APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2001, p. 2669–2676 Vol. 67, No. 6
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.6.2669–2676.2001

Induction of *bphA*, Encoding Biphenyl Dioxygenase, in Two Polychlorinated Biphenyl-Degrading Bacteria, Psychrotolerant *Pseudomonas* Strain Cam-1 and Mesophilic *Burkholderia* Strain LB400

EMMA R. MASTER AND WILLIAM W. MOHN*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Received 17 October 2000/Accepted 16 March 2001

We investigated induction of biphenyl dioxygenase in the psychrotolerant polychlorinated biphenyl (PCB) degrader *Pseudomonas strain* Cam-1 and in the mesophilic PCB degrader *Burkholderia strain* LB400. Using a counterselectable gene replacement vector, we inserted a *lacZ*-Gm^r fusion cassette between chromosomal genes encoding the large subunit (*bphA*) and small subunit (*bphE*) of biphenyl dioxygenase in Cam-1 and LB400, generating Cam-10 and LB400-1, respectively. Potential inducers of *bphA* were added to cell suspensions of Cam-10 and LB400-1 incubated at 30°C, and then beta-galactosidase activity was measured. Biphenyl induced beta-galactosidase activity in Cam-10 to a level approximately six times greater than the basal level in cells incubated with pyruvate. In contrast, the beta-galactosidase activities in LB400-1 incubated with biphenyl and in LB400-1 incubated with pyruvate were indistinguishable. At a concentration of 1 mM, most of the 40 potential inducers tested were inhibitory to induction by biphenyl of beta-galactosidase activity in Cam-10. The exceptions were naphthalene, salicylate, 2-chlorobiphenyl, and 4-chlorobiphenyl, which induced beta-galactosidase activity in Cam-10, although at levels that were no more than 30% of the levels induced by biphenyl. After incubation for 24 h at 7°C, biphenyl induced beta-galactosidase activity in Cam-10 to a level approximately four times greater than the basal level in cells incubated with pyruvate. The constitutive level of beta-galactosidase activity in LB400-1 grown at 15°C was approximately five times less than the level in LB400-1 grown at 30°C. Thus, there are substantial differences in the effects of physical and chemical environmental conditions on genetic regulation of PCB degradation in different bacteria.

Bioremediation of soil contaminated with polychlorinated biphenyls (PCBs) is an attractive clean-up strategy due to its potential to mineralize pollutants and to be inexpensive. Many PCB-degrading bacteria have been isolated and characterized (2, 3, 6, 8, 10, 22, 39). Some of these bacteria can grown on monochlorinated and dichlorinated biphenyls, and most cometabolize more highly chlorinated biphenyls while using biphenyl as a growth substrate (1, 7, 11). In some cases, the presence of biphenyl as a potential growth substrate and inducer of PCB metabolism (14) is important for maintaining PCB biodegradation activity in soil (5, 17). However, adding biphenyl to soil to stimulate PCB degradation activity is problematic due to the low water solubility of biphenyl and its possible adverse health effects (1, 19). Biphenyl is rare in natural environments, and it is possible that other, more common compounds also induce genes encoding biphenyl-degrading enzymes, termed *bph* genes (23). Such inducers may be less toxic and more water soluble than biphenyl, so that they could be added to soil to stimulate PCB degradation activity in bioremediation projects.

Several studies have investigated induction of PCB removal in cell suspensions of PCB-degrading bacteria by compounds other than biphenyl. Notably, cell suspensions of *Arthrobacter* sp. strain B1B grown on fructose medium supplemented with L-carvone, limonene, *p*-cymene, or isoprene remove Aroclor 1242 (27). *Alcaligenes eutrophus* H850 and *Corynebacterium* sp. strain MB1 grown on plant phenolic compounds and *Pseudomonas* sp. strain LB400 (10) (now a member of the genus *Burkholderia* [47]) grown on plant phenolic compounds, glucose, or glycerol degrade certain PCB congeners (9, 15). Also, other workers have amplified mRNA transcripts of 2,3-dihydroxybiphenyl dioxygenase (*bphC*) in *A. eutrophus* H850 grown on fructose plus L-carvone; however, these transcripts were not quantified to determine if there is a significant difference between the levels of *bphC* mRNA in cells grown on fructose alone and the levels of *bphC* mRNA in cells grown with carvone (38). Finally, *Cellulomonas* sp. strain T109 and *Rhodococcus rhodochrous* T100 grown on cymene and limonene, respectively, remove over 80% more Aroclor 1242 than these organisms grown on glucose (28). These studies support the hypothesis that certain compounds other than biphenyl may be used to stimulate PCB biodegradation. However, investigations so far have not shown that bacteria grown on substrates other than biphenyl remove PCBs as a result of induction of *bph* genes at levels above constitutive levels. Moreover, it is possible that the compounds used to induce bacterial PCB degradation activity did not induce *bph* genes but instead induced genes that encode other enzymes that also degrade PCBs or stimulated PCB degradation via mechanisms other than genetic regulation.

To determine if compounds other than biphenyl induce *bph* genes (Fig. 1), we constructed a chromosomal *bphA-lacZ* re-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, B.C., Canada V6T 1Z3. Phone: (604) 822-4285. Fax: (604) 822-6041. E-mail: wmohn@interchange.ubc.ca.

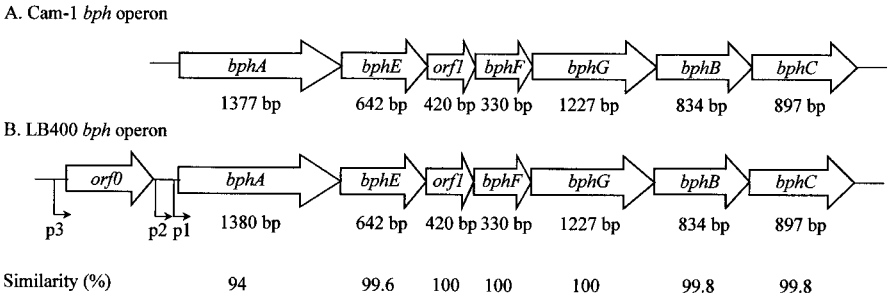

FIG. 1. Organization and similarity of the *bph* gene clusters in *Pseudomonas* sp. strain Cam-1 (A) and *Burkholderia* sp. strain LB400 (B). *bphA*, gene encoding the terminal dioxygenase large subunit; *bphE*, gene encoding the terminal dioxygenase small subunit; *bphF*, gene encoding ferredoxin; *bphG*, gene encoding ferredoxin reductase; *bphB*, gene encoding dihydrodiol dehydrogenase; *bphC*, gene encoding 2,3-dihydroxybiphenyl dioxygenase. In the LB400 operon the locations of promoter regions are indicated by p1, p2, and p3.

porter in the psychrotolerant PCB-degrading bacterium *Pseudomonas* sp. strain Cam-1 (34) to generate strain Cam-10. We also constructed a chromosomal *bphA-lacZ* reporter in the mesophilic PCB-degrading bacterium *Burkholderia* sp. strain LB400 to generate strain LB400-1. Construction of Cam-10 and LB400-1 allowed us to study the regulation of *bph* genes in a chromosomal context. We incubated Cam-10 and LB400-1 with compounds that previously have been shown to stimulate PCB degradation in other bacteria or that are structurally similar to biphenyl. Then we performed beta-galactosidase assays to determine if the *lacZ* reporter gene was induced. Induction of beta-galactosidase activity was correlated to induction of *bphA*. Our results suggest that regulation of *bphA* in Cam-1 is highly specific. In contrast, the beta-galactosidase activities were indistinguishable in LB400-1 cells incubated with biphenyl and LB400-1 cells incubated with pyruvate, suggesting that in LB400 *bphA* is expressed constitutively.

Few studies thus far have compared how different PCB-degrading bacteria regulate genes that encode enzymes involved in the biphenyl degradative pathway. We investigated induction of *bphA* in two PCB-degrading bacteria and found that *bph* genes in these organisms are regulated differently. This result has implications for PCB bioremediation strategies, as it suggests that the optimal methods for stimulating PCB degradation activity may depend on which PCB-degrading bacteria are present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, *Escherichia coli* was cultured at 37°C in Luria-Bertani (LB) medium, and *Pseudomonas* sp. strain Cam-1 and *Burkholderia* sp. strain LB400 were grown at 15 or 30°C in tryptic soy broth or mineral medium (6) containing 1.3 or 9 mM pyruvate as the growth substrate.

Chemicals. Biphenyl (99%), (±)-camphor (96%), (s)-(+)-carvone (96%), beta-citronellol (95%), cumene (99%), *p*-cymene (99%), anthracene (99%), benzoate (99%), fluorene (99%), naphthalene (99%), 2-methylnaphthalene (97%), 1,4-dimethylnaphthalene (95%), and phenanthrene (99.5%) were obtained from Aldrich Chemical Co. (+)-Limonene (97%), linoleic acid (60%), myricetin (85%), naringenin (95%), (+)-(α)-pinene (99%), salicylic acid (99%), and *o*-nitrophenyl-beta-D-galactopyranoside were obtained from Sigma. Benzene (99.9%) and toluene (99.8%) were obtained from Fisher Scientific. 2-Chlorobi-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
Pseudomonas sp. strain Cam-1	Wild type	34
Pseudomonas sp. strain Cam-10	*bphA-lacZ*-Gm^r	This study
Pseudomonas sp. strain Cam-20	*bphA-xylE*-Gm^r	This study
Burkholderia sp. strain LB400	Wild type	10
Burkholderia sp. strain LB400-1	*bphA-lacZ*-Gm^r	This study
Escherichia coli XL1-Blue MR	Δ(*mcrA)183* Δ(*mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
Escherichia coli DH5α	*endA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-argF*) *U169 deoR*[ϕ80*dlac (lacZ)M15*]	Gibco BRL
Escherichia coli S17-1	*recA pro thi hsdR* with integrated RP4-2-tc::Mu-kan::Tn*7*; Tra$^+$ Tr^r Sm^r	44
Plasmids		
pUC19	Cloning vector; Amp^r	49
pT7-7	Cloning vector; T7 promoter; Amp^r	46
pT7-6a	*bphAEFG* from *Burkholderia* sp. strain LB400 inserted into multiple cloning site of pT7-6	30
pEM1	SuperCosl cosmid library clone containing *bphAEFGBC* gene cluster from Cam-1	This study
pEM10	*bphAEFGBC* containing 8-kb *Sac*I fragment from pEM1 inserted into *Sac*I site of pUC19	This study
pT7-7a	4-kb *Bam*HI fragment PCR amplified from pEM10 inserted into *Bam*HI site of pT7-7	This study
pEX100T	*sacB* conjugable plasmid for gene replacement; Amp^r	43
pX1918GT	Plasmid containing the *xylE*-gentamicin resistance cassette; Amp^r Gm^r	43
pUCGm	Plasmid containing the gentamicin resistance cassette; Amp^r Gm^r	42
pIND/lacZ	Plasmid containing *lacZ*, Amp^r, Neo^r	Invitrogen
pEM2	4-kb *Bam*HI fragment from pT7-7a inserted into *Sma*I site of pEX100T	This study
pEM20	877-bp *Xba*I fragment from pUCGm inserted into *Xba*I site of pIND/lacZ	This study
pEM21	4-kb *Pme*I fragment from pEM20 inserted into *Eco*RI site of pEM2	This study

phenyl (99%), 3-chlorobiphenyl (99%), 4-chlorobiphenyl (99%), 2,2′-dichlorobiphenyl (99%), 4,4′-dichlorobiphenyl (99%), and Aroclor 1242 (99%) were obtained from AccuStandard.

Cloning *bph* genes from strain Cam-1. Total genomic DNA was isolated from strain Cam-1 by using hexadecyltrimethylammonium bromide (4) and was partially digested with *Sau*3A. The partially digested DNA was size fractionated with a 10 to 40% linear sucrose gradient. DNA fragments approximately 20 kb long were cloned into SuperCos by following the instructions of the manufacturer (Stratagene). In vitro packaging of the recombinant molecules was performed with GigapackII Gold packaging extract (Stratagene), and packaging reactions were used to infect *E. coli* XL1-Blue MR. The resulting cosmid library was amplified and screened for production of 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (a yellow *meta*-cleavage product) from biphenyl (32). Removal of biphenyl by yellow colonies was confirmed by adding 25 mg of biphenyl per liter to cell suspensions of the clones and then extracting the remaining biphenyl with hexane after incubation and analyzing the extracts by gas chromatography-mass spectrometry (34). Cosmid pEM1 was obtained from constructs that transformed biphenyl. Restriction fragments of cosmid pEM1 were separated on an agarose gel, transferred to a maximum-strength Nytran Plus nylon membrane (S&S Nytran Plus), and then hybridized with ^{32}P-labeled *bphA, bphF*, and *bphG* from pT7-6a (Table 1). A nick translation system from Life Technologies was used to label *bphA* and *bphG* with [α-^{32}P]dCTP. An Oligolabelling Kit from Pharmacia Biotech (Uppsala, Sweden) was used to label *bphF* with [α-^{32}P]dCTP. A *Sac*I restriction fragment that hybridized to all three probes was subcloned into pUC19, giving pEM10. The sequence of the cloned DNA from Cam-1 was obtained by generating successive unidirectional deletions of pEM10 with the double-stranded nested-deletion system from Pharmacia Biotech. Oligonucleotide primers synthesized at the Nucleic Acid and Protein Services Unit of the University of British Columbia were used to sequence DNA regions not covered by the deletions. DNA sequences were determined by the Nucleic Acid and Protein Services Unit by using AmpliTaq FS dyedeoxy terminator cycle sequencing chemistry (Applied Biosystems) and Centri-Sep columns (Princeton Separation, Adelphia, N.J.) to purify the extension products. ClustalX was used to align the cloned Cam-1 DNA sequence with the *bph* operon sequence from LB400 (Fig. 1). Vent polymerase (New England Biolabs) and PCR primers with 5′ extensions containing *Bam*HI recognition sites were used to subclone *bphAEFG* from pEM10 into pT7-7, which yielded pT7-7a.

Insertion of a *lacZ*-Gm^r cassette into the *bph* operon. The pEX100T gene replacement vector (42, 43) was used to insert a selectable *lacZ* reporter gene cassette between the *bphA* and *bphE* genes in Cam-1 and LB400 (Fig. 2). Plasmids pEM2, pEM20, and pEM21 were selected in *E. coli* DH5α grown on LB medium containing appropriate antibiotics and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmid pEM21 was transformed into the mobilizer strain *E. coli* S17-1 and conjugally transferred into Cam-1 and LB400 (24). Cam-1 and LB400 grow on pyruvate; however, *E. coli* S17-1 does not. Therefore, exconjugants were plated on minimal medium containing 9 mM pyruvate and 10 μg of gentamicin per ml, and colonies which appeared on this medium after 48 h of incubation at 30°C were streaked onto LB agar containing 10 μg of gentamicin per ml. Colonies of Cam-1 and LB400 in which double homologous recombination had occurred were selected on LB medium containing 10 μg of gentamicin per ml and 5% sucrose and were designated Cam-10 and LB400-1, respectively. Sucrose-resistant colonies were also sensitive to ampicillin, indicating that these colonies had lost the pEX100T vector-associated sequences. Gene insertions in Cam-10 and LB400-1 were also confirmed by performing colony PCR with 20-mer primers annealing to the 3′ region of *bphA* (5′-GACCTGGC AGAACAGCGACT) and the 5′ regions of *bphE* (5′-TCTGCACATGCACGT CCAGC-3′) and the *lacZ* reporter gene (5′-GTATCGCTCGCCACTTCAAC-3′) (50).

To verify that Cam-1 requires the *bph* genes for biphenyl degradation, we inserted the *xylE*-Gm^r cassette from pX1918GT between the chromosomal *bphA* and *bphE* genes in Cam-1 to form Cam-20. The *xylE*-Gm^r cassette contains a transcriptional termination sequence downstream of the gentamicin resistance gene. Consequently, transcription of genes downstream of the cassette is inhibited. The method used to generate Cam-20 was similar to that used to generate Cam-10, except that pEM20 was replaced by pX1918GT and the *xylE*-Gm^r cassette was ligated as an *Eco*RI fragment to the *Eco*RI site in pEM2. The resulting plasmid was transformed into the mobilizer strain *E. coli* S17-1 and conjugally transferred into Cam-1 (24). Exconjugants were selected as described above. Gene insertions in Cam-20 were confirmed by performing colony PCR with primers annealing to the 3′ region of *bphA* (5′-GCCGGCACAACATCC) and the 5′ region of *bphB* (5′-CCAGCTCTGCAAGGCGC-3′) (50).

Beta-galactosidase assays. Unless otherwise specified, Cam-10 and LB400-1 were grown at 30°C on 9 mM pyruvate in the presence of 10 μg of gentamicin per ml to the mid-log phase and then cooled on ice for 15 min. Cultures were centrifuged at 5,000 $\times$ *g* for 15 min at 4°C and washed with mineral buffer. Washed cells were suspended in mineral medium with 1 mM pyruvate and adjusted to a final optical density at 600 nm of 0.6. Cell suspensions (20 ml) were prepared in 125-ml Erlenmeyer flasks and then were inoculated with potential inducers of the *bphA* gene at concentrations of 0.001 to 1 mM. Unless otherwise specified, triplicate cell suspensions were incubated with potential inducers for 3 h at 30°C on a rotary shaker at 200 rpm. Beta-galactosidase assays were performed as described by Miller (35). Precise volumes of chloroform (20 μl) and 0.1% sodium dodecyl sulfate (10 μl) were used to permeabilize cells (25). Test samples without *o*-nitrophenyl-β-D-galactopyranoside were used as negative controls. Protein concentrations of cell suspensions were determined by a bicinchoninic acid protein assay (4).

Biphenyl removal by Cam-10 and LB400-1. Cell suspensions of Cam-10 and LB400-1 were prepared as described above, and then 2.5-ml aliquots were transferred to Teflon-lined screw-cap tubes. Duplicate cell preparations were inoculated with 0.1 mM biphenyl and then incubated on a tube roller at 30°C for 3 or 6 h. Boiled cells and mineral medium containing 0.1 mM biphenyl were used as negative controls. The remaining biphenyl was extracted from cell suspensions with hexane, and extracts were analyzed by gas chromatography as described previously (34).

Nucleotide sequence accession number. The Cam-1 nucleotide sequence determined in this study has been deposited in the GenBank database under accession no. AY027651.

RESULTS AND DISCUSSION

Optimization of *lacZ* reporter gene expression in Cam-10 and LB400-1. Maximum expression of the *lacZ* reporter gene in *Pseudomonas* sp. strain Cam-10 was observed after 3 h of incubation with 1 mM biphenyl (Fig. 3A). Other compounds that were studied to determine their abilities to induce beta-galactosidase activity in Cam-10 were tested under these conditions. Expression of the *lacZ* reporter gene in Cam-10 did not increase as the amount of biphenyl was increased above 1 mM. At concentrations of biphenyl less than 0.1 mM, beta-galactosidase activity was consistently higher when 1 mM pyruvate was also supplied. Pyruvate may provide cells with energy that allows greater beta-galactosidase production. Thus, unless otherwise stated, 1 mM pyruvate was added to all subsequent preparations in which potential inducers of *bphA* were tested.

At each concentration of biphenyl tested, the beta-galactosidase specific activity of Cam-10 initially increased with time and then decreased (Fig. 3A). This result suggested that biphenyl was depleted by Cam-10, thereby diminishing the concentration of the inducer. The utilization of biphenyl by Cam-10 was not surprising since the *lacZ*-Gm^r cassette did not contain a transcription termination sequence and was inserted between the *bphA* and *bphE* genes in Cam-1 without disrupting either gene. To verify that Cam-10 transformed biphenyl, 0.1 mM biphenyl was added to cell suspensions of pyruvate-grown Cam-10, and then the cell suspensions were incubated at 30°C. After 3 and 6 h of incubation, 30 and 100% of the biphenyl added to cell suspensions of Cam-10 was removed, respectively. Biphenyl was not removed by killed cells or from medium without cells. Cam-10 also grew on 1 mM biphenyl. Biphenyl degradation by Cam-10 requires the *bph* gene products, since insertion of the transcription termination sequence containing the *xylE*-Gm^r cassette from pX1918GT between *bphA* and *bphE* resulted in cells unable to grow on biphenyl. Thus, there do not appear to be any additional enzyme systems in Cam-1 catalyzing biphenyl degradation.

The observed decrease in beta-galactosidase activity in Cam-10 upon biphenyl depletion is consistent with observations by

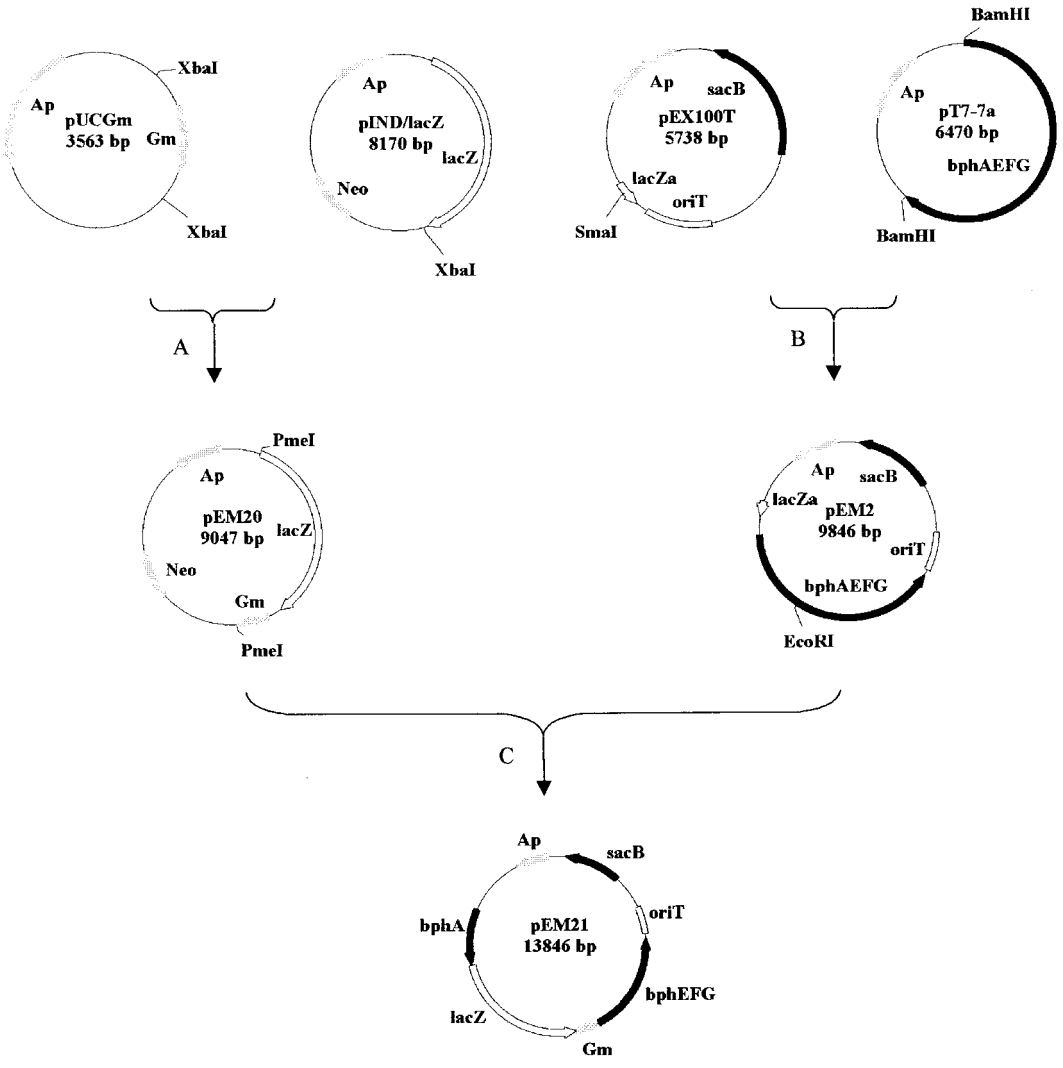

FIG. 2. Construction of a selectable *lacZ* reporter cassette and pEM21. Step A was construction of pEM20. The 877-bp *Xba*I fragment from pUCGm was gel purified and ligated into the *Xba*I site of pIND/lacZ. Restriction digests obtained with *Eco*RV were used to isolate plasmids containing *lacZ* and the gene encoding gentamicin acetyltransferase 3-1 in the same transcriptional orientation. Step B was construction of pEM2. The 4-kb *Bam*HI fragment from pT7-7a was treated with the large fragment of DNA polymerase I (PolK) and then ligated into the *Sma*I site of pEX100T. Restriction digests obtained with *Sac*I and *Sac*II were used to isolate plasmids containing *lacZ*α and *bphAEFG* in the same transcriptional orientation. Step C was construction of pEM21. The 4-kb *Pme*I fragment of pEM20 was gel purified and ligated into the PolK-treated *Eco*RI site of pEM2. Colonies containing *bphAEFG* and *lacZ*-Gm^r in the same transcriptional orientation were detected by formation of a blue color when the organisms were grown on LB medium supplemented with gentamicin, ampicillin, and X-Gal. The locations of restriction sites and genes and their transcriptional orientations are shown. Ap, β-lactamase-encoding gene; Gm, gentamicin acetyltransferase 3-1-encoding gene; Neo, neomycin resistance gene; oriT, origin of transfer.

other workers suggesting that repeated addition of biphenyl to soil microcosms is necessary for PCB biodegradation (5). The decrease in induction of *bphA* with biphenyl depletion may also explain why pure cultures of certain PCB-degrading bacteria remove more PCBs when cells are growing on biphenyl than when resting cells are used (33). Interestingly, although the solubility of biphenyl is approximately 0.044 mM (19), greater induction of beta-galactosidase activity was consistently observed in cell suspensions of Cam-10 containing 1 mM biphenyl than in cell suspensions containing 0.33 mM biphenyl (Fig. 3A). This result suggests that bacteria may use biphenyl via direct contact with the crystals instead of, or in addition to, via uptake of dissolved biphenyl.

In contrast to induction of beta-galactosidase activity in Cam-10, the level of beta-galactosidase activity in *Burkholderia* sp. strain LB400-1 did not depend on the presence of biphenyl (Fig. 3B). This suggests that regulation of the *bphA* gene in LB400 is constitutive. A gradual increase in beta-galactosidase specific activity over time was consistently observed. This result may reflect recovery from a decrease in beta-galactosidase activity during harvesting and preparation of cell suspensions of LB400-1. Like Cam-10, LB400-1 completely transformed 0.1 mM biphenyl after 6 h. Biphenyl degradation by LB400-1 is believed to require the *bph* gene products, since many attempts by other workers to find more than one biphenyl dioxygenase in LB400 have not been successful (26).

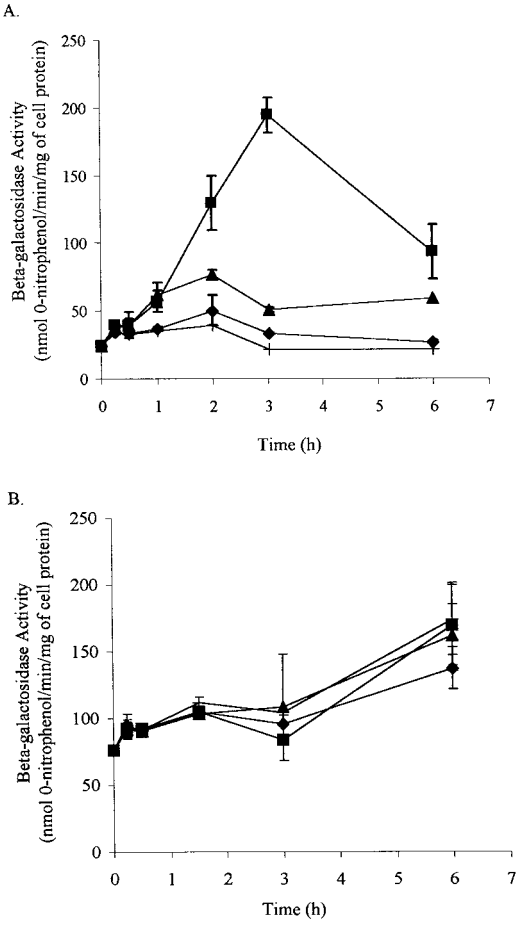

FIG. 3. Induction of beta-galactosidase activity at 30°C in Cam-10 (A) and LB400-1 (B). The error bars indicate standard deviations (n = 3). The treatments consisted of 1 mM pyruvate alone (+), 1 mM pyruvate plus 0.01 mM biphenyl (◆), 1 mM pyruvate plus 0.33 mM biphenyl, (▲), and 1 mM pyruvate plus 1 mM biphenyl (■).

Generally, PCB-degrading bacteria are prepared for bioaugmentation of PCB-contaminated soil by growing the bacteria on biphenyl. The rates of growth and the final cell densities of bacteria are often lower when the organisms are grown on biphenyl than when they are grown on certain alternative substrates, such as pyruvate. Our results demonstrate that a PCB-degrading bacterium can be grown on pyruvate (or a cheaper substrate) quickly and to high optical densities and then induced within hours to remove biphenyl. Thus, it is possible that this method can be used to prepare bacterial inocula for bioremediation of PCB-contaminated soil, particularly in cases where the bacterial inoculum is defined and where catabolic genes are located on chromosomes rather than on plasmids which can be lost during growth on substrates other than biphenyl (20, 29). However, it may be important to determine the effect of biphenyl on other physiological parameters, such as membrane composition, and to determine how these parameters affect PCB biodegradation.

Inducers of beta-galactosidase activity in Cam-10. Biphenyl induced beta-galactosidase activity in Cam-10 to a level approximately six times greater than the basal level of expression in cells grown with pyruvate (Fig. 4A). At a concentration of 1 mM, 2-chlorobiphenyl, 4-chlorobiphenyl, salicylate, and naphthalene induced beta-galactosidase activity to levels greater than the basal levels. Thus, these compounds appear to be inducers of *bphA* in Cam-1. However, none of them appears to be as strong an inducer as biphenyl, as none of them induced beta-galactosidase to the same level of activity in Cam-10 as biphenyl did. At noninhibitory concentrations (Table 2), none of the other potential inducers tested induced beta-galactosidase activity to levels greater than the basal levels in Cam-10. The levels of beta-galactosidase activity after exposure to benzene, carvone, 3-chlorobiphenyl, and pyruvate (Fig. 4A) were typical of those observed after exposure to other noninducing aromatic compounds, terpenoids, chlorobiphenyls, sugars, alcohols, and organic acids (data not shown) (the compounds tested are listed in Table 2).

Since Cam-10 grew with naphthalene and since salicylate is a metabolite of naphthalene degradation, it is possible that the observed induction by naphthalene of beta-galactosidase activ-

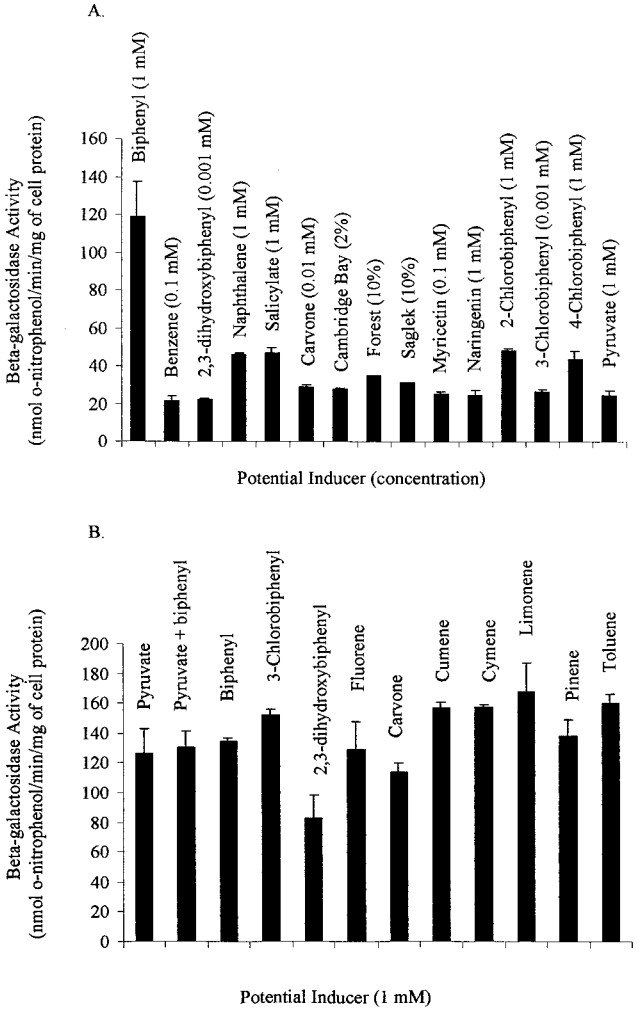

FIG. 4. Induction of beta-galactosidase activity for 3 h at 30°C in Cam-10 (A) and LB400-1 (B). The error bars indicate standard deviations (n = 3). The concentrations of potential inducers used to test induction of beta-galactosidase activity in Cam-10 are indicated in parentheses. All preparations were supplemented with 1 mM pyruvate.

TABLE 2. Percentages of inhibition of beta-galactosidase activity in Cam-10 by potential inducers at various concentrations[a]

Potential inducer	% Inhibition at the following concn:			
	1 mM	0.1 mM	0.01 mM	0.001 mM
Aromatic compounds				
Benzene	98	0	—[b]	—
Toluene	97	70	5	—
Benzoate	51	0	—	—
Catechol	100	64	15	0
2,3-Dihydroxybiphenyl	100	70	52	2
Acenaphthalene	50	19	0	—
Fluorene	100	69	68	10
Dioxin	100	100	85	43
2-Methylnaphthalene	100	58	0	—
1,4-Dimethylnaphthalene	100	67	0	—
Anthracene (crystals)	41	0	—	—
Phenanthrene	100	37	0	—
Terpenoids				
Camphor	100	55	10	—
(s)-(+)-Carvone	99	76	0	—
beta-Citronellol	95	70	5	—
Cumene	99	0	0	—
p-Cymene	100	56	8	—
Dehydroabietic acid	45	0		—
(+)-Limonene	97	33	24	—
Linoleic acid	81	0	—	—
Pinene	88	49	0	—
Soil extract and flavenoids				
Cambridge Bay soil (10%, 2%)	100	30	—	—
Forest soil (10%)	52	0	—	—
Saglek soil (10%)	55	0	—	—
Myricetin	90	90	—	—
Chlorinated biphenyls				
3-Chlorobiphenyl	100	100	32	0
2,2′-Dichlorobiphenyl	—	20	—	—
4,4′-Dichlorobiphenyl	—	0	—	—
Aroclor 1242 (100 ppm)	60	23	—	—

[a] Inhibition of beta-galactosidase activity in Cam-10 by potential inducers was determined by incubating cells with 1 mM pyruvate plus 1 mM biphenyl plus the potential inducer and then determining beta-galactosidase activity. Percentages of inhibition were determined by comparing the beta-galactosidase activities in cell suspensions containing pyruvate and biphenyl without a potential inducer to the activities in cell suspensions containing pyruvate and biphenyl plus the potential inducers.

[b] —, Not measured.

ity in Cam-10 was due to salicylate or its catabolites (41). Induction of beta-galactosidase activity by salicylate is consistent with the observation that certain *Pseudomonas* species readily oxidize biphenyl when they are grown on salicylate and readily oxidize salicylate when they are grown on biphenyl (21).

Other workers have proposed that naphthalene could be used as a growth substrate for PCB-degrading bacteria (40). Naphthalene is a natural component of soil and has been used in solvents and motor oils. Consequently, naphthalene frequently occurs as a co-contaminant at PCB-contaminated sites (31). Pellizari et al. (40) found that bacteria isolated on biphenyl remove more PCBs than bacteria isolated on naphthalene. In these experiments, PCB removal was assayed with resting cells of bacteria grown on the substrate used for isolation. Our results are consistent with the findings of Pellizari et al. (40) since naphthalene induced *bphA* in Cam-1, although at lower levels than biphenyl did. As has been proposed previously (40), the stimulatory effect of naphthalene on PCB degradation may be sufficient for PCB bioremediation in cases where extensive initial dechlorination has occurred.

Inducers of beta-galactosidase activity in LB400-1. In contrast to the results obtained with Cam-10, the beta-galactosidase activities in cell suspensions of LB400-1 containing 1 mM biphenyl, carvone, cumene, cymene, pinene, limonene, fluorene, 3-chlorobiphenyl, or toluene were similar to the beta-galactosidase activity observed in cell suspensions containing only pyruvate (Fig. 4B). Interestingly, 1 mM 2,3-dihydroxybiphenyl had a slight inhibitory effect on induction of beta-galactosidase activity in LB400-1 (Fig. 4B). These results are consistent with S1 nuclease mapping studies of *bph* genes in LB400 done by other workers which identified three transcriptional initiation sites (16). Activation from the promoter region furthest upstream from the biphenyl dioxygenase translation start site (p3) is dependent on biphenyl. However, activation from the two proximal promoter regions (p2 and p1) is constitutive (16).

Despite constitutive expression of the *bph* genes in LB400 from p1 and p2, Mondello (36) showed that LB400 grown on biphenyl is able to degrade di-*para*-substituted PCBs and tetra- and pentachlorobiphenyls more effectively than LB400 grown on succinate or on biphenyl plus succinate. However, Brazil et al. (12) observed that expression of *bphC*, which is located downstream of p1, p2, and *bphA* and encodes 2,3-dihydroxybiphenyl 1,2-dioxygenase, was similar in LB400 grown on mineral medium supplemented with succinate and in LB400 grown in mineral medium supplemented with biphenyl. To examine the effect of pyruvate plus biphenyl on *bphA* gene induction in LB400, we compared the beta-galactosidase activities in cell suspensions of LB400-1 containing pyruvate alone, pyruvate plus biphenyl, and biphenyl alone. Similar levels of beta-galactosidase activity were detected for all treatments (Fig. 4B). These results suggest that *bphA* and *bphC* are coordinately and constitutively expressed. Constitutive expression of genes encoding the initial enzymes for biphenyl degradation by LB400 may explain why LB400 grown on glucose, glycerol (9), or terpenoid compounds (15) removes PCBs. It would be interesting to determine if regulation of the *bph* genes in other organisms that have been shown to be induced for PCB removal when they are grown on substrates other than biphenyl (9, 15, 27) is constitutive.

The activation of p3 by biphenyl in LB400 is correlated with increased efficiency of degradation of certain PCBs (36). Transcriptional activation from p3 results in transcription of *orf0* (16). We verified the presence of *orf0* in LB400 by PCR. The translation product of *orf0* is 58% similar to BphS, a GntR-like negative regulator of *bph* genes in *Ralstonia eutropha* A5 (37). Recently, other investigators found that in the PCB degrader *Pseudomonas pseudoalcaligenes* KF707 the translation product of *orf0* is autoregulated and is necessary for expression of genes encoding enzymes in the biphenyl degradation pathway downstream of *bphC* (48). Induction of genes downstream of *bphC* allows cells to grow on biphenyl and minimizes the accumulation of metabolites resulting from biphenyl and PCB catabolism. Decreased accumulation of metabolites from PCB transformation may explain why LB400 grown on biphenyl degrades di-*para*-substituted PCBs and tetra- and pentachlorobiphenyls more effectively than LB400 grown on other substrates (36). Also, other physiological effects of growth on biphenyl, such as changes in membrane composition, may be necessary for transformation of certain PCB congeners.

Inhibition effects of potential inducers. At a concentration of 1 mM, most of the potential inducers tested actually inhibited induction by biphenyl of beta-galactosidase activity in Cam-10 (Table 2). The exceptions were compounds previously found to be inducers, naphthalene, salicylate, 2-chlorobiphenyl, and 4-chlorobiphenyl, as well as naringenin, fructose, glucose, and glycerol. With the exception of benzoate, acenaphthalene, fluorene, dioxin, anthracene, 3-chlorobiphenyl, 2-methylnaphthalene, and dimethylnaphthalene, compounds that inhibited induction also inhibited cell growth. At concentrations less than 0.1 mM, none of the potential inducers were inhibitory to cell growth, yet at such concentrations several of these compounds substantially inhibited induction of beta-galactosidase. Clearly, in complex environments, inhibitory effects such as those found here can be expected to modulate expression of genes essential for PCB biodegradation. The inhibitory effect of soil extracts (Table 2) is consistent with this expectation.

Metabolites of several potential inducers may have had a role in inhibition of beta-galactosidase induction in Cam-10. Transformation of 3-chlorobiphenyl by Cam-10 to 3-chlorocatechol was apparent from the formation of black catecholic polymers in cell suspensions (13). Since 3-chlorocatechol is a potent inhibitor of 2,3-dihydroxybiphenyl 1,2-dioxygenase (18, 45), inhibition of beta-galactosidase induction in Cam-10 by 3-chlorobiphenyl may result from negative regulation by accumulated metabolites. Cam-10 rapidly transformed 2,3-dihydroxybiphenyl to the *meta*-cleavage product, as indicated by production of a bright yellow metabolite. Thus, inhibition of beta-galactosidase induction in Cam-10 by 2,3-dihydroxybiphenyl may also result from negative regulation by accumulated metabolites. Fluorene, catechol, dioxin, and 2-methylnaphthalene were also transformed by Cam-10, as indicated by the production of colored metabolites. Interestingly, the compounds that were transformed by Cam-10 include the most potent inhibitors of beta-galactosidase induction (Table 2). Detailed biochemical studies will be necessary to determine if inhibition of *bphA* induction by particular compounds involves negative genetic regulation.

Temperature dependence of *bphA* induction in Cam-1 and LB400. Cam-1 was isolated from PCB-contaminated arctic soil and was studied to determine the feasibility of bioremediating PCB-contaminated arctic soil with indigenous soil bacteria. We found that at 7°C Cam-1 removed PCBs at higher rates than LB400 removed PCBs (34). To investigate the role of *bphA* induction in the efficiency of PCB removal at low temperatures, we compared the beta-galactosidase activities in cell suspensions of Cam-10 and LB400-1 incubated at 7°C with pyruvate or biphenyl plus pyruvate. Cell suspensions of Cam-10 were prepared by using cells grown on pyruvate at 7°C. Since LB400 does not grow at 7°C (34), cell suspensions of LB400-1 were prepared by using cells grown on pyruvate at 15°C. Samples of the cell suspensions were obtained at several time points over 24 h and transferred to 28°C to measure beta-galactosidase activity.

After 24 h, the beta-galactosidase activity of Cam-10 cells incubated at 7°C with biphenyl was four times greater than that of cells incubated at 7°C with pyruvate (Table 3). Thus, *bphA* appears to be induced by biphenyl in Cam-1 at 7°C, which is consistent with Cam-1 being cold adapted. Interestingly, the initial beta-galactosidase activity was significantly less in LB400-1 cells grown at 15°C than in LB400-1 cells grown at 30°C (Table 3). As observed at 30°C, biphenyl did not induce beta-galactosidase activity in cell suspensions of LB400-1 at 7°C. These results further support the conclusion that *bphA* expression in LB400 is constitutive and indicate that the level of constitutive expression is temperature dependent.

TABLE 3. Effect of temperature on induction of beta-galactosidase activities in Cam-10 grown at 7°C on pyruvate and in LB400-1 grown at 15°C on pyruvate

Strain	Time	Beta-galactosidase activity (nmol of *o*-nitrophenol/min/mg of cell protein)[a]	
		7°C	30°C
Cam-10	Initial[b]	18.0 ± 2.6	24.0 ± 0.6
	Final	74.0 ± 8.3[c]	118.6 ± 18.5[d]
LB400-1	Initial[b]	15.5 ± 4.0	76.3 ± 4.5
	Final	16.8 ± 1.0[c]	83.9 ± 0.7[d]

[a] Mean ± standard deviation ($n = 3$).
[b] Activity at zero time.
[c] Activity at 24 h.
[d] Activity at 36 h.

Our research shows that regulation of the *bphA* gene is remarkably different in two PCB-degrading bacteria. The *bphA* gene in Cam-1 is inducible at 7 and 30°C, and induction is greatest with biphenyl. In contrast, expression of *bphA* in LB400 is constitutive and is lower at a lower temperature. These results indicate that available chemical inducers, as well as physical environmental conditions, can affect *bphA* expression in PCB-degrading bacteria. Consequently, knowledge of how physical and chemical environmental variables affect *bphA* induction in particular bacteria in a treatment system will be necessary to determine the optimal conditions for PCB bioremediation.

ACKNOWLEDGMENTS

We thank V. J. J. Martin and L. D. Eltis for helpful discussions and L. D. Eltis for providing *Burkholderia* strain LB400, pT7-7, and pT7-6a.

This research was supported by the Natural Science and Engineering Research Council of Canada, the Canadian Department of National Defense, and a Natural Science and Engineering Research Council graduate scholarship to E.R.M.

REFERENCES

1. **Abramowicz, D. A.** 1990. Aerobic and anaerobic biodegradation of PCBs: a review. Crit. Rev. Biotechnol. **10:**241–251.
2. **Ahmad, D., R. Masse, and M. Sylvestre.** 1990. Cloning and expression of genes involved in 4-chlorobiphenyl transformation by *Pseudomonas testosteroni*: homology to polychlorobiphenyl-degrading genes in other bacteria. Gene **86:**53–61.
3. **Ahmed, M., and D. D. Focht.** 1972. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19:**47–52.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology, 2nd ed. Greene Publishing Associates and John Wiley & Sons, New York, N.Y.
5. **Barriault, D., and M. Sylvestre.** 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. Can. J. Microbiol. **39:**594–602.
6. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.
7. **Bedard, D. L., and M. L. Haberl.** 1990. Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. **20:**87–102.
8. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown, Jr.** 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094–1102.

9. **Billingsley, K. A., S. M. Backus, C. Juneson, and O. P. Ward.** 1997. Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can. J. Microbiol. **43:**1172–1179.
10. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.
11. **Boyle, A. W., C. J. Silvin, J. P. Hassett, J. P. Nakas, and S. W. Tanenbaum.** 1992. Bacterial PCB biodegradation. Biodegradation **3:**285–298.
12. **Brazil, G. M., L. Kenefick, M. Callanan, A. Haro, V. de Lorenzo, D. N. Dowling, and F. O'Gara.** 1995. Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. Appl. Environ. Microbiol. **61:**1946–1952.
13. **Brenner, V., J. J. Arensdorf, and D. D. Focht.** 1994. Genetic construction of PCB degraders. Biodegradation **5:**359–377.
14. **Brunner, W., F. H. Sutherland, and D. D. Focht.** 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. J. Environ. Qual. **14:**324–328.
15. **Donnelly, P. K., R. S. Hedge, and J. S. Fletcher.** 1994. Growth of PCB-degrading bacteria on compounds from photosynthetic plants. Chemosphere **28:**981–988.
16. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
17. **Focht, D. D., and W. Brunner.** 1985. Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. Appl. Environ. Microbiol. **50:**1058–1063.
18. **Focht, D. D.** 1995. Strategies for the improvement of aerobic metabolism of polychlorinated biphenyls. Curr. Biol. **6:**341–346.
19. **Foreman, W. T., and T. F. Bidleman.** 1985. Vapor pressure estimates of individual polychlorinated biphenyls and commercial fluids using gas chromatographic retention data. J. Chromatogr. **330:**203–216.
20. **Furukawa, K., and A. M. Chakrabarty.** 1982. Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. **44:** 619–626.
21. **Furukawa, K., J. R. Simon, and A. M. Chakrabarty.** 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154:**1356–1362.
22. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166:**392–398.
23. **Furukawa, K.** 1994. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5:**289–300.
24. **Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg.** 1994. Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
25. **Giacomini, A., V. Corich, F. J. Ollero, A. Squartini, and M. P. Nuti.** 1992. Experimental conditions may affect reproducibility of the beta-galactosidase assay. FEMS Microbiol. Lett. **100:**87–90.
26. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561–4564.
27. **Gilbert, E. S., and D. E. Crowley.** 1997. Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B. Appl. Environ. Microbiol. **63:**1933–1938.
28. **Hernandez, B. B., S.-C. Koh, M. Chial, and D. D. Focht.** 1997. Terpene-utilizing isolates and their relevance to enhance biotransformation of polychlorinated biphenyls in soil. Biodegradation **8:**153–158.
29. **Higson, F. K.** 1992. Microbial degradation of biphenyls and its derivatives. Adv. Appl. Microbiol. **37:**135–164.
30. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene **130:**47–55.
31. **Jones, K. C., J. A. Stratford, K. S. Waterhouse, and N. B. Vogt.** 1989. Organic contaminants in Welsh soils: polynuclear aromatic hydrocarbons. Environ. Sci. Technol. **5:**540–550.
32. **Kiyohara, H., K. Nagao, and K. Yana.** 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. **43:**454–457.
33. **Kohler, H.-P. E., D. Kohler-Staub, and D. D. Focht.** 1988. Cometabolism of polychlorinated biphenyls: enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl. Environ. Microbiol. **54:**1940–1945.
34. **Master, E. R., and W. W. Mohn.** 1998. Psychrotolerant bacteria isolated from arctic soil that degrade polychlorinated biphenyls at low temperature. Appl. Environ. Microbiol. **64:**4823–4829.
35. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725–1732.
37. **Mouz, S., C. Merlin, D. Spingael, and A. Toussaint.** 1999. A GntR-like negative regulator of the biphenyl degradation genes of the transposon Tn4371. Mol. Gen. Genet. **262:**790–799.
38. **Park, Y.-I, J.-S. So, and S.-C. Koh.** 1999. Induction by carvone of the polychlorinated biphenyl (PCB)-degradative pathway in *Alcaligenes eutrophus* H850 and its molecular monitoring. J. Microbiol. Biotechnol. **9:**804–810.
39. **Parsons, J. R., and D. T. H. M. Sijm.** 1988. Biodegradation kinetics of polychlorinated biphenyls in continuous cultures of *Pseudomonas* strain. Chemosphere **17:**1755–1766.
40. **Pellizari, V. H., S. Bezborodnikov, J. F. Quensen III, and J. M. Tiedje.** 1996. Evaluation of strains isolated by growth on naphthalene and biphenyl for hybridization of genes to dioxygenase probes and polychlorinated biphenyl-degrading ability. Appl. Environ. Microbiol. **62:**2053–2058.
41. **Schell, M. A.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. Gene **36:**301–309.
42. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance cassette for site specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
43. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:** 15–22.
44. **Simons, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–790.
45. **Sondossi, M., M. Sylvestre, and D. Ahmad.** 1992. Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl. Environ. Microbiol. **58:**485–495.
46. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
47. **Viallard, V., I. Poirier, B. Cournoyer, J. Haurat, S. Wiebkin, K. Ophel-Keller, and J. Balandreau.** 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of *Pseudomonas phenazinium, Pseudomonas pyrrocinia* and *Pseudomonas glathei* as *Burkholderia*. Int. J. Syst. Bacteriol. **48:**549–563.
48. **Watanabe, T., R. Inoue, N. Kimura, and K. Furukawa.** 2000. Versatile transcription of biphenyl catabolic *bph* operon in *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **275:**31016–31023
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
50. **Zon, L. I., D. M. Dorfman, and S. H. Orkin.** 1989. The polymerase chain reaction colony miniprep. BioTechniques **7:**696–698.