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Three Different 2,3-Dihydroxybiphenyl-1,2-Dioxygenase Genes in the Gram-Positive Polychlorobiphenyl-Degrading Bacterium *Rhodococcus globerulus* P6

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***Rhodococcus globerulus* P6 (previously designated *Acinetobacter* sp. strain P6, *Arthrobacter* sp. strain M5, and *Corynebacterium* sp. strain MB1) is able to degrade a wide range of polychlorinated biphenyl (PCB) congeners. The genetic and biochemical analyses of the PCB catabolic pathway reported here have revealed the existence of a PCB gene cluster—*bphBC1D*—and two further *bphC* genes—*bphC2* and *bphC3*—that encode three narrow-substrate-specificity enzymes (2,3-dihydroxybiphenyl dioxygenases) that *meta* cleave the first aromatic ring. None of the *bphC* genes show by hybridization homology to each other or to *bphC* genes in other bacteria, and the three *bphC* gene products have different kinetic parameters and sensitivities to inactivation by 3-chlorocatechol. This suggests that there exists a wide diversity in PCB *meta* cleavage enzymes.**

Polychlorinated biphenyls (PCBs) are a family of human-made compounds of exceptional stability that were in the past produced in large quantities for use as dielectric fluids, plasticizers, flame retardants, and hydraulic fluids. The extensive use of these fat-soluble, toxic xenobiotic compounds has resulted in their widespread contamination of the environment. Commercial mixtures of PCBs, such as Aroclors, can contain more than 60 of the possible 209 different congeners that differ from one another in the number and position of the chlorine substituents on the biphenyl rings. Surprisingly perhaps, given the chemical stability, toxicity, and poor bioavailability of PCBs, a number of organisms able to degrade such compounds (at least partially) have been isolated. These organisms differ both in the extent of degradation of typical PCB mixtures they are able to effect and in the number and type of congeners attacked (1).

One attractive means of removing PCBs from the environment is therefore microbial degradation. In this regard, two types of PCB-degrading organisms have received considerable attention over the past few years, because of the high levels of transforming activity they exhibit and the wide spectrum of PCB congeners attacked, namely, *Pseudomonas* sp. strain LB400 and *Alcaligenes eutrophus* H850, which preferentially degrade PCBs carrying *ortho* chlorines on both rings, and *Rhodococcus globerulus* P6 (previously designated *Acinetobacter* sp. strain P6 [22], *Arthrobacter* sp. strain M5 [20], and *Corynebacterium* sp. strain MB1 [10] but now reclassified [5]), which preferentially transforms more planar congeners (such as 4,4′-chlorobiphenyl and 3,3′-chlorobiphenyl) and congeners carrying a single *ortho* chlorine (8, 48). The fact that the spectra of substrates transformed by these two types of organisms are distinct and complementary suggests that they or their pathways may be combined to accomplish degradation of the majority of the less-chlorinated congeners in typical PCB mixtures. However, three major hurdles to such applications have emerged, namely, the poor competitiveness and robustness of the best degraders, the poor stability of the PCB degradation phenotype in the absence of selection on biphenyl as sole carbon source, and the inability of such strains to degrade the second aromatic ring of PCBs, which results in accumulation of chlorobenzoates.

The major pathway for PCB and biphenyl degradation is a four-step process initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 of one of the aromatic rings by biphenyl dioxygenase, the product of *bphA* genes. The resulting 2,3-dihydrodiol is then dehydrogenated by a dihydrodiol dehydrogenase, the product of *bphB*, to 2,3-dihydroxybiphenyl. The resulting dihydroxylated ring is cleaved in the *meta* position by 2,3-dihydroxybiphenyl-1,2-dioxygenase (2,3-DHBD), encoded by the *bphC* gene. The resulting 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid is then hydrolyzed by a hydrolase, encoded by *bphD*, to the corresponding benzoic acid (3, 23, 35, 39).

The cloning and characterization of the genes responsible for PCB degradation will facilitate the development of bacterial strains possessing a superior ability to mineralize different PCB congeners, and such genes have been cloned from several gram-negative organisms (3, 23, 35, 39). Genetic analysis of the biphenyl degradation pathway of gram-positive microorganisms has not, however, thus far been reported. In this communication, we describe the cloning of genes encoding three enzymes of the biphenyl catabolic pathway of *R. globerulus* P6. Interestingly, this organism was found to possess two additional *bphC* genes that are not clustered with the *bph* operon. Preliminary characterization of the three distinct ring cleavage enzymes encoded by the *bphC* genes is reported.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this study are listed in Table 1. The cosmid vector pLAFR3 (46) and the plasmid vectors pBluescript II SK and II KS (Stratagene, Heidelberg, Germany) were used for cloning. The broad-host-range cosmid vector pLAFR3 (46) was used to construct a genomic library of *R. globerulus* P6. The vector is not self-transmissible but can be

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TABLE 1. Bacterial strains used in this work

Strain	Relevant characteristic(s)	Source or reference[a]
Rhodococcus globerulus P6	Polychlorobiphenyl degrader	22
Pseudomonas sp. strain LB400	Polychlorobiphenyl degrader	12
Sphingomonas paucimobilis Q1	Biphenyl-naphthalene degrader	47
Sphingomonas sp. strain RW1	Dibenzofuran-dioxin degrader	50
Pseudomonas putida ATCC 17453	Carries CAM plasmid specifying camphor degradation	ATCC
Pseudomonas putida KT2442	*Pseudomonas putida mt-2 hsdR1 hsdM*$^+$ Rif^r	18
Escherichia coli DH5α	F$^-$ *lacZΔM15 recA1 hsdR17 supE44* Δ(*lacZYA argF*)	BRL

[a] ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories.

mobilized and contains a tetracycline resistance selection marker and a polylinker located between a *lac* promoter and a β-galactosidase structural gene. Clones containing recombinant cosmids carrying inserts in the polylinker appear as white colonies. *R. globerulus* P6 was grown on phosphate-buffered mineral salts medium as described previously (9). Biphenyl (approximately 1.0%), used as the sole carbon and energy source, was added directly to liquid medium or provided as vapor by placing biphenyl crystals on the lid of the petri dish. The dish was sealed with polyethylene tape. *Escherichia coli* and *Pseudomonas putida* strains were grown in either Luria-Bertani (LB) broth or M9 (38) containing 0.5% succinate and supplemented with required amino acids and vitamins. Solid medium contained 1.7% purified agar (Difco Laboratories, Detroit, Mich.). Where appropriate, the antibiotics tetracycline, chloramphenicol, and ampicillin were used for selection at concentrations of 20, 25, and 100 μg/ml, respectively; isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at concentrations of 110 and 40 μg/ml, respectively. Cultures were incubated at 30°C for *R. globerulus* and *P. putida* strains and 37°C for *E. coli* strains. Liquid cultures were incubated in a rotary shaker-incubator at 250 rpm.

Preparation of total DNA. *R. globerulus* cells grown in phosphate-buffered mineral salts medium plus biphenyl were harvested at mid-log phase. A total of 600 mg of cells was resuspended in 1.5 ml of buffer (10 mM Tris-hydrochloride, 50 mM EDTA, pH 8.0), containing 3 mg of lysozyme, and incubated at 37°C for 60 min. Proteinase K was then added to a final concentration of 750 mg/ml, and the cell suspension was incubated for a further 30 min. Lysis was achieved by the addition of a one-seventh volume of 10% sarcosyl. Following incubation at 37°C for another 60 min, the NaCl concentration of the solution was adjusted to 0.7 M. A total of 0.25 ml of hexadecyltrimethyl ammonium bromide (CTAB)–NaCl solution (10% CTAB–0.7 M NaCl) was added, and a final incubation at 60°C for 20 min was performed. The solution was phenol-chloroform extracted three times, and the final aqueous phase was extracted with chloroform-isoamyl alcohol. The DNA was then precipitated with 2 volumes of −20°C absolute ethanol, collected by spooling onto a glass rod, and dissolved in Tris-EDTA buffer (43).

Genomic DNA was partially digested with *Sau*3AI and size fractionated through a 20% NaCl gradient (31). Gradient centrifugation was carried out in a TH-641 rotor (Dupont Instruments) at 37,000 rpm, 20°C, for 4.5 h. Fractions containing genomic DNA fragments between 20 and 30 kilobase pairs (kb) in size were identified by agarose gel electrophoresis.

Arms of the cosmid cloning vector pLAFR3 were prepared as previously described (46), and the cosmid vector DNA was digested to completion with *Eco*RI or *Hin*dIII in two separate aliquots, treated with alkaline phosphatase, and digested with *Bam*HI.

Library construction. Fragments of *R. globerulus* P6 DNA (2 μg) were joined to the individual pLAFR3 arms (1 μg each) with T4 DNA ligase in a total reaction volume of 12 μl and incubated at 15°C for 16 h. In vitro packaging of the recombinant molecules was performed with a commercially available extract (Stratagene) as recommended by the manufacturer. Aliquots of the packaging reaction mixture were used to infect cells of *E. coli* DH5α that were then plated onto LB agar plates containing tetracycline, IPTG, and X-Gal to select recombinant infectants. The efficiency of infection was 38,000 clones per μg of ligated DNA, 96% of which were recombinants. Randomly selected recombinant clones were found to contain DNA inserts ranging from 10 to 30 kb in size.

Isolation of plasmid DNA. Cosmid pLAFR3 and its hybrids carrying *bph* genes were isolated from *E. coli* DH5α or *P. putida* KT2442 by the alkaline lysis method (43). Plasmid isolation from *R. globerulus* P6 was performed as described by Schreiner et al. (44). Minipreparation of plasmid pBluescript II SK and its hybrid derivatives was done by the boiling method (43).

Detection of recombinants and measurement of enzymatic activities. Strains containing recombinant plasmids were grown as described above, washed with 0.05 M phosphate buffer (pH 7.5) containing 10% acetone, and resuspended in the same buffer. The cells were disrupted by a single passage through a French press (Aminco) operated at a pressure of 20,000 lb/in^2. The cell debris was removed by centrifugation at 17,000 rpm for 20 min in an SS-34 rotor (Sorvall), and the clear supernatant fluid was carefully decanted and used as raw extract.

The presence of 2,3-DHBD activity was assayed by the appearance of the yellow metabolite, 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid, which is formed by *meta* cleavage of 2,3-dihydroxybiphenyl. This reaction was exploited to detect the expression of *bphC* in recombinant colonies by spraying plates with a 1-g/liter solution of 2,3-dihydroxybiphenyl (Wako Chemical Co., Tokyo, Japan) (23), as well as to assay the activity of 2,3-DHBD in raw extracts. Enzymatic activity was measured by following the formation of reaction products at 434 nm with a Beckman DU-70 spectrophotometer equipped with a thermojacketed cuvette holder and a Haake circulating water bath. Activity assays were performed at 25°C in 50 mM phosphate buffer (pH 7.5) containing 165 μM 2,3-dihydroxybiphenyl as previously described (15, 24). One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min. The molar extinction coefficient of the

product under the assay conditions was taken to be 13,200 cm^{-1} M^{-1} (15). Data were fitted to a substrate inhibition equation (2) with MINSQ (Micromath Inc.), a least-squares fitting routine. The effect of 3-chlorocatechol on the 2,3-DHBD activity was checked by simultaneous incubation with 3-chlorocatechol and 2,3-dihydroxybiphenyl.

The relative ring cleavage activities were determined by the extinction coefficients of the ring fission products formed from the following substrates: catechol, λ_{max} = 375 nm and ε = 36,000 cm^{-1} M^{-1}; 3-methylcatechol, λ_{max} = 388 nm and ε = 32,000 cm^{-1} M^{-1}; 4-methylcatechol, λ_{max} = 382 nm and ε = 17,000 cm^{-1} M^{-1}; 4-chlorocatechol, λ_{max} = 379 nm and ε = 40,000 cm^{-1} M^{-1} (42); and 1,2-dihydroxynaphthalene, λ = 331 nm and ε = 2,600 cm^{-1} M^{-1} (37). The extinction coefficient of the unstable *meta* cleavage product of 2,2',3-trihydroxybiphenyl was calculated to be 16,200 cm^{-1} M^{-1} at 434 nm.

The BphB product was detected in strains concomitantly expressing *bphC* by the formation of the *meta* cleavage product following addition of a solution containing 2,3-dihydroxy-1-phenylhexa-4,6-diene. This compound was enzymatically prepared from biphenyl with a resting cell culture of an engineered *E. coli* strain that hyperexpresses the *bphA1A2A3A4* genes of *Pseudomonas* sp. strain LB400 (28).

The BphD hydrolase activity was assayed both by following spectrophotometrically the disappearance of the *meta* cleavage compound at 434 nm and by monitoring the appearance of benzoic acid by reverse-phase high-performance liquid chromatography with a Lichrosphe RP8 column (Bischoff Chromatography, Leonberg, Germany). For strains expressing *bphC*, a coupled 2,3-DHBD–hydrolase assay was used. In strains that did not express *bphC*, the *meta* cleavage compound used as the substrate was produced from 2,3-dihydroxybiphenyl with purified 2,3-DHBD from *Pseudomonas* sp. strain LB400 (15).

Hybridization experiments. Southern blots (45) were performed with a Biodyne B nylon membrane (Pall, Glen Cove, N.Y.). Hybridizations were carried out under the conditions described by Anderson and Young (4). High-stringency hybridization experiments were performed at 42°C in the presence of 50% formamide–5× SSC (750 mM sodium chloride and 75 mM sodium citrate, pH 7.0 [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), and filters were washed at 68°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS). Low-stringency hybridizations were carried out at 35°C in the same hybridization buffer, and filters were washed at 50°C in 5× SSC containing 0.1% SDS. The DNA fragments used as probes were isolated from low-melting-point agarose. The probes were labelled by incorporation of digoxigenine-dUTP, with a random-primer DNA labelling kit (Boehringer Mannheim Biochemicals, Mannheim, Germany).

Mating experiments. *E. coli* cells containing the cosmid gene library or specific recombinant cosmids were mated with *P. putida* KT2442 with the help of the plasmid pRK600 (33). pRK600 is a derivative of pRK2013 (16) that contains the RK2 *tra* functions and a chloramphenicol resistance gene ligated to a ColE1 replicon. This construct can mobilize recombinant pLAFR3 derivatives into various gram-negative hosts. Triparental matings, in which *E. coli* HB101 (pRK600) was used as a source of the mobilizing plasmid pRK600, were performed by a filter mating technique (27) employing a mixture of donor and recipient cells at a ratio of 1:2. Filters were incubated for at least 8 h at 30°C on the surface of LB agar plates; cells were then resuspended in M9 liquid medium, and appropriate dilutions were plated onto M9 supplemented with 0.2% citrate.

Subcloning procedures and restriction analysis. The DNA fragments specifying 2,3-DHBD activity were subcloned into pBluescript II SK digested with the appropriate enzymes and treated with alkaline phosphatase. The ligation mixture was used to transform cells of *E. coli* DH5α by the rubidium chloride method (26) or by electroporation (14). Cells were plated onto LB agar containing ampicillin, X-Gal, and IPTG. Clones expressing 2,3-DHBD activity were identified by the spray method as described above. Restriction analysis and recombinant DNA techniques were performed by standard methods (43) or as recommended by the manufacturer.

RESULTS

Screening of library for 2,3-DHBD expression. A cosmid library containing approximately 68,000 clones was prepared as described in Materials and Methods, resuspended in LB broth containing 20 μg of tetracycline per ml and 20% glycerol, and frozen at −70°C. Approximately 1,600 recombinant clones were tested for 2,3-DHBD activity by spraying with a solution containing 2,3-dihydroxybiphenyl. Thirteen colonies which turned bright yellow when sprayed were then purified. Plasmid DNAs were prepared from these clones, digested with various restriction enzymes, and on the basis of the restriction patterns obtained were classified into three groups: group I (pJA2, pJA6, and pJA8), group II (pJA9, pJA10, pJA11, pJA13, pJA14, and pJA16), and group III (pJA1, pJA3, pJA4, and pJA7). Throughout this work, the three 2,3-DHBDs are referred to as follows: 2,3-DHBDI is encoded by the gene *bphC1* of restriction group I, 2,3-DHBDII is encoded by the gene *bphC2* of restriction group II, and 2,3-DHBDIII is encoded by the gene *bphC3* of restriction group III.

Subcloning of the genes encoding 2,3-DHBDs. Cosmid DNA from *E. coli* strains DH5α(pJA3), DH5α(pJA6), and DH5α(pJA9) was isolated and characterized by restriction endonuclease digestion and agarose gel electrophoresis. The sizes of the inserts in pJA3, pJA6, and pJA9 were 25.6, 19.0, and 28.8 kb, respectively. Restriction maps for the three inserts (Fig. 1) were established from single and double digestions with restriction endonucleases and from Southern hybridizations.

The *bphC1* gene was subcloned from pJA6 as a 5.1-kb *Cla*I fragment into pBluescript II SK, yielding plasmid pJA61. Further subcloning experiments localized the *bphC1* gene to a 1.65-kb *Bst*EII-*Bam*HI fragment (Fig. 1A). Subclones pJA65 and pJA66 carried the same 2.7-kb *Cla*I-*Bam*HI fragment in opposite orientations, but only the former expressed detectable 2,3-DHBD activity. This suggests that the *bphC1* gene is expressed in *E. coli* by transcription from the *lac* promoter of the pBluescript vector and that either the *bphC1* gene does not contain its own transcriptional signals or the *Rhodococcus* promoter is not active in *E. coli*. The orientation of the *bphC1* gene suggested by this result is indicated in Fig. 3.

The gene encoding the 2,3-DHBDII activity of pJA9 was subcloned as a 2.5-kb *Bgl*II fragment into pBluescript II SK, yielding plasmid pJA91. This fragment included 1.2 kb of the cosmid vector pLAFR3. Further subcloning experiments revealed that the *bphC2* gene is contained within a 1.0-kb *Bam*HI fragment (Fig. 1B). Subclones pJA94 and pJA95 carried the same 1.0-kb *Bam*HI fragment in opposite orientations, and both expressed the *bphC2* gene.

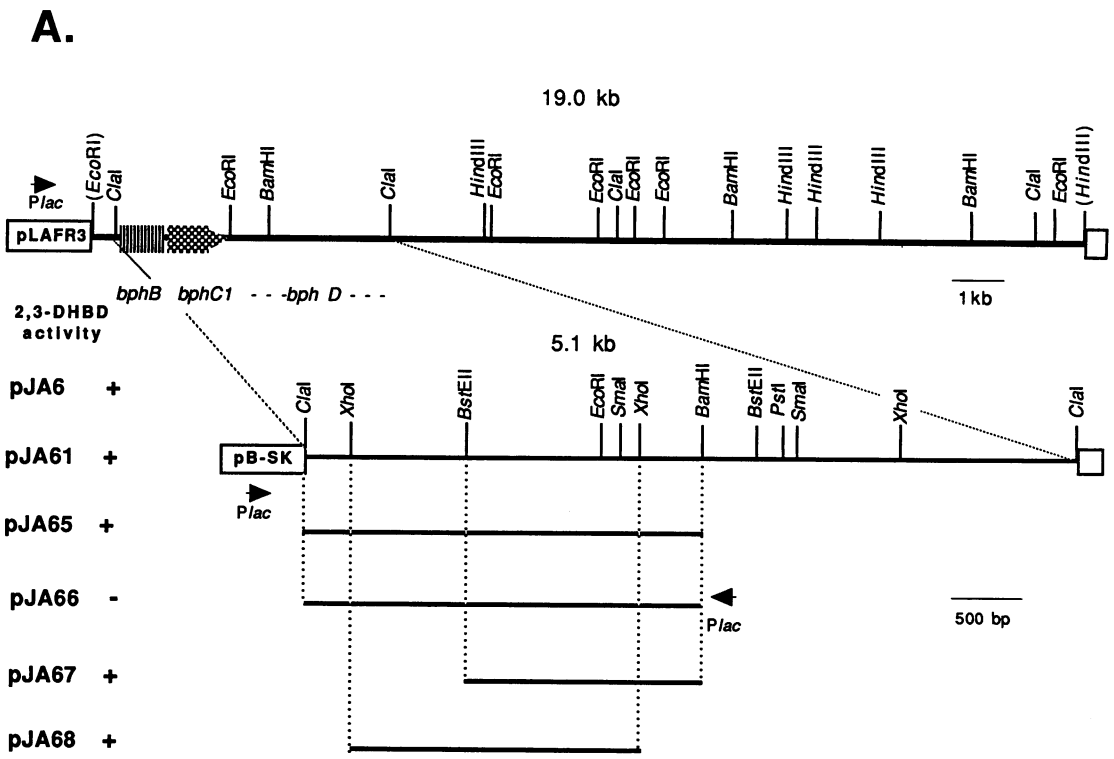

FIG. 1. Cloning of *R. globerulus bphC* genes. Cosmid pLAFR3 was used in *E. coli* and *P. putida*, whereas pBluescript II SK or pBluescript II KS was used in *E. coli*. (A) Cloning of *bphB*, *bphC1*, and *bphD* genes; (B) cloning of *bphC2*; (C) cloning of *bphC3*. Restriction sites shown in parentheses are located in the vector. Only restriction sites of relevant enzymes are shown. The asterisk indicates that the restriction site is not unique. The location and direction of the *lac* promoter presented on pLAFR3 or pBluescript are indicated by an arrow. +, activity; −, no activity.

The *bphC3* gene was subcloned from pJA3 as a 7.3-kb *Eco*RI fragment into pBluescript II SK, giving rise to plasmid pJA31. To more precisely localize this gene, successive deletions were made with single restriction enzyme sites in the fragment. Plasmids pJA32 and pJA34, which contain the same 1.6-kb *Eco*RI-*Pst*I insert but in opposite orientations, both expressed 2,3-DHBD activity (Fig. 1C). Unlike earlier findings with cloned *Rhodococcus* genes, *bphC2* and *bphC3* were both expressed well in *E. coli* independent of vector transcriptional signals, suggesting that their native *Rhodococcus* promoters function in *E. coli* or that their native promoters are not typical *Rhodococcus* promoters.

2,3-DHBD activities in bacteria carrying recombinant cosmids. Recombinant cosmids pJA6, pJA9, and pJA3 representing restriction groups I, II, and III, respectively, were mobilized into *P. putida* KT2442 with the help of the plasmid pRK600. 2,3-DHBD activity was analyzed for raw extracts of recombinant bacteria grown in LB broth supplemented with tetracycline and IPTG. Enzyme activities were 3.05 U/mg in extracts from KT2442(pJA3) bacteria, 2.30 U/mg in extracts from KT2442(pJA9), and 1.52 U/mg in extracts from KT2442(pJA6). No activity was observed for extracts from pLAFR3-carrying *P. putida* KT2442 cells. All three cloned 2,3-DHBDs exhibited a narrow substrate specificity: none showed activity towards 4-methylcatechol, 3-chlorocatechol, 4-chlorocatechol, 3,4-dihydroxybiphenyl, or 1,2-dihydroxynaphthalene. 2,2′,3-Trihydroxybiphenyl was a good substrate for all of the enzymes, as was 3-methylcatechol for 2,3-DHBDI. This latter substrate was, however, only poorly transformed by the other two enzymes, as was catechol by 2,3-DHBDI (Table 2). These results indicate that all of these ring cleavage enzymes are bona fide DHBDs.

Lineweaver-Burke representations of typical kinetic data obtained with raw extracts are shown in Fig. 2. All dioxygenases display strong substrate inhibition with 2,3-dihydroxybiphenyl: i.e., at high substrate concentrations, a lower initial catalytic turnover was observed. This phenomenon has been described previously by the polynomial expression $[S]/V = K_m/V_{max} + (1/V_{max})[S] + (1/K_{ss} \cdot V_{max})[S]^2$, where $[S]$ is the substrate concentration (in micromolar), V is the observed rate (in nanomoles per minute per milligram of protein), V_{max} is the maximum rate, K_m is the Michaelis-Menten constant (in micromolar), and K_{ss} is the inhibition constant (in micromolar) (2). 2,3-DHBDI also displayed substrate inhibition with 3-methylcatechol, but only at substrate concentrations greater than 0.7 mM. The calculated best-fit parameters are presented in Table 3.

The three 2,3-DHBDs have significantly different kinetic parameters (Table 3). 2,3-DHBDI has the greatest affinity for 2,3-dihydroxybiphenyl, having a K_m value between four and eight times lower than that of the other two enzymes, and has the highest inhibition constant, which indicates that this enzyme is less sensitive to substrate inhibition than 2,3-DHBDII and 2,3-DHBDIII. On the other hand, the V_{max} of 2,3-DHBDI is two to three times lower than that of 2,3-DHBDII and 2,3-DHBDIII. The K_m value of 2,3-DHBDI for 3-methylcatechol is around 25 times higher than the corresponding one for 2,3-dihydroxybiphenyl.

The irreversible inactivation of 2,3-DHBD and catechol-2,3-dioxygenases by 3-chlorocatechol is a well-documented

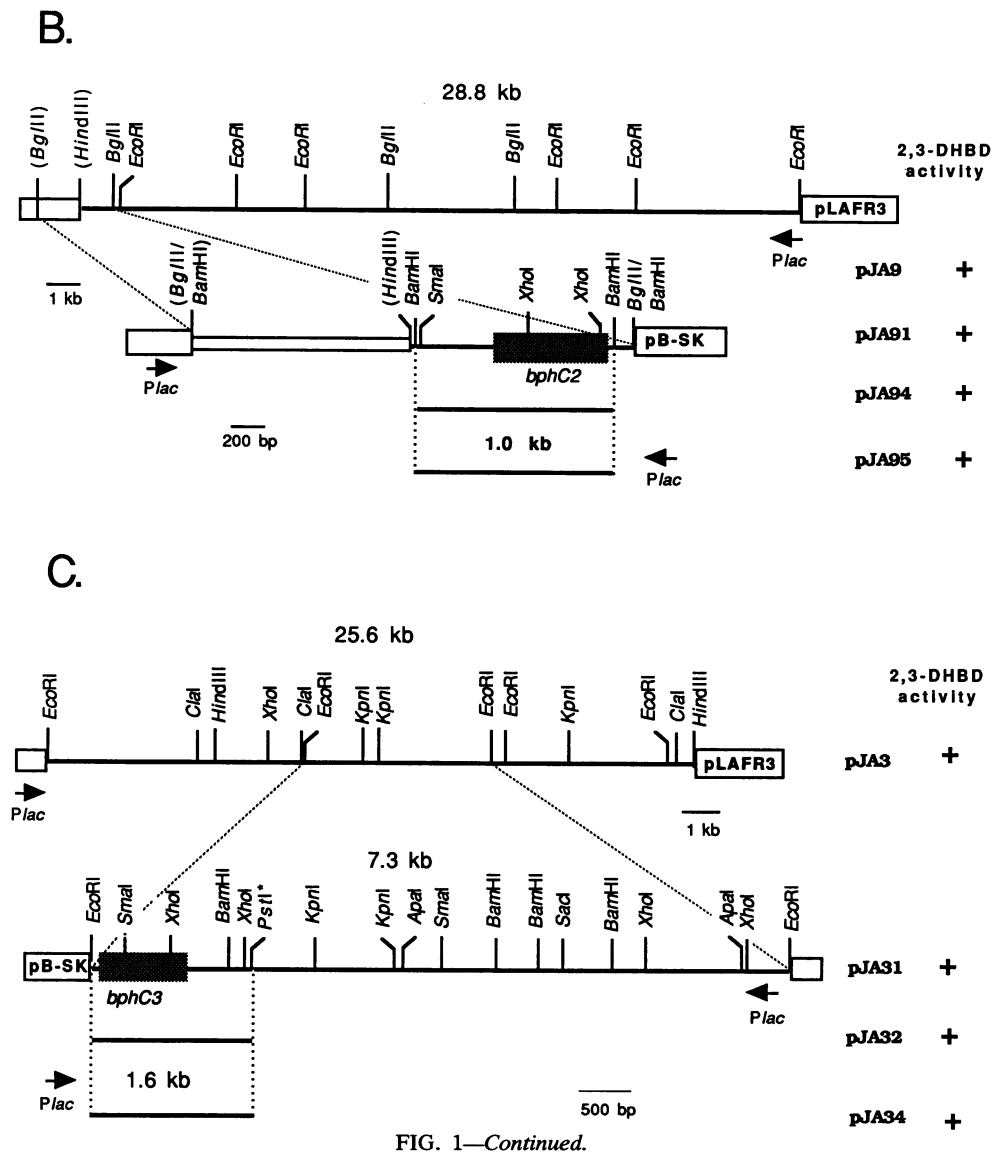

FIG. 1—*Continued.*

phenomenon (2, 6). The activity of the three 2,3-DHBDs was measured after incubating the raw extracts in the presence of different concentrations of 3-chlorocatechol and the substrate 2,3-dihydroxybiphenyl. These measurements confirm that all three enzymes are inhibited by 3-chlorocatechol but that 2,3-DHBDII is significantly less sensitive than the other two, requiring an approximately three-times-higher concentration of this compound to be inhibited to the 95% level (Table 4).

Localization of other genes involved in biphenyl degradation. Whether the cloning of genes for three different 2,3-DHBDs reflects the existence of three complete biphenyl pathways or only segments thereof was investigated by assaying other enzymatic activities associated with biphenyl degradation. None of the recombinant cosmids was sufficient to endow upon *E. coli* or *P. putida* the ability to grow on biphenyl or catabolize biphenyl to the *meta* cleavage compound. This indicates either that none of the clones contain the complete set of genes for the degradation of biphenyl or that some genes are not expressed in *E. coli* or *P. putida*.

TABLE 2. Substrate specificities of the different 2,3-DHBDs cloned from *R. globerulus* P6

Substrate	Substrate concn (μM)	Activity with enzyme[a]:		
		DHBDI	DHBDII	DHBDIII
2,3-Dihydroxybiphenyl	165	100 (1.46)	100 (2.02)	100 (3.40)
3,4-Dihydroxybiphenyl	330	0	0	0
2,2',3-Trihydroxybiphenyl	100	32	35	34
1,2-Dihydroxynaphthalene	600	0	0	0
3-Methylcatechol	330	58	5	3
4-Methylcatechol	330	0	0	0
Catechol	330	5	0	0
3-Chlorocatechol	330	0	0	0
4-Chlorocatechol	330	0	0	0

[a] Activities were measured in raw extracts of *P. putida* recombinant strains carrying the cosmids pJA6, pJA9, and pJA3 for detection of DBHDI, DHBDII, and DHBDIII activities, respectively. The reaction rates are expressed as percentages of that measured with 2,3-dihydroxybiphenyl as substrate, taken as 100%. Absolute specific activities (units per microgram of protein, as defined in Materials and Methods) are given in parentheses for the relative rates reported as 100%. The increase of the absorbances of the products was monitored at their respective wavelengths.

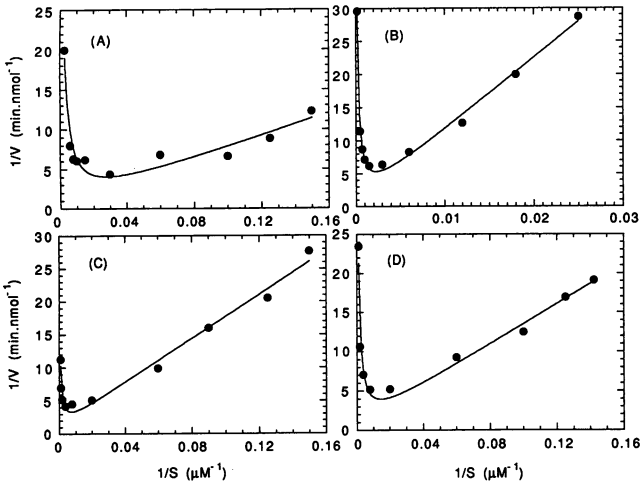

FIG. 2. Lineweaver-Burke plot of 2,3-dihydroxybiphenyl or 3-methylcatechol cleavage by the three different 2,3-DHBDs. (A) 2,3-DHBDI with 2,3-dihydroxybiphenyl as substrate; (B) 2,3-DHBDI with 3-methylcatechol as substrate; (C) 2,3-DHBDII with 2,3-dihydroxybiphenyl as substrate; (D) 2,3-DHBDIII with 2,3-dihydroxybiphenyl as substrate.

The activity of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase, which converts the *meta* cleavage compound to benzoic acid and is encoded by the *bphD* gene, was assayed both spectrophotometrically and by high-performance liquid chromatography analysis. Raw extracts of *P. putida* KT2442(pJA6) converted 2,3-DHBP to a compound that has the same retention time as benzoic acid (activity, 0.22 U/mg of protein). This compound was not detected in the reactions performed with extracts of cells containing pJA3 or pJA9, although in analogous reactions monitored spectrophotometrically, a slight decrease in A_{434} was observed.

Similarly, only *P. putida* and *E. coli* bacteria carrying recombinant cosmids belonging to restriction group I exhibited dihydrodiol dehydrogenase activity (encoded by the *bphB* gene; Table 5). In cosmids pJA6 and pJA8, the *bphB* gene appears to be transcribed from the *lac* promoter. Extracts of recombinant KT2442(pJA2) bacteria exhibited no dihydrodiol dehydrogenase activity, even though pJA2 should also include the *bphB* gene (Table 5). Dihydrodiol dehydrogenase activity was also detected in extracts of *E. coli* bacteria containing pJA61 and pJA65, but not in bacteria containing pJA67 and pJA68. This suggests that deletion of the 0.3-kb *Cla*I-*Xho*I fragment prevents the expression of the *bphB* gene and that the *bphB* gene of *R. globerulus* is located just upstream of the *bphC1* gene (Fig. 1A).

TABLE 3. Kinetic parameters of the different 2,3-DHBDs cloned from *R. globerulus* P6[a]

Enzyme	Substrate	K_m (μM)	V_{max} (nmol/min/mg)	K_{ss} (μM)
DHBDI	2,3-DHBP	9.9 ± 0.7	1,873	592.8 ± 57.6
DHBDI	3-MC	251.2 ± 7.9	1,852	520.6 ± 11.5
DHBDII	2,3-DHBP	84.2 ± 4.0	5,237	153.3 ± 7.6
DHBDIII	2,3-DHBP	42.8 ± 5.3	5,878	123.9 ± 2.5

[a] Activities were measured in raw extracts of *P. putida* recombinant strains carrying the cosmids pJA6, pJA9, and pJA3 for detection of DHBDI, DHBDII, and DHBDIII activities, respectively. 3-MC, 3-methylcatechol; 2,3-DHBP, 2,3-dihydroxybiphenyl; K_{ss}, inhibition constant.

TABLE 4. 3-Chlorocatechol inactivation of the different cloned 2,3-DHBDs

Enzyme	2,3-DHBD activity[a] (nmol/min/mg) with following concn of 3-chlorocatechol (μM):		
	0	166	500
DHBDI	805	142 (83)	NT
DHBDII	872	593 (32)	39 (95)
DHBDIII	1,784	76 (96)	NT

[a] Activities were measured in raw extracts as described in footnote *a* of Table 2 after incubation with both 3-chlorocatechol and 2,3-dihydroxybiphenyl. Percent inhibition is indicated in parentheses. NT, not tested.

Localization of *bphC1* and *bphC3* on the chromosome. Restriction endonuclease cleavage and Southern hybridization analysis showed that a total of 65 kb of *Rhodococcus globerulus* P6 chromosomal DNA had been cloned in six overlapping cosmids. As is shown in Fig. 3, *bphC3* and *bphBC1D* are situated in this region and are separated by a DNA stretch approximately 27 kb long.

A single plasmid 80 kb in length and designated pKF1 has been isolated from *R. globerulus* P6 (20). While no fragment of the 65-kb stretch of DNA encompassing *bphC1* and *bphC3* hybridized to undigested pKF1 plasmid DNA, all such fragments hybridized to the undigested chromosomal DNA of *R. globerulus* P6. These results indicate that *bphBC1D* and *bphC3* are chromosomally located.

Lack of homology of *R. globerulus* P6 *bphC* genes to each other and to other *bphC* genes. To determine whether the three different *bphC* genes from *R. globerulus* P6 are related to one another and to genes of other *meta* cleavage enzymes, hybridization experiments were performed with the *bphC* gene probes. Hybridizations were performed under high- and low-stringency conditions, as described in Materials and Methods, in order to distinguish between strong and weak sequence homologies. A 5.1-kb *Cla*I fragment containing *bphC1* and *bphB* hybridized to *Cla*I-digested pJA6 DNA (positive control) and *Cla*I-digested *R. globerulus* P6 total

TABLE 5. Catabolic enzyme activities of *E. coli* or *P. putida* recombinant clones[a]

Plasmid	BD[b,c]	DHDH[b,c]	2,3-DHBD[b,c]	HOPDAH[c]
pJA3	−	−	+	−
pJA7	−	−	+	−
pJA4	−	−	+	−
pJA9	−	−	+	−
pJA16	−	−	+	−
pJA6	−	+	+	+
pJA8	−	+	+	+
pJA2	−	−[d]	+	+
pJA61[e]	−	+	+	NT
pJA65[e]	−	+	+	NT
pJA67[e]	−	−	+	NT
pJA68[e]	−	−	+	NT

[a] BD, biphenyl dioxygenase; DHDH, dihydrodiol dehydrogenase; HOPDAH, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase; NT, not tested.
[b] *E. coli.*
[c] *P. putida.*
[d] Has no activity but contains the *bphB* gene.
[e] Replication only in *E. coli.*

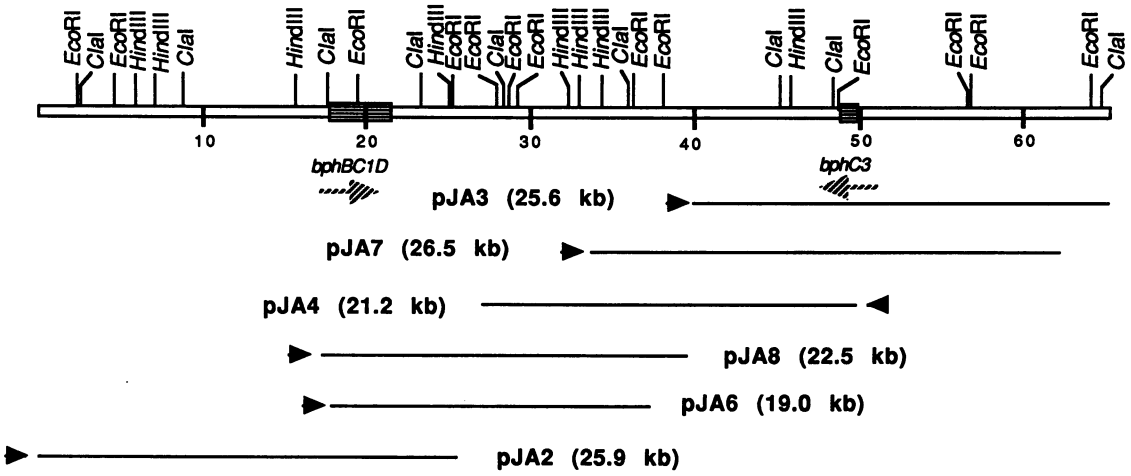

FIG. 3. Region of the *R. globerulus* chromosome cloned in the overlapping cosmids pJA2, pJA3, pJA4, pJA6, pJA7, and pJA8 and containing the *bph* cluster and a second *bphC* gene. Arrows indicate the direction of the transcription from the *lac* promoter of the vector pLAFR3. The order and locations of the genes for PCB-biphenyl degradation as deduced from the cloning and expression studies are shown. The direction of transcription of *bphC1* and *bphC3* is indicated by a striped arrow.

DNA, but not to pJA3, pJA9, or total DNA of other tested strains, even under low-stringency conditions (Fig. 4A). Similarly, the 1.0-kb *bphC2* gene-containing *Bam*HI fragment from pJA94 hybridized to the positive control pJA9, which was double digested with *Eco*RI and *Hin*dIII to liberate a fragment of 1.5 kb containing the *bphC2* gene, and to a 5.5-kb *Eco*RI fragment of *R. globerulus* DNA. No hybridization to other fragments was found even when using low-stringency conditions (Fig. 4B). The 7.3-kb *bphC3* gene-containing *Eco*RI fragment from pJA31 hybridized to a 7.3-kb *Eco*RI fragment of the positive control pJA3 and of *R. globerulus*, and more weakly to two further fragments (4.0 and 2.7 kb) also present in pJA9. This hybridization pattern was conserved under high-stringency conditions. No other bands were revealed by the probe (Fig. 4C). This hybridization profile changed when the smaller 1.2-kb *bphC3* gene-containing *Eco*RI-*Pst*I fragment of pJA32 was used as a probe, and the weak 4.0- and 2.7-kb hybridization bands of *R. globerulus* genomic DNA and of the pJA9 cosmid disappeared (Fig. 4D).

DISCUSSION

Gram-positive organisms exhibit a wealth of catabolic activities and are considered to be environmentally important for the mineralization of organic pollutants (e.g., see reference 17). The degradation of PCBs in gram-positive microorganisms is well-known. A *Micrococcus* sp. used 4-chlorobiphenyl as a growth substrate (11). *Nocardia* spp. are capable of selectively degrading mono- through tetrachlorobiphenyls in Aroclor 1254 (7) whereas *Arthrobacter* sp. strain B1B can degrade 12 capillary gas chromatographic peaks of this mixture, including tetra- and pentachlorobiphenyls (36). *R. globerulus* P6 is able to transform 25 of the 40 major components of Aroclor 1254 (25, 36). Despite the importance of gram-positive organisms in general and the interest in *R. globerulus* P6 in particular, no genetic studies on PCB degradation by such bacteria have so far been described. In this report, we describe the cloning and characterization of some of the PCB degradation determinants of *R. globerulus* P6. Although we have identified a *bphBCD* gene cluster that we can express in *E. coli*, we have

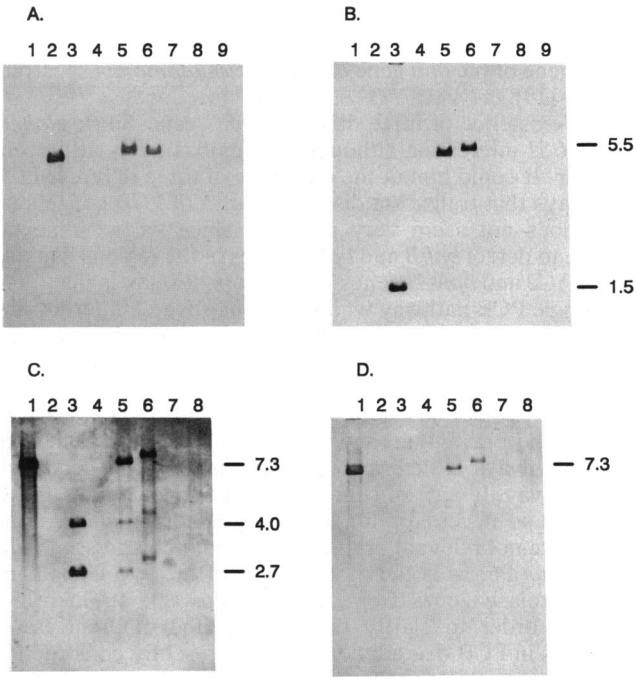

FIG. 4. Hybridization of the different *bphC* genes with genomic DNAs from various strains. (A) Genomic DNAs were digested with *Cla*I, electrophoresed, and probed with the 5.1-kb *Cla*I fragment from pJA61 containing the *bphC1* and *bphB* genes. (B) Genomic DNAs were digested with *Eco*RI, except *Sphingomonas* sp. strain RW1 DNA, which was digested with *Cla*I, and pJA9, which was double digested with *Eco*RI and *Hin*dIII. The probe used was the 1.0-kb *Bam*HI fragment from pJA94 that contains the *bphC2* gene. (C) Genomic DNAs were digested as in panel B and probed with the 7.3-kb *Eco*RI fragment from pJA31 that contains the *bphC3* gene. (D) Shown is the same blot as in panel C but hybridized with the 1.2-kb *Eco*RI-*Pst*I fragment from pJA32. Lanes: 1, pJA3; 2, pJA6; 3, pJA9; 4, *P. putida* ATCC 17453; 5 and 6, *R. globerulus* P6; 7, *Pseudomonas* sp. strain LB400; 8, *Sphingomonas* sp. strain RW1; 9, *S. paucimobilis* Q1. The slightly different migration of the bands corresponding to lanes 5 and 6 was also observed in the ethidium bromide-stained gel and was due to different migration of the digested DNA.

not so far localized the corresponding *bphA* gene, presumably because of the fact that it is poorly expressed in the clones we have. Other approaches currently being taken should, however, soon identify this gene so that a convenient *bphABCD* gene cassette with native as well as foreign gene expression signals can be constructed. This in turn should lead to the development of better-performing degraders able to mineralize the important spectrum of congeners transformed by *R. globerulus* P6.

Interestingly, we have demonstrated the existence of determinants for two additional 2,3-DHBDs distinct from that of the *bphBC1D* gene cluster. The evidence for three different *bphC* genes is that they map at different locations (the locations of two were determined), their DNA sequences are not homologous as determined by Southern hybridization, and their products exhibit distinct properties in terms of their substrate specificities, kinetic parameters, and inhibition by 3-chlorocatechol. Though distinct, the enzymes are similar in that they have narrow substrate spectra and prefer 3-methylcatechol over 4-methylcatechol, which is consistent with the enzymes' inability to cleave 3,4-dihydroxybiphenyl. Similar enzymes with narrow substrate specificity have been isolated from *Pseudomonas* sp. strain LB400 (15), *Pseudomonas pseudoalcaligenes* KF707 (19), and *P. putida* OU83 (34). The K_m value of 2,3-DHBDI is close to that of the analogous enzyme encoded by the *bphC* gene of the *bph* gene cluster in *Pseudomonas* sp. strain LB400 (15).

The existence of three different *bphC* genes in *R. globerulus* P6 is interesting although its significance is at present unclear. It could hint at the existence of three different PCB pathways that transform distinct spectra of PCB congeners. This does not seem very probable since we were so far unable to detect *bphB* and *bphD* genes in the regions flanking the *bphC2* and *bphC3* genes. Another possibility is that there is a single PCB pathway which exploits several different ring cleavage enzymes for the broad spectrum of substrates. Or perhaps the different ring cleavage enzymes are expressed under different environmental conditions. If these latter possibilities turn out to be correct, it will be necessary to modify the prevailing view that the substrate specificity of PCB pathways is determined by the initial dioxygenase of the pathway. It may, of course, be that only one of the BphC enzymes is necessary for PCB degradation and that the others are not relevant or indeed may not be expressed in *R. globerulus* P6, as is the case for one of the two haloalkane halohydrolase genes from *Rhodococcus* sp. strain m15-3 (40). In order to clarify the roles of the different BphC enzymes in PCB degradation, we are presently constructing deletion mutant derivatives of each of the *bphC* genes which will then be separately and in combination introduced into *R. globerulus* P6 to determine the influence of such mutations on the degradation phenotype of the organism.

Amplifications and rearrangements of genetic material are a well-documented phenomena in actinomycetes (13). Duplications are considered to be an important means of evolving new catabolic activities. Once duplicated, the second gene copy is free of selective constraints and can thus diverge much faster than genes subjected to such constraints. There are several examples of duplications of genes involved in biodegradation of toxic compounds, in particular of genes encoding ring cleavage enzymes. Nonhomologous catechol-2,3-dioxygenase genes have been found in *A. eutrophus* JMP222 (29, 30), in *Rhodococcus rhodochrous* CTM (44), and in the TOL plasmid pWW15 of *P. putida* (32). Many naturally occurring TOL plasmids carry duplications of the upper- and/or lower-pathway operons, and/or the *xylS* and *xylR* regulatory genes (49); any selective advantage that these duplications might confer upon the plasmid has not yet been defined. The only example of duplication of genes specifying the degradation of biphenyl is *P. putida* OU83, which has two 2,3-DHBDs, one encoded by the *bph* operon and exhibiting a narrow substrate specificity, and another, not specified by the operon, exhibiting a wide substrate specificity (34, 35). In this context, it is interesting to note that there is homology in the flanking regions of genes *bphC2* and *bphC3* (Fig. 4C and D), downstream of *bphC2* and upstream of *bphC3* (data not shown), which may hint at the existence of insertion sequences in the neighborhood of these *bphC* genes; whether or not the homologous regions are insertion elements that have mediated duplication and transposition of the *bphC* genes requires further investigation.

Several studies have investigated the molecular relationships between the genes of PCB-biphenyl catabolic pathways (3, 21, 51). Such studies could in principle provide fundamental information regarding the evolution and horizontal transfer of these pathways, as well as facilitating the design of appropriate hybridization probes to detect PCB-biphenyl catabolic pathways in culturable and nonculturable bacteria. The usefulness of this latter technique in evaluating the PCB-degrading potential of different microbial ecosystems is, however, limited by the diversity of PCB genotypes (41). The hybridization experiments described here demonstrate that the genes encoding the *bph* catabolic pathway of *R. globerulus* P6 are not closely related to analogous genes from other aerobic PCB-degrading bacteria (Fig. 4). Although the precise degree of homology between these different cloned *bph* clusters awaits nucleotide sequence comparisons, hybridization experiments to date indicate that they fall into four different categories represented by the *bph* cluster of *Pseudomonas* sp. strain LB400 (including those of *A. eutrophus* H850, *P. pseudoalcaligenes* KF707, and *Pseudomonas* sp. strain KF705), of *Sphingomonas paucimobilis* Q1, of *Pseudomonas testosteroni* B-356, and of *R. globerulus* P6.

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