.

Growth on 4-CB. The recombinant strain RHA1 grew with 4-CB (1 mM nominal concentration) as the only C source and released nearly stoichiometric amounts of chloride (Figure 3.4). The molar growth yield of the recombinant strain on biphenyl and 4-CB was 177 ± 6 and 189 ± 9 g dry weight of cells/mol of substrate, respectively, which is similar to the theoretical value of 173 g dry weight of cells/mol for complete 4-CB oxidation. An initial long lag-phase of approximately 50 h was followed by a sharp increase in growth to OD_{600} = 0.46. No transient formation of 4-CBA was detected during growth.

Growth in soil. Direct bacterial counts of the soil before inoculation averaged to 9.9×10^8. Since we could not distinguish our strain by color and colony morphology in comparison to the indigenous bacteria on mineral medium amended with 4-CBA or biphenyl, we selected a rifampicin-resistant mutant for tracking our recombinant strain. No indigenous bacteria grew on this medium. Recombinant cells grew in both sterile and non-sterile soil concurrent with 4-CB removal (Figure 3.5), but did not grow in the non-contaminated, non-sterile control soil. Most significant, however, was the growth of the recombinant strain in the inoculated non-sterile 4-CB contaminated soil and 4-CB removal. Furthermore, analysis of the growth stoichiometry in the inoculated non-sterile soil is consistent with the yield expected from growth on PCBs. The initial growth (0-2 days) may have been partially due to carry over of endogenous reserves of the cells but the growth from day 2 to 10 ($2 \cdot 10^5 \pm 5051$ to $6 \cdot 10^5 \pm 3464$ cells/g), which was 67 % of the 10 day total growth, corresponds to the cell yield of 22.7 μg dry weight of cells/g. Using the molar growth yield, the 22.6 ppm of PCB removed between days 2 and 10 should have produced 25.3 μg of cells on a dry weight basis, similar to the 22.7 μg of dry

weight of cells found. Additional growth would have been expected after 10 day from the remaining PCB consumed but this was not observed. This could be due to the restoration of endogenous reserves, oxidation of some intermediates by other populations, or loss of cells to grazing by soil protozoa. The non-inoculated soil showed no removal of PCB providing further evidence that the inoculated strain was responsible for PCB degradation (Figure 3.5).

The *fcb* operon appeared stable in both the sterile and non-sterile soil since during 60 days only one of the 700 colonies examined did not yield a *fcb*B amplicon (Figure 3.5). Furthermore, the same isolated colonies grew on solid medium containing 4-CBA as the only carbon source. All colonies also grew on plates containing biphenyl as the C source.

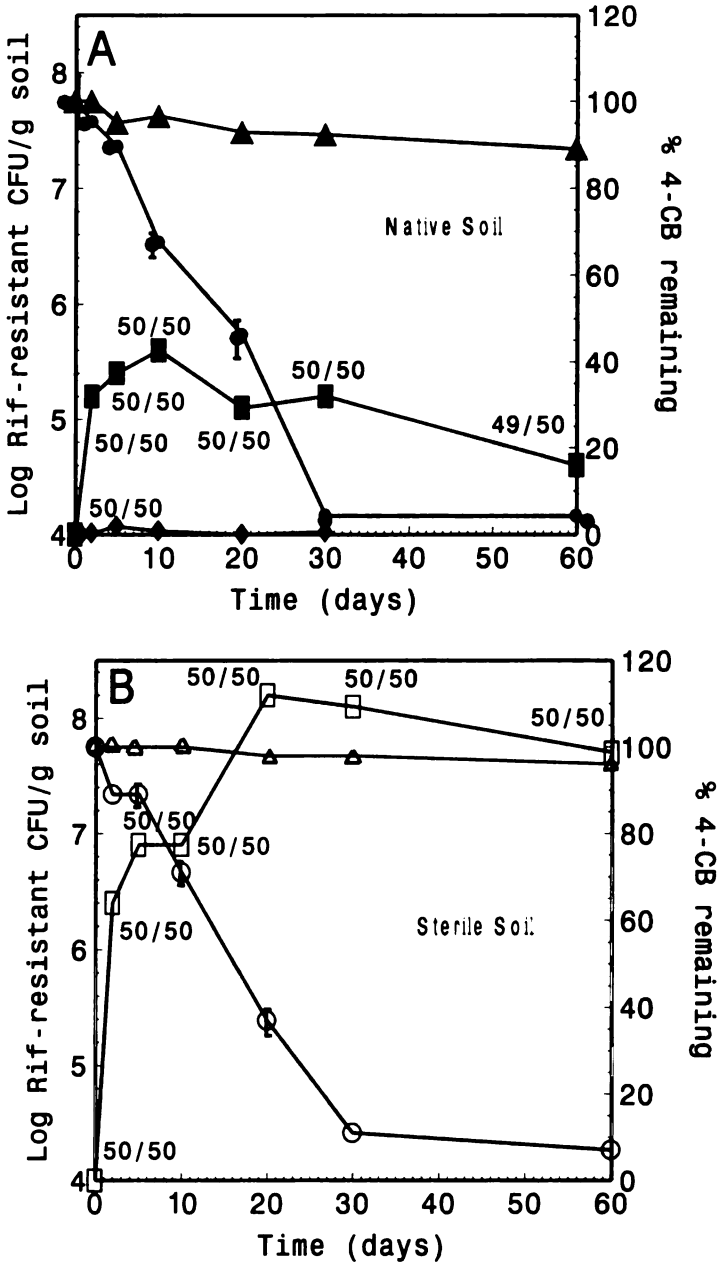

Figure 3.5. Population density of recombinant *Rhodococcus* sp. strain RHA1 containing the *fcb* operon and 4-CB remaining in non-sterile soil (A-closed symbols) and sterile soil (B-open symbols). Symbols: (■,□) population of recombinant in 4-CB contaminated soil and (◆) the same soil without 4-CB, (●,○) 4-CB concentration in inoculated soils, and (▲,△) 4-CB in soils with no inoculant. Values above each sampling time represent stability of the *fcb* operon in 50 randomly chosen colonies. Where the error bars are not shown, excluding controls, the standard error of triplicates was smaller than the size of the symbol.

DISCUSSION

The Gram-positive, biphenyl-degrading *Rhodococcus* sp. strain RHA1, transformed with a plasmid harboring the 4-chlorobenzoate degradation pathway grew on 4-CBA and 4-CB without accumulating 4-CBA and produced the stoichiometric amount of Cl^- and protein. Furthermore, when inoculated into non-sterile soil at a low density (10^4/g), the recombinant strain both grew and degraded 100 ppm of the appropriate PCB. Growth in the non-sterile soil agrees reasonably well with the amount of 4-CB consumed during the first 10 days. Effectiveness of a low inoculum density and growth in soil typical of a contaminated site are two key components needed for a practical remediation technology. This stage, however, represents only proof of a concept since the model tested involves a single congener, which was not aged in soil. Nonetheless, achieving growth and PCB removal in non-sterile soil is a major step towards the practical goal.

Attempts to engineer PCB degrading microorganisms have primarily focused on increasing the spectrum of PCB congeners degraded (Havel and Reineke 1991, McCullar et al. 1994, McKay et al. 1997), and few have paid attention to the problem of intermediate formation and their influence on growth (Stratford et al. 1996, Hickey et al. 1992, Kimbara et al. 1989, Seeger et al. 1995). Previous workers have taken advantage of naturally occurring CBA-degrading microorganisms and introduced the *bph* pathway without knowing the toxic effects of different PCB degradation intermediates to the engineered strain. This study took advantage of the strong PCB degrading capability of *Rhodococcus* strain RHA1, which transforms 45 congeners from different Kanechlor mixtures with accumulation of CBAs (Seto et al. 1996). In addition, this Gram-positive bacterium grows on other toxic compounds such as *o*-xylene, ethylbenzene, toluene,

benzene, propylbenzene, and butylbenzene (Hatta et al. 1998), which can be co-contaminants at PCB sites. Hence, this strain seemed to be at least among the most suitable for introduction of dehalogenase genes.

In the past, other PCB recombinant strains have been obtained through intergeneric mating (Stratford et al. 1996, Havel and Reineke 1991, Hickey et al. 1992, McCullar et al. 1994, McKay et al. 1997, Reineke 1998). Mating for PCB recombinants has involved combined chemostats or the presence of a third parental strain during transconjugation. It is also dependent on selective conditions, and can result in a low frequency of transconjugate recovery if those conditions are not maintained (Brener et al. 1994). The electrotransformation procedure used in this study yielded 5×10^4 tetracycline-resistant transformants per μg of plasmid DNA. The broad host range shuttle vector, which contained the entire *Rhodococcus* replication origin of pRC1 (Hashimoto et al. 1992) in vector pSP329, a low copy number plasmid, was stably maintained in both *E. coli* (data not shown) and *Rhodococcus* sp. hosts. In this plasmid the *fcb* operon is under control of the *lac* promoter, demonstrating the functionality of this promoter in this *Rhodococcus* species.

Since the 4-CBA degradation pathway is not expected to be under constant selection, information on the stability of degradation in the absence of any selective factor is important for environmental applications. The *fcb* operon was stable for 60 days after growth in both non-sterile and sterile soil, the later under primarily non-selective conditions. The long-term effect of carrying exogenous DNA sequences or constitutive expression of new pathways on the fitness of the recombinant strain was not studied

(Lenski and Nguyen 1988), however, the period in which aerobic PCB treatment is needed in the field would not normally be lengthy.

Previously, Maltseva et al. (1999) showed that the strain RHA1 degrades 60% of PCBs present in the anaerobic dechlorination pattern represented in mix M with production of CBAs. The recombinant strain had similar efficiency in utilizing mix M, but with advantageous characteristics of completely degrading 4-CBA and reducing the accumulation of the *meta*-cleavage products.

Growth on 4-CB was possible only when cells were previously grown on 4-CBA. Our failure in trying to grow recombinant cells in 4-CB after growth in biphenyl is believed to be due to the rapid turnover of 4-CB with accumulation of intermediate compounds such as HOPDA when the *bph* pathway is fully induced. Seto et al. (1996) have suggested that chlorinated HOPDA or its metabolites inhibited growth of wild type RHA1. In support of this interpretation, we observed that when our strain was inoculated into 4-CB-containing medium from biphenyl cultures, formation of HOPDA was rapid and intense, but no growth was detected. In contrast, the OD_{600} and protein concentration increased over time when inoculation was done with recombinant cultures grown in 4-CBA and only a light yellow color was seen indicating a low concentration of HOPDA. This result suggests that the RHA1 inoculum for a soil application should be grown on 4-CBA.

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CHAPTER IV

DETECTION AND QUANTITATION OF THE PCB DEGRADER *RHODOCOCCUS* SP. STRAIN RHA1 IN SOIL BY REAL TIME PCR

ABSTRACT

A real time PCR assay using fluorescently labeled oligonucleotides (TaqMan probes) was used to detect and quantify the recombinant *Rhodococcus* sp. strain RHA1 in soil. We developed two sets of primers/probes: The TaqMan-16S rDNA probe targeted the hypervariable region of the 16S rRNA gene and the TaqMan-*fcb* was directed toward the recombinant 4-chlorobenzoate degradation (*fcb*) operon. The TaqMan-16S rDNA probe detected RHA1 and phylogenetically related species while TaqMan-*fcb* probe was specific for the recombinant strain. The method had a 6-log dynamic range of detection (10^2 to 10^7) for both probes when tested cultures. Although the sensitivity of the method decreased in environmental samples, the estimated number of cells by real time PCR was similar to the measured number of RHA1 cells determined by colony forming units. The real time PCR is easy to perform, has high throughput, and was reliable for enumerating the recombinant strain RHA1 in soil.

INTRODUCTION

The success of any bioremediation process depends upon establishing favorable conditions for microorganisms to degrade an undesirable chemical. Failures in bioremediation using a particular bacterium might result more from our lack of knowledge about ecological constraints than from the information about the genetic capabilities of the strain of interest. The investigation of colonization by, fate of, and ecological consequences of releasing organisms is important for providing insights into better methods of inoculation, maintenance, and management of contaminated sites.

Markers for detection of microorganisms range from morphological to molecular. Morphological characteristics are frequently insufficient to distinguish a microorganism from other species in the environment. Molecular methods have been used for detection of microorganisms containing antibiotic resistance genes (Smith and Tiedje 1980), transposons (Recorbet et al. 1992), engineered catabolic genes (Hwang and Farrand, 1997), bacterial luciferase (*luxAB* genes) (Burlage et al. 1990) and green fluorescent protein (GFP) encoding genes (Blomberg et al. 1997, Leef and Leef 1996). However, addition or alteration of genetic traits may have deleterious effects on the environmental fitness of microorganisms (Lenski 1991). Other molecular tracking methods such as amplified ribosomal DNA restriction analysis (ARDRA) (Nüsslein and Tiedje, 1998), single-strand conformation polymorphism (SSCP) (Lee et al. 1996, Schwieger and Tebbe 1998), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al 1993, Muyzer et al. 1998), and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997) lack a quantitative component. *In situ* hybridization with fluorescently labeled oligonucleotide probes (FISH) targeted to the rRNA sequences has been used to detect

and quantify microbial groups in their environment and to study microbial population dynamics (DeLong et al. 1989). Despite its great utility in linking population structure to the function of a specific group of microorganisms in a community, FISH is labor intensive and has been more difficult to use in a complex environment as soil.

Real time PCR has recently been developed for estimating the number of copies of a targeted gene (Dölken et al. 1998, Heid et al. 1996, Higuchi et al. 1993). In this method, a double-labeled probe is used to measure the accumulation of fluorescence of the released reporter dye during PCR. The fluorescence is then correlated to the amount of product formed in real time during amplification when PCR is more quantitative (Higuchi et al. 1993).The method has been developed for quantifying several clinically important bacteria: *Listeria monocytogenes* (Bassler et al. 1995), *Yersinia pestis* (Higgins et al. 1998), *Mycobacterium tuberculosis* (Desjardin et al. 1998), and *Borrelia burgdorferi* (Pahl et al. 1999), and for a functional gene of ecological importance (Grüntzig et al. 2000).

Here, we have applied this technique to study the population dynamics of the polychlorinated biphenyl (PCB) degrader, *Rhodococcus* sp. strain RHA1 (Seto et al. 1995), containing the 4-chlorobenzoate (*fcb*) degradation operon (Rodrigues et al. 2000) in soil from an industrial area.

MATERIALS AND METHODS

DNA isolation. Bacterial strains, primers, probes, and plasmids used in this study are shown in Table 4.1. Genomic DNA from pure cultures was prepared from 50 ml

liquid cultures harvested by centrifugation. DNA was isolated by using of the Qiagen Blood and Cell Kit (Qiagen Inc., Valencia, CA).

Sequencing the 16S rRNA gene. The 16S rRNA gene of strain RHA1 was amplified by PCR with eubacterial primers (Table 4.1.). Amplifications were performed in a 20 μl reaction volume containing 10 pmol of primers, 200 μM each deoxynucleoside triphosphate, 400 ηg/ml of Bovine Serum Albumin, 1 X *Taq* Buffer, 1.5 U *Taq* DNA polymerase (Sigma Co., St Louis, MO), and 50 ηg of DNA template. The PCR was initiated with a 3 min denaturation step at 94°C; followed by 30 cycles at a denaturation temperature of 94°C for 1 min, primer annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension for 5 min. PCR products were analyzed on 1% agarose gels.

Cloning and transformation were carried out according to instructions provided with an Invitrogen TOPO Kit (Invitrogen Corp., San Diego, CA). *Escherichia coli* transformants were screened further by PCR with primers provided with the kit. Cell pellets were prepared from overnight growth of transformed *E. coli* in LB liquid medium containing ampicilin (50 μg/ml). Plasmid isolation was performed using a Promega Wizard Kit (Promega Corp., Madison, WI). Sequencing was performed at Michigan State University Sequencing Facility (East Lansing, MI). The sequences were aligned and edited using the Sequencher software version 3.0 (Gene Codes Corp., Ann Arbor, MI).

TABLE 4.1. Plasmids, bacterial strains, probes, and primers used in this study.

Plasmids, bacterial strains, and primers	Characteristics, catalog number, or sequence	Reference or source
Plasmids		
pRHD34	pRC1 replicon, *fcb*ABC	Rodrigues et al 2000
pCR 2.1-TOPO	Amp^r Kan^r α*lacZ* ColE1 origin	Invitrogen
pRHA16S	same as pCR 2.1-TOPO containing 16S rDNA gene from strain RHA1.	This work
Strains		
Escherichia coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- *Δ(lac-proAB) [F'(traD36)] proAB lacI ZΔM15*	Promega
Rhodococcus sp. RHA1(*fcb*)	Recombinant 4-chlorobenzoate degrader, Rif^r	This work
R. globerulus	ATCC 25714	
R. marinonascens	ATCC 35653	
R. opacus	DSM 43206	
R. percolatus	BMS 1	
R. erythropolis	ATCC 19369	
R. fascians	ATCC 35014	
Streptomyces griseus	ATCC 10137	
Primers and probes		
fD1 eubacterial	5' AGAGTTTGATCCTGGCTCAG 3'	Weisburg et al. 1991
rD1 eubacterial	5' AAGGAGGTGATCCAGCC 3'	Weisburg et al. 1991
Forward 16S rDNA	5' GCAGTACTCAAGTCTGCCCGTAT 3'	This work
Taqman 16S rDNA	5'FAM-CACAGTTGAGCTGTGAGTTTTCACAAACG-TAMRA 3'	This work
Reverse 16S rDNA	5' AAGGATTCGTAGGCGGTTTGTC 3'	This work
Forward *fcb*	5' GTTGATCGCCGCCAATG 3'	This work
TaqMan *fcb*	5' FAM-CGGCTTCTCGATCCGCGCC-TAMRA 3'	This work
Reverse *fcb*	5' TGGTACGGCACTAGGTGTA 3'	This work

Phylogenetic analysis and primer/probe design. The determined 16S rDNA sequence was submitted to the programs SEQUENCE MATCH and SEQUENCE ALIGN provided by the Ribosomal Database Project II (RDP) (Maidak et al. 1999) and aligned with the ARB software package (Strunk and Ludwig, 1995), which takes into consideration the secondary structure of the molecule. Design and checking of species specific probes were performed by searching 16,000 sequences of the ARB database for unique sequence regions. Primer/probe sequences were also tested under the CHECK PROBE program provided by the RDP as well as the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990).

Specific primers for the 16S rRNA gene were tested against template DNA obtained from species phylogenetically related to strain RHA1 and *E. coli* (Table 4.1). Amplifications were performed as above with annealing temperature of 68°C.

Soil experiment. Soil samples were obtained from a non-contaminated area near a PCB contaminated site at Picatinny Arsenal, New Jersey. The soil, classified as a loamy sand (84.6 % sand, 12.1 % silt, and 3.4 % clay), 3.5 % organic matter, pH 7.6, was sieved through a 4-mm mesh and stored at 4 °C until used. The soil contained 75 ppm of PCBs from artificial mix M (Maltseva et al. 1999) at the start of the 30-day experiment. Recombinant RHA1 Rif^r was grown on 3 mM 4-CBA, and washed twice with 50 mM phosphate buffer (pH 7.0), resuspended in K1 medium, and added in different 10-fold dilutions (1 ml) into 5 g of soil. Soil was brought to 30% water content with sterile K1 medium, mixed well, and incubated in duplicate at room temperature for 1 h. Total soil DNA was extracted with Soil DNA Extraction Kit (MoBio Laboratories, Inc., Solana

Beach, CA) according to manufacturer's instructions. DNA samples were stored at -20 °C until use.

Cells were recovered from soil by vortexing 1 g of soil with phosphate buffer (9 mL) for 10 min. Appropriate dilutions were spread on LB plates containing rifampicin (50 μg/mL). CFUs were determined after one week of incubation.

Real Time Quantitative PCR. The probe and primer sequences for real time PCR were designed by using the Primer Express software (Perkin Elmer Applied Biosystems, Foster City, CA). The probe contained FAM (6-carboxy-fluorescein) as a reporter fluorochrome on the 5' end and TAMRA (*N,N,N',N'*- tetramethyl-6-carboxy-rhodamine) as quencher on the 3' end of the nucleotide sequence. The 30 μl reaction volume contained 300 ηM of forward primer, 900 ηM of reverse primer, 275 ηM of TaqMan probe, 1 X TaqMan Buffer, 1.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA), and 30 ηg of DNA template from each appropriately diluted sample. Experiments were performed in triplicate for each time point. The cycle started with 2 min at 50 °C for optimal AmpErase uracil-N-glycosylase enzyme activity, followed by one cycle of denaturation at 95 °C for 10 min and by 40 cycles of amplification of 15 s at 95 °C for melting and 1 min at 64°C of annealing and extension. PCR was carried out in a spectrofluorimetric thermal cycler, ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA).

RESULTS

Signature probe design. The development of 16S specific internal probe for real time PCR was necessary for the *Rhodococcus* sp. strain RHA1. The design of the probe

was based on the phylogeny of the *Rhodococcus* sp. strain RHA1 and close relatives inferred by 16S rRNA gene alignment using the RDP and ARB (Figure 4.1). We were able to design three 16S rRNA oligonucleotide PCR primers for this purpose. PCR primer names were given according to the Oligonucleotide Probe Database (ODP) and their sequences are listed in Figure 4.2. These primers were intended to be as specific as possible for detecting strain RHA1 in single-cell assays in soil. However, all primers included more than one species of the genus *Rhodococcus*, because of the inherent difficulty in designing a strain-specific probe. The PCR primer S-*-Rco-0183-a-A-20 was designed by taking in consideration the presence of a longer stem loop in the *Rhodococcus* sp. 16S rRNA secondary structure from the positions 183 to 193 when compared to *E. coli* numbering system (Gutell 1993). The primer proved to be a perfect match for the species *Rhodococcus percolatus* and *R. opacus*. When one mismatch was allowed, one additional species, *R. marinonascens*, was detected. This primer had mismatches of two nucleotides for the non-target species *Nocardia asteroides*, but both mismatches (positions 189 and 196) are located at the central region of the primer, allowing mismatch discrimination due to the stringency of PCR conditions. The PCR primer S-*-Rco-0599-b-A-21 is located in the region 599-619 according to the *E. coli* 16S rRNA sequence. This primer targeted a signature region of the 16S rRNA shared by *Rhodococcus marinonascens* and *R. globerulus*. Mismatch of one nucleotide (C:U) was found for *R. opacus* at the position 599. The third primer S-*-Rco-0828-c-A-18 is positioned in a secondary stem loop and matches with the rDNA sequence of the following species: *R. marinonascens*, *R. globerulus*, *R. erythropolis*, and *R. fascians*. One

nucleotide mismatch was found at position 837 for a *Streptosporangium* and an *Actinomadura* (U:G) species.

Specificity studies. The three primer sequences were used as PCR primer pairs for specificity studies. When S-*- Rco-0599-b-A-21 and S-*-Rco-0828-c-A-18 were used as amplification primers at annealing temperature of 64 °C, amplifications were observed for the strain RHA1, *R. globerulus*, *R. marinonascens*, and *R. opacus*, yielding an amplification product of 283 bp (Figure 4.3). PCR with annealing temperatures higher than 66 °C did not improve specificity, but rather decreased the amplification resulting in weaker bands. The *Rhodococcus* 16S rRNA gene sequence correspondent to the *E. coli* position 599-619 was chosen for designing the Taqman-16S rDNA probe for real time PCR. Our choice was based on the presence of only two other perfect matches for this primer, *R. marinonascens* and *R. globerulus*. Because of the internal probe design requirements, the S-*- Rco-0599-b-A-21 primer had to be extended to 29 nucleotides, yielding the Taqman-16S rDNA probe (Figure 4.2). To test the specificity of the newly designed probe, real time PCR was performed against genomic DNA isolated from different *Rhodococcus* species and *E. coli*. Fluorescence during PCR amplification was observed for the strain RHA1, *R. globerulus*, *R. marinonascens*, *R. opacus*, and *R. percolatus* (Figure 4.4).

When the Taqman probe specific for the *fcb*B gene (Taqman-*fcb*) was used against total DNA from the same closely-related species, quantitative amplification of the sequence was possible only for the recombinant strain RHA1 (Figure 4.4).

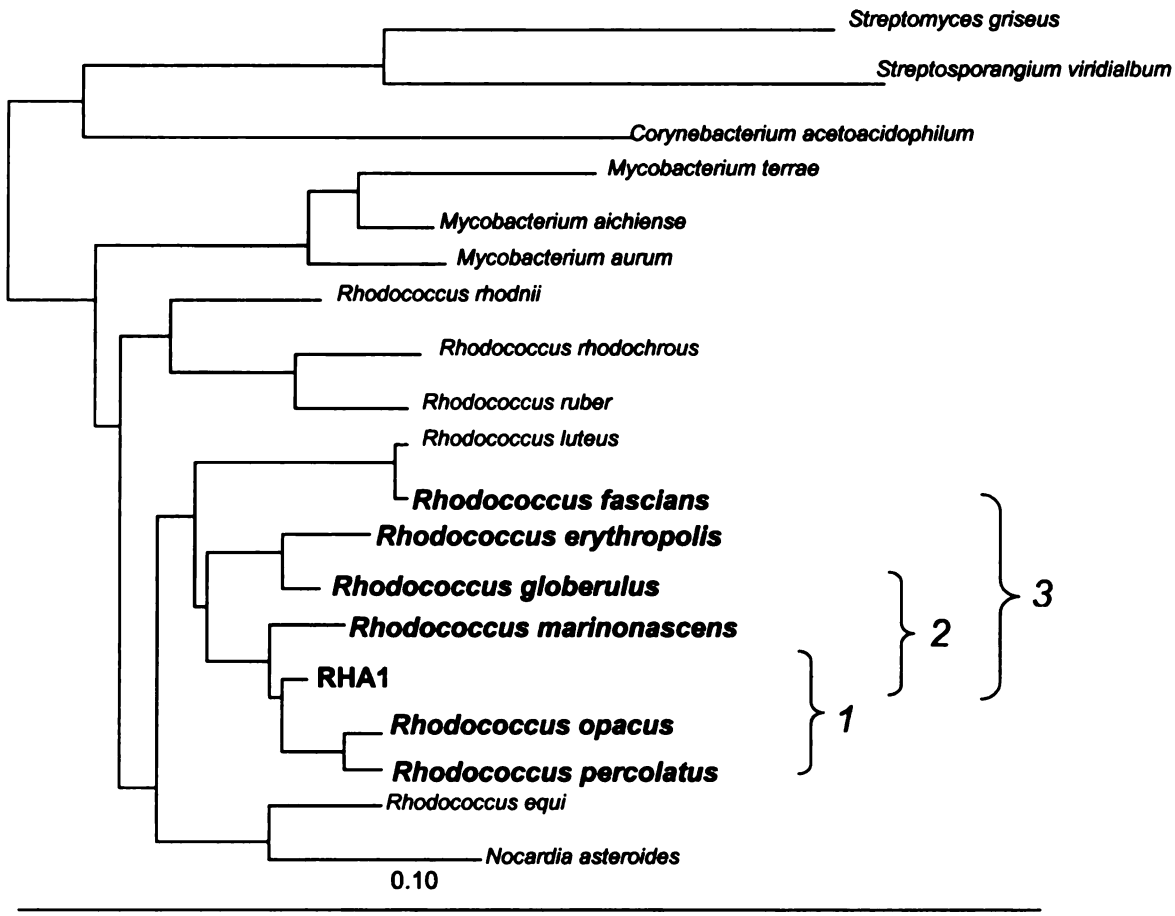

Figure 4.1. Neighbor-joining phylogenetic tree (Saitou and Nei 1996) based on the 16S rRNA gene of *Rhodococcus* sp. strain RHA1 and phylogenetically related bacteria. The scale is the expected number of substitutions per position. Numbers represent oligonucleotide probes with their respective target group: 1. S-*-Rco-0183-a-A-20, 2. S-*-Rco-0599-b-A-21, and 3. S-*-Rco-0828-c-A-18.

S-*-Rco-0183-a-A-20	3'	G	A	A	G	C	C	G	A	C	G	T	A	C	C	G	A	C	T	C	C	5'
Target Sequence	5'	C	T	T	C	G	G	C	T	G	C	A	T	G	G	C	T	G	A	G	G	3'
Rhodococcus sp. RHA1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus marinonascens		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	
Rhodococcus globerulus		.	.	C	.	T	A	T	C	.	.	.	.	.	.	T	A	.	G	T	.	
Rhodococcus opacus		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus percolatus		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus erythropolis		.	.	C	.	.	.	.	T	.	.	.	.	.	A	.	.	T	G	.	.	
Rhodococcus fascians		.	A	C	A	.	C	A	.	.	.	.	.	.	T	G	.	T	G	T	.	
Nocardia asteroides		.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	
Rhodococcus rhodochrous		.	.	C	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Sphingomonas sp.		A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C	.	

S-*-Rco-0599-b-A-21	3'	C	A	A	A	C	A	C	T	T	T	T	G	A	G	T	G	T	C	G	A	G	5'
Target Sequence	5'	G	T	T	T	G	T	G	A	A	A	A	C	T	C	A	C	A	G	C	T	C	3'
Rhodococcus sp. RHA1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus marinonascens		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus globerulus		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus opacus		T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus percolatus		.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	
Rhodococcus erythropolis		.	.	.	.	.	.	.	.	.	.	.	.	C	A	G	.	.	.	.	.	.	
Rhodococcus fascians		.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	G	G	.	.	.	.	

S-*-Rco-0828-c-A-18	3'	C	C	A	C	A	C	C	C	A	A	G	G	A	A	G	G	T	G	5'
Target Sequence	5'	G	G	T	G	T	G	G	G	T	T	C	C	T	T	C	C	A	C	3'
Rhodococcus sp. RHA1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus marinonascens		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus globerulus		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus opacus		.	.	.	.	.	.	.	.	.	.	T	.	C	.	T	.	C	A	
Rhodococcus percolatus		.	T	G	T	G	.	.	T	.	.	.	.	.	.	.	.	.	.	
Rhodococcus erythropolis		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rhodococcus fascians		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Streptosporagium viridialbum		.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	
Actinomadura verrucosospora		.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	

Figure 4.2. Variable region sequence of the 16S rRNA gene from *Rhodococcus* sp. strain RHA1 used for primer/probe sequence design. Sequences are aligned with corresponding regions of phylogenetically related species. A dot indicates sequence identity to the 16S rDNA region of the strain RHA1.

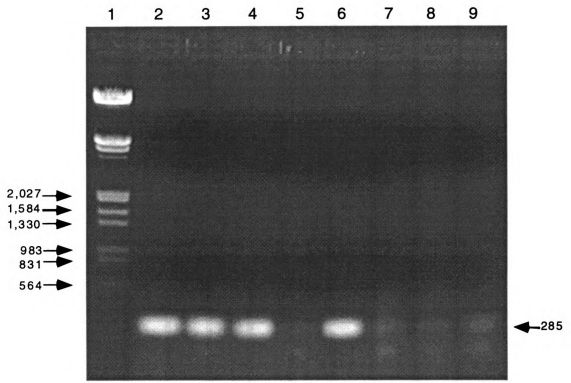

Figure 4.3. Ethidium bromide-stained agarose gel of PCR products after amplification of the 16S rRNA gene of phylogenetically related *Rhodococcus* species and *E. coli* with *Rhodococcus*-targeted 16S specific primers. Numbers above lanes refer to: 1 - Molecular marker λ restricted with *Hind* III and *Eco* RI, 2 - *Rhodococcus* sp. strain RHA1, 3 - *R. marinonascens*, 4 - *R. globerulus*, 5 - *E. coli*, 6 - *R. opacus*, 7 - *R. percolatus,* 8 - *R. erythropolis*, and 9 - *R. fascians*.

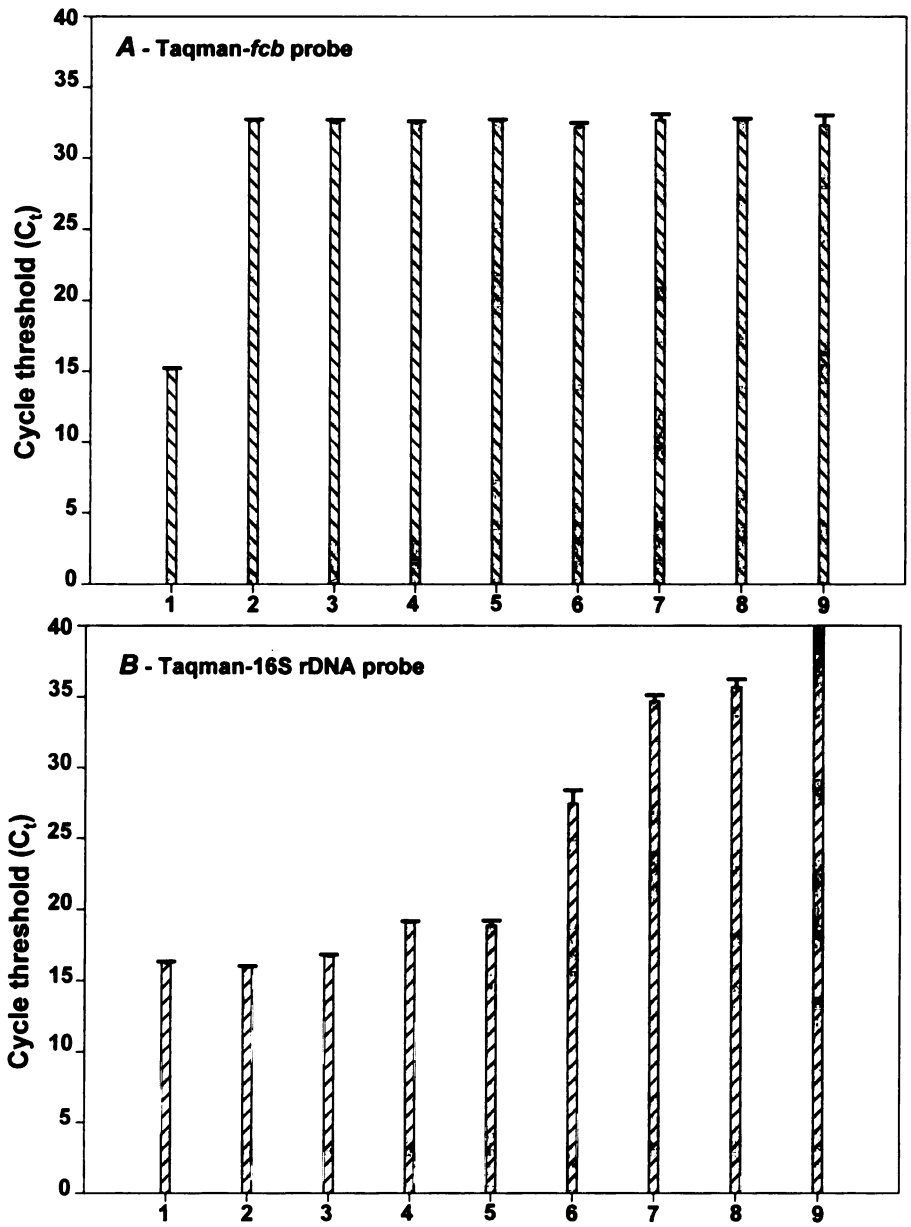

Figure 4.4. Averaged cycle threshold values (C_t) for real time PCR performed with A. Taqman-*fcb* probe, and B. Taqman-16S probe and a fixed DNA template concentration (30 ηg) from the recombinant strain RHA1, its phylogenetically related *Rhodococcus* species, and *E. coli*. Data are mean values and standard error from triplicates. Numbers bellow bars refer to: 1 - *Rhodococcus* sp. strain RHA1, 2 - *R. marinonascens*, 3 - *R. globerulus*, 4 - *R. opacus*, 5 - *R. percolatus*, 6 - *R. erythropolis*, 7 - *R. fascians*, 8 - *E. coli*, 9 - Negative control (no DNA).

Sensitivity of real time PCR. The sensitivity of the Taqman-16S rDNA and Taqman-*fcb* probe and primer sets for real time PCR was evaluated with 1:10 serial dilutions (from 10^2 to 10^7 copies) of the strain RHA1 genome and the *fcb*-containing plasmid pRHD34, respectively. A standard curve was generated from changes in fluorescence reporter signal (ΔRn) versus cycle number during PCR, allowing determination of the threshold cycle (C_t). When the Taqman-*fcb* was used against isolated *fcb*-containing plasmid in different dilutions, a linear relationship between C_t and the log of copy number (r^2 = 0.998) was obtained. The C_t values decreased linearly as target quantity increased (Figure 4.5). Increments in fluorescence intensity were also observed when genomic DNA extracted from strain RHA1 was used against the internal probe TaqMan-16S rDNA (r^2 = 0.999) (Figure 4.5). The same fluorescence intensity values for genomic DNA were obtained when serial dilutions of the RHA1-16S rRNA gene-containing plasmid pRHA16S were also used as target for amplification with the Taqman-16S rDNA probe. Strain RHA1 was found to have four copies of the 16S rRNA gene per genome, and this value was taken into account for data reported in Figures 4.5-4.7.

Detection and sensitivity in soil samples. To test whether the recombinant strain RHA1 could be detected in a background of soil community DNA, soil samples were spiked with a known number of cells. Real time PCR with total DNA extracted from non-inoculated soil did not result in fluorescence increase above the threshold value. Increments in fluorescence intensity occurred when soil was inoculated with recombinant RHA1 cells. Increase in isolated CFUs per gram of soil linearly correlated with a decrease in C_t values for both probes; TaqMan-16S rDNA (r^2 = 0.998) and TaqMan-*fcb*

(r^2 = 0.981) (Figure 4.6). This represents a decrease in sensitivity of 1.1 and 1.3 orders of magnitude for the TaqMan-*fcb* and -16S rDNA probes, respectively, when DNA isolated from soil rather than culture was used.

When TaqMan probes were tested with soil DNA extracted from the 30 day-microcosms experiment, the estimated numbers of RHA1 cells per g of soil were similar to the values obtained for culturable rifampicin-resistant RHA1 cells (CFUs g^{-1} soil) (Figure 4.7).

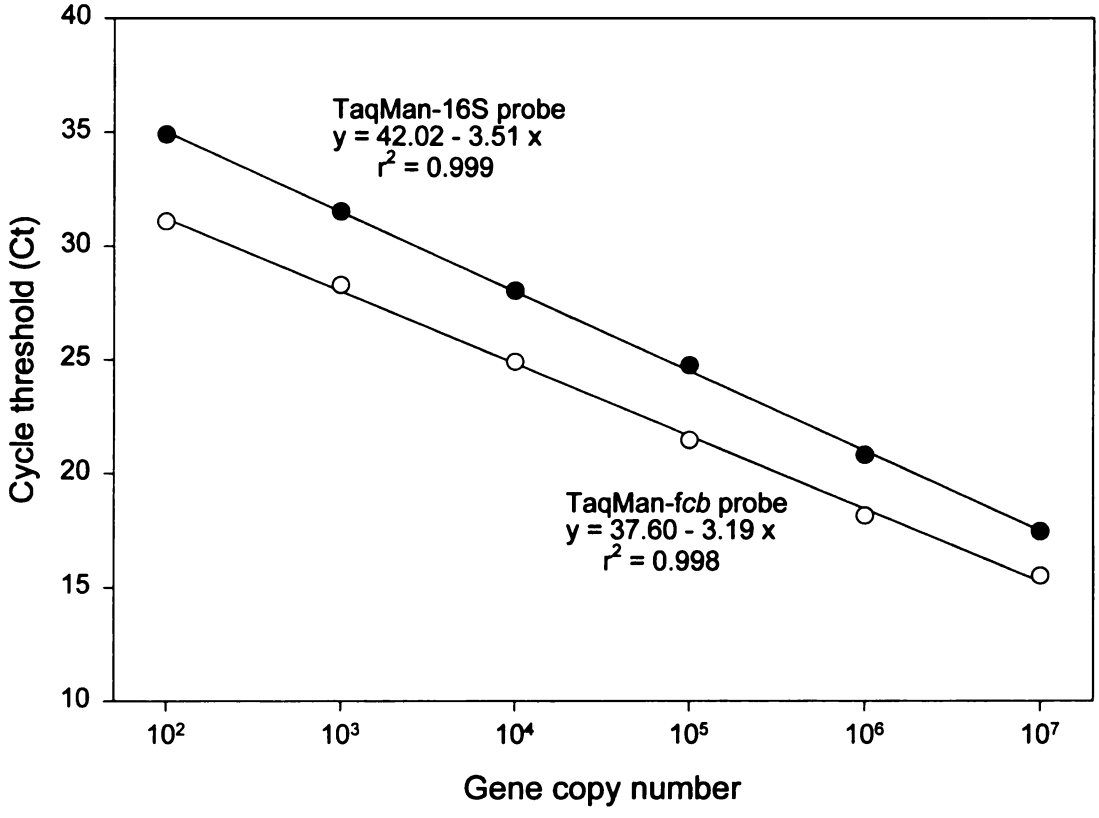

Figure 4.5. Standard curves showing the threshold cycle (C_t) values plotted versus log of dilution series of RHA1 genomic DNA and the plasmid pRHD34. The equation and correlation coefficient (r^2) for each curve is indicated. Each dot represents the result of triplicate PCR amplification for each dilution. Standard error of triplicates was smaller than the size of the symbol.

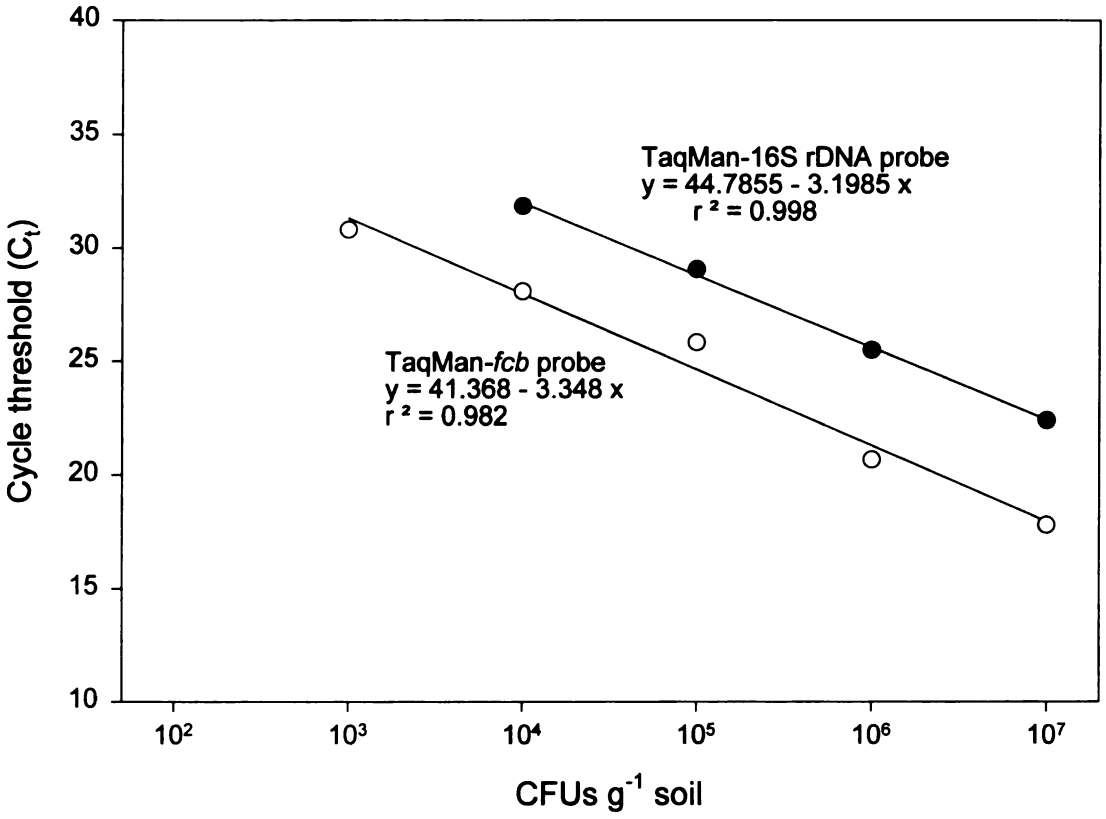

Figure 4.6. Standard curves showing the threshold cycle (C_t) values plotted versus log of spiked RHA1 cells (CFUs/g of soil) isolated from soil. The equation and correlation coefficient (r^2) for each curve is indicated. Each dot represents the result of triplicate PCR amplification for each dilution. Standard error of triplicates was smaller than the size of the symbol.

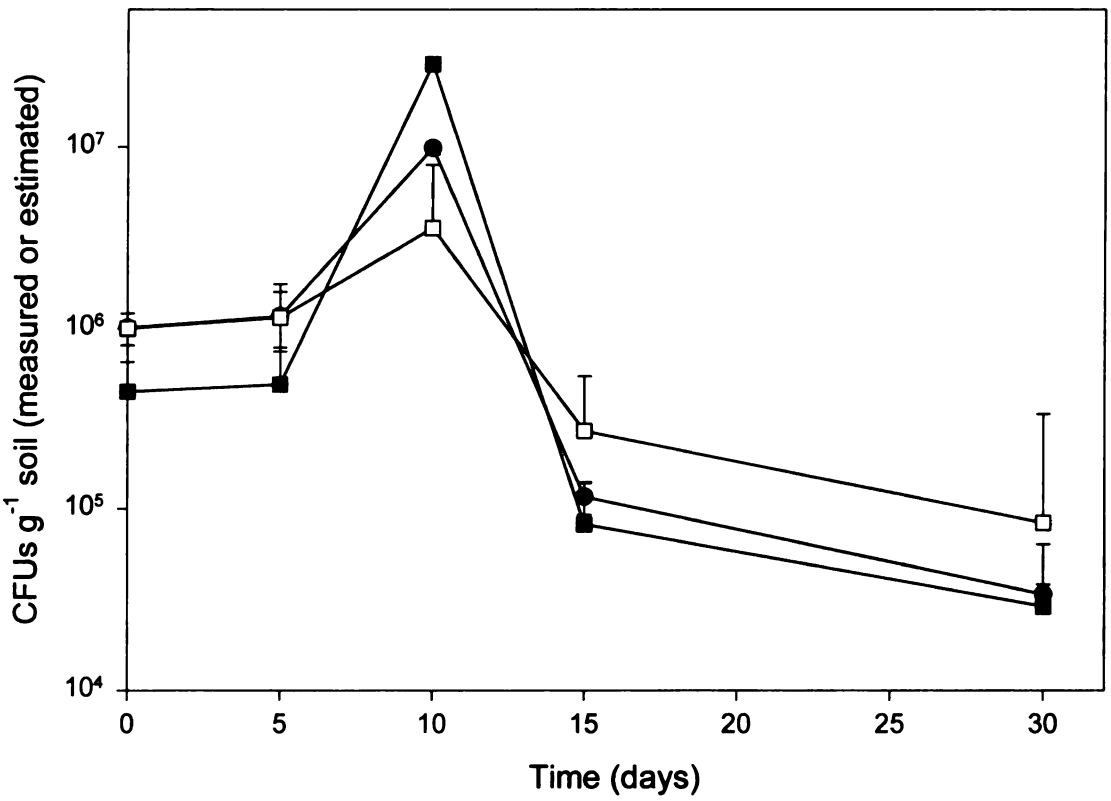

Figure 4.7. Comparison of numbers of CFU (□) on rifampicin-containing Luria-Bertani medium and estimated number of cells by real time PCR with TaqMan-*fcb* (●) and TaqMan-16S rDNA (■) probes. Where error bars are not shown, the standard error of triplicates was smaller than the size of the symbol.

DISCUSSION

To date, many of the techniques used in molecular microbial ecology lack a quantitative response. FISH as well as competitive PCR in soil and sediment samples (Johnsen et al. 1999, Lee et al. 1996, Löffler et al. 2000), when possible, are labor intensive and require very skilled workers. The real time PCR method is automated, sensitive, and has high quantitative reproducibility for both a functional gene and the 16S rRNA gene in the soil.

A critical factor for the real time PCR method is to assess the specificity of the probes and to avoid the contamination issues especially when a eubacterial 16S rRNA gene TaqMan probe is used (Corless et al. 2000). Due to the conserved nature of the rRNA genes, we were able to identify only three 16S rRNA-targeted oligonucleotide sequences reasonably specific for our *Rhodococcus* sp. strain, RHA1. Primer S-*-Rco-0599-c-A-21 was choosen as the TaqMan-16S rDNA probe, because of its perfect match with only two other *Rhodococcus* species, *R. globerulus* and *R. marinonascens*. *R. marinonascens* is a marine strain with specific growth conditions, unlikely to be found in soil (Helmke and Weyland 1984).

Besides the inherent difficulty in designing a strain-specific oligonucleotide sequence, the extension of the primer to 29 nucleotides (Table 4.1) due to the Taqman-16S rDNA probe requirements reduced the specificity of the probe (Figure 4.4), allowing the detection of two other *Rhodococcus* species, *R. opacus* and *R. percolatus*. This is likely due to the annealing temperature limitation of 64°C for the outside primers, which was already four degrees above the recommended value (Perkin Elmer Applied

Biosystems 1998). The decrease in overall specificity with one or two mismatches does not abolish reliable quantitation (Klein et al. 1999).

Theoretically, PCR should detect one copy of the 16S rRNA gene (van Kuppeveld et al. 1992), but precision becomes limiting below 100 gene copies (Grüntzig et al. 2000). But, our purpose was to develop a method for tracking and quantifying the recombinant strain RHA1 during bioaugmentation of a PCB contaminated soil, and inoculation at a cell density of 10^4 per gram of soil was shown to be appropriate for PCB removal (Rodrigues et al. 2000). Hence standard curves ranging from 10^2 to 10^7 gene copies were sufficient. Highly linear standard curves were found through out this range with pure RHA1 genomic DNA, *fcb*-containing plasmid pRHD34, and with both in soil (Figures 4.5 and 4.6). However, when RHA1 cells were added to soil, the sensitivity of the assay decreased (Figure 4.6), suggesting that the soil DNA recovery, was not 100% efficient or that PCR inhibition occurred.

The different factors affecting detection limits in PCR-based methods, include: the type and composition of the matrix, the type of target organism, the number of other bacteria present in the same material, the *Taq* polymerase used, and the DNA extraction protocol (Löffler et al. 2000). Our experiments indicate that the target sequence can also be an important factor in the detection as seen by the different standard curves for TaqMan probes targeting the *fcb*- versus the 16S rRNA- genes. One explanation for different sensitivities might be the size of the strain RHA1 genome (3.0 Mb) (Tonso 1997) in comparison to the plasmid pRHD34 size (14.4 Kb), limiting the TaqMan-16S rDNA probe from finding its target. However, chromossomal and plasmidial targets of RHA1's 16S rRNA gene gave the same C_t. The lower C_t values observed when the

TaqMan-*fcb* probe was used is probably due to the probe efficiency and not the difficulty of genome strand separation during the heat denaturation PCR step when the TaqMan-16S rDNA probe was used. Penalty score analysis for the two TaqMan probes indicated differences between the two probes; values of 250 for the TaqMan-16S rDNA and 49 for TaqMan-*fcb* (Perkin Elmer Applied Biosystems 1998), suggesting that the probe targeting the *fcb* operon met most of the criteria required for real time PCR, while the other did not. The phylogenetic probe is limited by the range of specific regions within the hypervariable sequence of the 16S rRNA gene (Stackebrandt and Rainey 1995). Thus, the TaqMan-16S probe design had to be constructed from a pre-determined position, while the TaqMan-*fcb* probe was chosen from many different possibilities within the entire *fcb* operon.

We recognize that other species and yet-to-be isolated microorganisms could also be targeted by the same TaqMan 16S rDNA probe (Amann et al. 1995). However, the high C_t values obtained in soil control treatments suggest such organisms are not present above the detection limit in soil from this site. Our microcosm experiment shows strikingly similar RHA1 cell numbers whether measured by a culturable or a molecular method (Figure 4.7). While we found the real time method using the two probes to be sensitive, specific, quantitative, and had high capacity (96 samples per assay), the probe and reagent costs make the method relatively expensive.

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CHAPTER V

DEGRADATION OF PRODUCTS FROM ANAEROBIC DECHLORINATION OF AROCLOR 1242 IN CONTAMINATED SEDIMENT USING TWO RECOMBINANT BACTERIA

ABSTRACT

Rhodococcus sp. strain RHA1 and *Burkholderia cepacia* strain LB400, both naturally possessing the biphenyl pathway were engineered to contain the 4-chlorobenzoate (*fcb*) and 2-chlorobenzoate (*ohb*) degradation operons, respectively. These strains were inoculated into microcosms with Aroclor 1242-contaminated sediment that had undergone anaerobic dechlorination to evaluate their efficacy in removing the remaining PCBs. The population dynamics of both strains was followed by selective plating. Both recombinant strain populations increased in contaminated sediment. The recombinant RHA1 cell number and *fcb* gene copy were quantified over the experimental period by real time PCR. These results agreed well with plate counts of this strain. Inoculation at cell densities of 10^4 and 10^6 cells g^{-1} sediment resulted in equivalent PCB removals, 57% and 54%, respectively, although the initial rate of removal was faster with the high inoculum dose. The residual PCB congener profile after 30 days was the same for both high and low cell density inoculation. The effectiveness of the low density inoculum (10^4 organisms g^{-1}) makes bioaugmentation a less costly process.

INTRODUCTION

Approximately 635 million kg of PCBs were produced in the United States from 1929 to 1978 and a similar amount was manufactured in Japan, Europe, and the former USSR (Hutzinger and Veerkamp 1981). From this total amount, several tons currently contaminate soils and sediments. The high hydrophobicity of PCB molecules contribute to their long-term environmental persistence and accumulation in higher trophic levels.

Over the past 30 years, PCB research has shown that these compounds, previously thought to be recalcitrant, could be degraded under two environmental situations: 1) anaerobic conditions, in which reductive dechlorination by microorganisms takes place, converting highly chlorinated congeners into lesser chlorinated biphenyls, leaving the rings intact (Brown et al. 1984, Bedard and Quensen 1995, Quensen et al. 1988, Quensen et al. 1990) and 2) aerobic conditions, in which some microorganisms with more versatile oxidative capabilities can breakdown PCBs to chlorinated benzoates and pentanoic acid derivatives (Mondello 1989). Hence, sequential anaerobic-aerobic treatment could result in both natural attenuation as well as be developed into a PCB bioremediation technology (Unterman 1996).

Experiments involving inoculation of selected aerobic PCB-degrading bacteria for xenobiotic removal have been described. However, most of the work employed only one congener (Mokross et al. 1990), a freshly applied contaminant (Lajoie et al. 1993) and/or was carried out under resting cell conditions (McCullar et al. 1994, Adams et al. 1992). In fact, PCB contaminated sites usually contain a full spectrum of congeners coming from Aroclor spills, other co-contaminants might be present, and PCBs are aged in soil organic matter making then less bioavailable. Furthermore, added PCB-degrading

bacterial strains have to compete effectively with highly adapted indigenous soil microorganisms.

In this paper, we combined both anaerobic and aerobic steps for biodegradation of Aroclor 1242 in contaminated sediments. This is the first instance in which recombinant strains containing the entire pathway for mineralization of chlorobenzoates are tested after reductive dechlorination has occurred.

MATERIAL AND METHODS

Bacterial strains and culture conditions. Strains used in this study were *Rhodococcus* sp. RHA1 (Masai et al. 1995) and *Burkholderia cepacia* LB400 (Bopp 1986). Recombinant cells were grown on synthetic medium K1 (Zaitzev et al. 1991) containing 2 mM, 2- or 4-CBA or 3 mM biphenyl (nominal concentration). Luria-Bertani (LB) agar plates containing rifampicin (50 μg/mL) were used for re-isolating cells from 10-fold dilutions of sediments (Rodrigues et al. 2000b).

Sediment samples. Sediment samples were obtained from the Red Cedar River (Michigan) and contaminated with Arochlor 1242. The sediment, classified as sand (91.1 % sand, 8.1 % silt and clay), 6.7 % organic matter, 0.71% total nitrogen, pH 7.2, was passed through a 4-mm mesh sieve and stored at 4 °C until used. The sediment was inoculated with microorganisms eluted from the River Raisin sediment and incubated under anaerobic conditions for 1 year before being used. Successful pattern M (Bedard and Quensen 1995) anaerobic dechlorination was documented by congener specific PCB analysis. Total sediment bacterial counts before inoculation were performed by staining with 5-(4,6-dichlorotriazine-2-yl) aminofluoroscein (DTAF) followed by epifluorescence

microscopy (Bloem 1995). Naturally occurring rifampicin-resistant variants of recombinants RHA1(*fcb*) and LB400(*ohb*) were obtained and tested on gradient plates (Smith and Tiedje 1980). These rif^r variants were used to aid tracking the inoculum. They were grown on 3 mM (nominal concentration) biphenyl-containing medium. Cells were washed once with K1 medium, resuspended, and diluted in the same medium, and 1 ml was added to 1 g of contaminated sediment to give a density of 10^4 (low density treatment) or 10^6 (high density treatment) cells g^{-1} of sediment for each recombinant strain. Non-contaminated sediment as well as non-inoculated contaminated sediment incubated under the same conditions were used as controls. Flasks were continually shaken at 150 rpm and incubated in duplicate at 30°C for a period of 30 days. Each flask was opened once a week to ensure proper aeration of samples. Sediment samples were stored at – 20 °C for soil DNA and PCB extractions.

Samples were taken periodically, and immediately diluted. Appropriate dilutions were spread on Luria-Bertani agar plates containing rifampicin (50 μg/ml). Numbers of colony forming units (CFUs) for both strains were determined after one week of incubation.

DNA extraction and real time-PCR analysis. Total sediment DNA was extracted according to manufacturer's instructions using the Soil DNA Extraction Kit (MoBio, Inc., Solana Beach, CA) and used for monitoring strain RHA1(*fcb*) by real time PCR. Probe and primer sequences for real time PCR were designed by using the Primer Express software (Perkin Elmer, Foster City, CA). The probe contained FAM (6-carboxy-fluorescein) as a reporter fluorochrome on the 5' end and TAMRA (*N,N,N',N'*-tetramethyl-6-carboxy-rhodamine) as quencher on the 3' end of the nucleotide sequence

(Rodrigues et al. 2000a). The 30 µl reaction volume contained 300 ηM of forward primer, 900 ηM of reverse primer, 275 ηM of TaqMan probe, 1 X TaqMan Buffer, 1.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA), and 3 µl of DNA template from each appropriately diluted sample. Experiments were performed in duplicate for each time point. The cycle started with 2 min at 50 °C for optimal AmpErase uracil-N-glycosylase enzyme activity, followed by one cycle of denaturation at 95 °C for 10 min and by 40 cycles of amplification of 15 s at 95 °C for melting and 1 min at 58°C of annealing and extension. PCR was carried out in a spectrofluorimetric thermal cycler ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA). A standard curve was generated from changes in fluorescence reporter signal (ΔRn) versus cycle number during PCR, allowing determination of the threshold cycle (C_t). A maximum threshold cycle (C_t) 32 for negative amplification was used based on previous results ((Rodrigues et al. 2000a).

Stability of the *fcb* and *ohb* genes. The number of *fcb*- and *ohb*-containing colonies was determined by picking 10 colonies of each strain periodically and screening for *fcb* or *ohb* genes by PCR amplification. Template DNA for PCR was prepared by lysing whole cells in 100 µl NaOH 0.05N at 95°C for 15 min. Primers were specifically designed for the *fcb*B and *ohb* genes to yield PCR products of 599 and 580 bp, respectively. Amplifications were performed in a 20 µl reaction volume containing 10 pmol of primers, 200 µM each deoxynucleoside triphosphate, 400 ηg/ml of Bovine Serum Albumin, 1 X *Taq* Buffer, 1.5 U *Taq* DNA polymerase (Sigma, St Louis, MO), and 2 µl of DNA template. The PCR was initiated with a 3 min denaturation step at 94°C; followed by 30 cycles at denaturation temperature of 94°C for 1 min, primer annealing at

55°C for *fcb*B and 58°C for *ohb* for 30 s, extension at 72°C for 2.1 min, and final extension for 5 min. Three µl aliquots of the PCR products were analyzed on 1% agarose gels.

PCB analysis. Duplicate samples of each treatment were sacrificed at predetermined time intervals. The entire contents were extracted, the PCBs purified, and analyzed for congener specific PCB content as previously described (Quensen et al. 1990).

RESULTS

Population dynamics of recombinant RHA1(*fcb*) and LB400(*ohb*) strains. Direct bacterial counts of the sediment before inoculation averaged to 4.82×10^8 cells g^{-1} sediment. No indigenous sediment bacteria could be isolated on rifampicin-containing medium bellow our limit of detection (10^2 cells g^{-1} soil) (Figure 5.1D). The *Rhodococcus* sp. RHA1(*fcb*) and *Burkholderia cepacia* LB400(*ohb*) strains could be easily distinguished because of their distinct color and morphological characteristics on plates. Bacterial counts of both strains increased after both high and low density inoculation treatments (Figures 5.1A and 5.1B). In the high inoculation treatment, RHA1(*fcb*) cell number increased to 7.8×10^6 rifampicin resistant cells g^{-1} sediment while LB400(*ohb*) cell number increased to 1.4×10^7 cells g^{-1} sediment. We found approximately 3.8×10^6 and 6.2×10^6 cells g^{-1} sediment for RHA1(*fcb*)and LB400(*ohb*), respectively, in the low density treatment at day 15. No LB400(*ohb*) colonies could be detected on agar medium in the PCB-free sediment, while RHA1(*fcb*) colonies increased from 1.0×10^4 to 2.2×10^5 cells g^{-1} soil (Figures 5.1C).

Monitoring RHA1(*fcb*) by real time PCR. Increase in numbers of 16S rRNA- and *fcb*- genes was also observed, consistent with the plate count data. The calculated CFUs g^{-1} of sediment for RHA1(*fcb*) using the TaqMan-*fcb* and -16S rDNA probes were 5.3×10^6 and 1.1×10^7 cells g^{-1} of sediment, respectively, at the day 15 (Figures 5.1A and 5.1B). Sediment samples taken from the non-inoculated control did not yield any increment of fluorescence above the threshold.

Stability of the *fcb* and *ohb* operons. The *fcb* and *ohb* operons in the strains RHA1 and LB400, respectively, appeared to be stable under non-selective conditions. All screened colonies, but one LB400 colony, resulted in PCR amplified products of the expected size from these two genes (Figures 5.1A and 5.1B), 599 bp (*fcb*) and 580 bp (*ohb*).

PCB removal and congener profile. After 30 days, PCB removals for high and low inoculation densities were 57% and 54% after aerobic treatment, respectively, while only a small amount (4%) were degraded in the non-inoculated treatment (Figure 5.2). Degradation was slightly faster with the higher density inoculum. The profiles of degradation were similar for both low and high inoculation treatments (Figure 5.3). The major congeners not degraded were: 2,2'-/2,6-, 2,4'-, 2,4,2'-, 2,6,2'-, 2,6,4'-, and 2,4,2',4'-chlorobiphenyl (CB), which are the ones known to be most resistant to the *meta* and *ortho* directed attacks, respectively, on the biphenyl ring by these two strains (Maltseva et al. 2000).

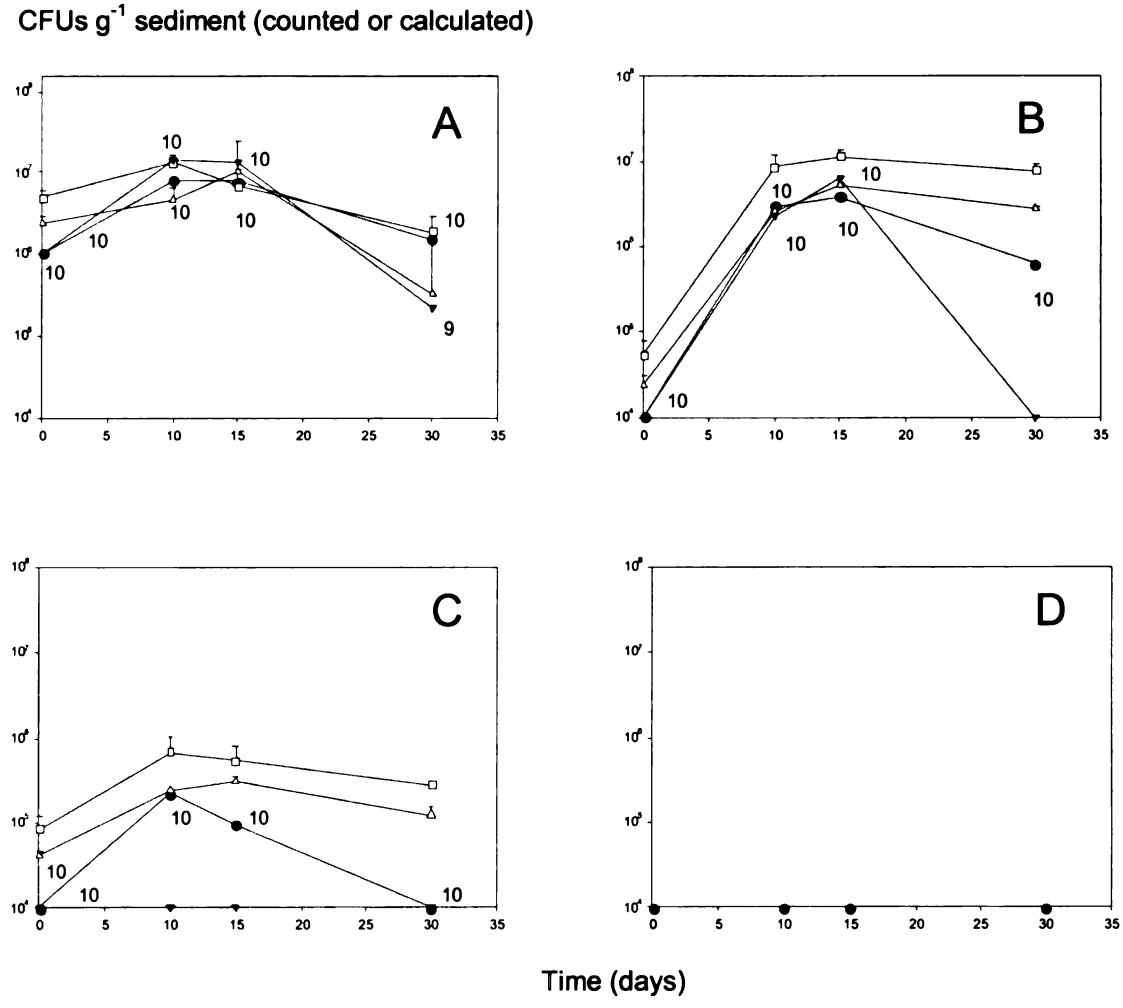

Figure 5.1. Population density of recombinant strains *Rhodococcus* sp. RHA1 (●) and *Burkholderia cepacia* strain LB400 (▼) containing the *fcb* and *ohb* operons, respectively, in sediment containing dechlorinated Aroclor. RHA1(*fcb*) cell numbers were also estimated by real time PCR with TaqMan-*fcb* (△) and TaqMan-16S *Rhodococcus* rDNA (□) probes. (A) Inoculation density of 10^6 cells g^{-1} sediment for each strain. (B) Inoculation density of 10^4 cells g^{-1} sediment for each strain. (C) PCB-free sediment control with inoculation density of 10^4 cells g^{-1} sediment for each strain, and (D) Non-inoculated control. Values above each sampling time represent stability of the *fcb* and *ohb* operons in 10 randomly chosen colonies from each strain when isolation was possible above detection limit. Where error bars are not shown, the standard error of triplicates was smaller than the size of the symbol.

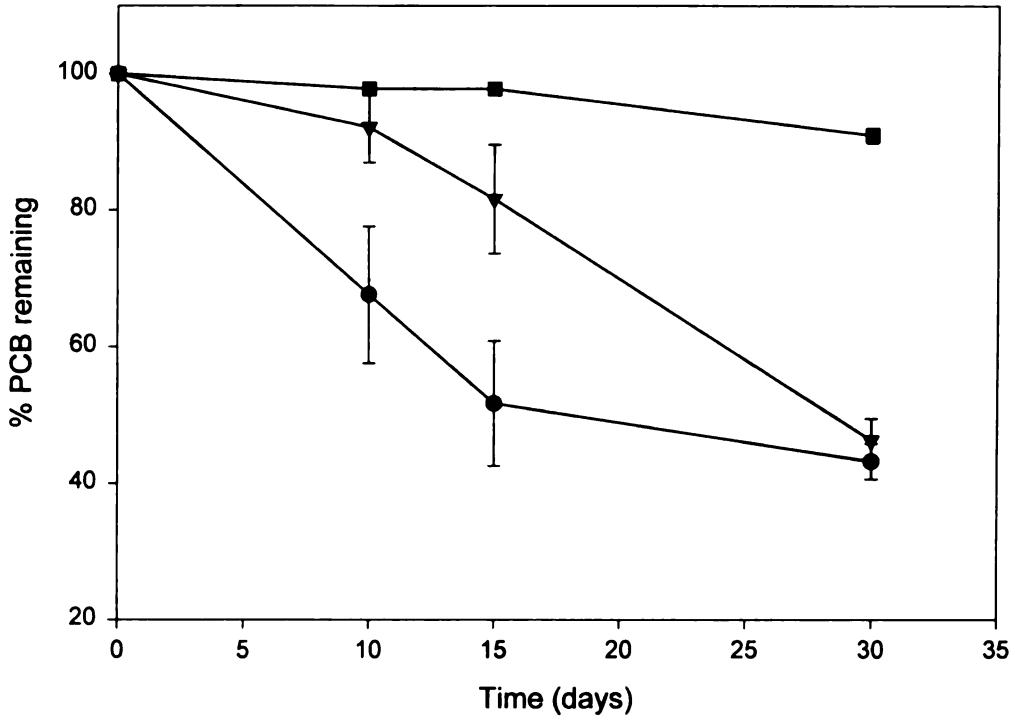

Figure 5.2. Percentage of PCBs remaining during aerobic treatment with recombinant strains RHA1(*fcb*) and LB400(*ohb*) at two different inoculation densities: Symbols: (●) 10^6 cells of each strain g^{-1} of sediment, (▼)10^4 cells of each strain g^{-1} of sediment, and (■) non-inoculated control. Duplicate samples were used for non-inoculated control. Where error bars are not shown, the standard error of triplicates was smaller than the size of the symbol.

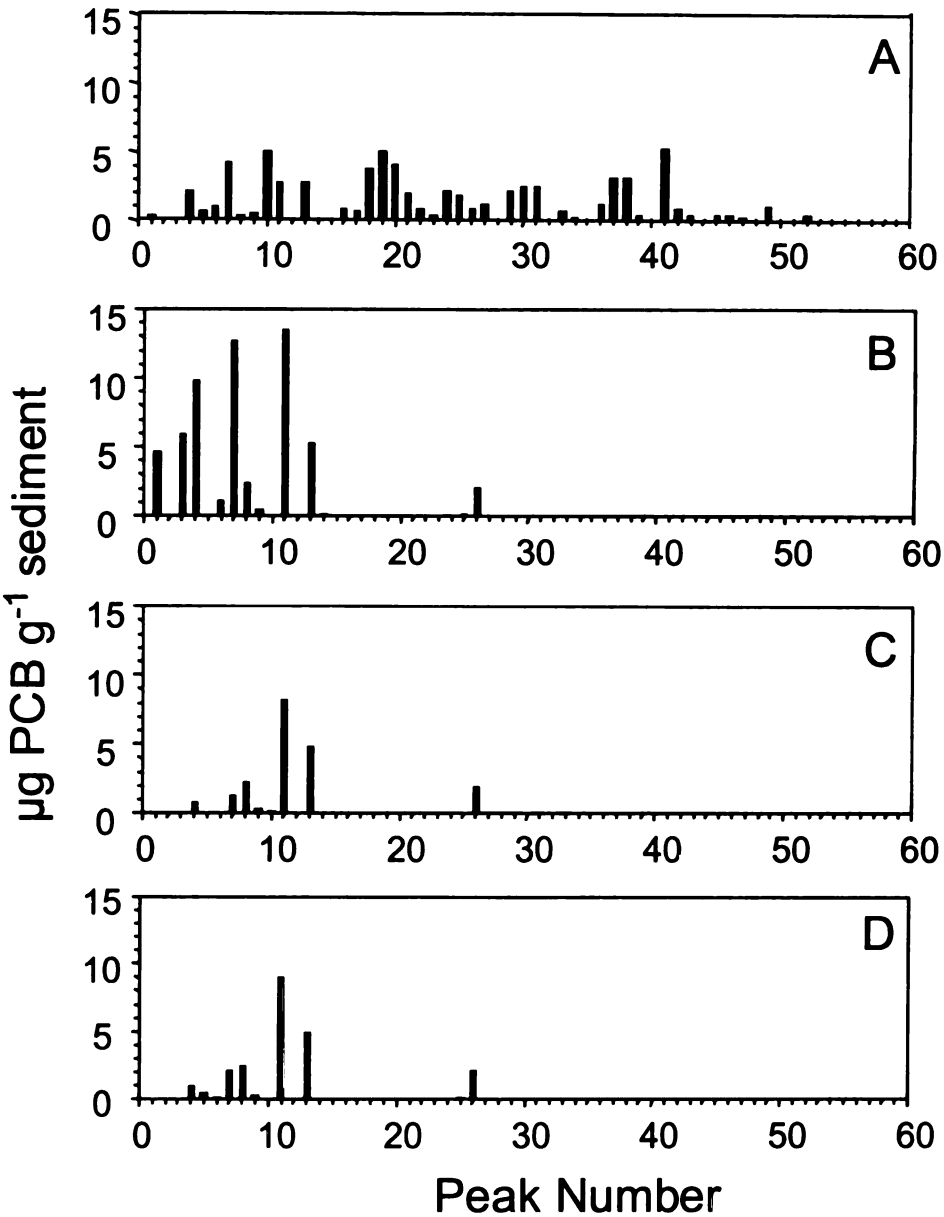

Figure 5.3. Aroclor 1242 congener distribution and concentration (µg g^{-1}) in contaminated sediment at time zero (A), after one year incubation under anaerobic conditions (B) followed by aerobic incubation for 30 days with recombinant strains RHA1(*fcb*) and LB400(*ohb*) with 10^6 cells g^{-1} of sediment (C), or 10^4 cells g^{-1} (D).

DISCUSSION

The entire 2- (*ohb*) and 4-chlorobenzoate (*fcb*) degradation operons were successfully inserted into *Burkholderia cepacia* LB400 and *Rhodococcus* sp. RHA1, respectively, as indicated by the stability of both operons. Strain RHA1(*fcb*) had been previously shown to grow in soil freshly contaminated with a single PCB, 4-CB, and that the growth correlated with disappearance of this contaminant (Rodrigues et al. 2000b). In the current study, we used Aroclor 1242-contaminated sediments that had undergone extensive anaerobic dechlorination for testing the effectiveness of these strains as inoculum for the aerobic phase. The pattern M dechlorination observed is the most common in nature and hence represents the suite of PCB substrates to be expected from the anaerobic stage. Furthermore, the PCB products have been in sediments for at least several months making their bioavailability more typical of the natural case.

Although RHA1(*fcb*) cells grew one order of magnitude in PCB-free microcosm samples, probably on sediment carbon, they grew to much higher densities in sediments containing PCBs. More impressive still is the population increase for LB400(*ohb*) under the same conditions, confirming that products of anaerobic dechlorination of Aroclor 1242 are selective substrates for only the recombinant populations. PCB removal was consistent with growth of the PCB-degrading strains. No PCB degradation occurred in the non-inoculated sediment.

Real time PCR data confirmed the same RHA1(*fcb*) population trend observed by plate counts. Real time PCR has been shown to be more advantageous in complex environments such as sediments or soils in comparison to other molecular tracking methods (Grüntzig et al 2000, Rodrigues et al 2000a). It avoids the need of nested PCR

for the detection of fewer copies of the target gene and it does not require addition of another DNA sequence to be amplified as does competitive PCR. The difference between plate counts and the estimated number of RHA1(*fcb*) cells was no more than an order of magnitude, attesting the efficacy of the molecular method. The recombinant bacteria counted in the rifampicin-containing medium were the culturable ones. Thus, the viable but non-culturable state might have affected recovery of the strains (Ducrocq et al. 1999). Bacteria when subjected to incubation in soil or water are shown to become non-culturable, as determined by plate counts, while the total number of cells remains unchanged (Bogosian et al. 1996). Although one could claim that non-culturable bacteria to be the same as dead for bioremediation purposes, and our experiment could not confirm or ruled out this possibility, we found the molecular method to be specific, rapid (in comparison to ten days for plate counts), and reliable for our purposes.

Another important issue addressed by our experiments was the inoculum density required for effective PCB removal. Xenobiotic degradation and survival of strains in soils have usually been studied under inoculation densities much higher than the soil carrying capacity for that population. Inevitably, cell numbers decrease, often by four to six orders of magnitude, in a short period of time (Havel and Reineke 1993, Leung et al. 1997, Huertas et al. 1998, Tchelet et al. 1999). This was not the case for our experiments. Since we inoculated at relatively low cell densities, we were able to see an increase in cell number. The rationale for using a low cell density is that for bioremediation of contaminated soils, scaling up the amount of inocula required for treatment could be prohibitively costly. Our results indicate that a similar extent of biodegradation (Figures 5.2 and 5.3) can be obtained in 30 days with very a low density inoculum. In practice, as

a safety factor one may want to increase the inoculum to 10^5 organisms g^{-1} or even more, but these levels are still inexpensive.

A large scale model using the same recombinant strains and conditions is currently being tested for PCB bioremediation in reactors with different solids loading.

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