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Construction of a Rhizosphere Pseudomonad with Potential To Degrade Polychlorinated Biphenyls and Detection of *bph* Gene Expression in the Rhizosphere

G. M. BRAZIL,[1] L. KENEFICK,[1] M. CALLANAN,[1] A. HARO,[2] V. DE LORENZO,[2] D. N. DOWLING,[1] AND F. O'GARA[1]*

Department of Microbiology, University College, Cork, Ireland,[1] and Centro de Investigaciones Cientificas del Consejo Superior de Investigaciones Cientificas, 28006 Madrid, Spain[2]

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The genetically engineered transposon Tn*PCB*, contains genes (*bph*) encoding the biphenyl degradative pathway. Tn*PCB* was stably inserted into the chromosome of two different rhizosphere pseudomonads. One genetically modified strain, *Pseudomonas fluorescens* F113pcb, was characterized in detail and found to be unaltered in important parameters such as growth rate and production of secondary metabolites. The expression of the heterologous *bph* genes in F113pcb was confirmed by the ability of the genetically modified microorganism to utilize biphenyl as a sole carbon source. The introduced trait remained stable in laboratory experiments, and no *bph*-negative isolates were found after extensive subculture in nonselective media. The *bph* trait was also stable in nonselective rhizosphere microcosms. Rhizosphere competence of the modified F113pcb was assessed in colonization experiments in nonsterile soil microcosms on sugar beet seedling roots. F113pcb was able to colonize as efficiently as a marked wild-type strain, and no decrease in competitiveness was observed. In situ expression of the *bph* genes in F113pcb was found when F113pcb bearing a *bph'lacZ* reporter fusion was inoculated onto sugar beet seeds. This indicates that the *bph* gene products may also be present under in situ conditions. These experiments demonstrated that rhizosphere-adapted microbes can be genetically manipulated to metabolize novel compounds without affecting their ecological competence. Expression of the introduced genes can be detected in the rhizosphere, indicating considerable potential for the manipulation of the rhizosphere as a self-sustaining biofilm for the bioremediation of pollutants in soil. Rhizosphere bacteria such as fluorescent *Pseudomonas* spp. are ecologically adapted to colonize and compete in the rhizosphere environment. Expanding the metabolic functions of such pseudomonads to degrade pollutants may prove to be a useful strategy for bioremediation.

The introduction of modified bacteria capable of in situ bioremediation into a competitive nonsterile environment requires that the microorganism be able to survive in high numbers and also express the desired catabolic phenotype. However, a general decline in introduced bacteria into such environments has been observed (1). To effect significant biodegradation of pollutants, introduced strains often need to be reinoculated to compete with the indigenous flora. Alternatively, positive selection is required to maintain the introduced bacteria in the soil (1, 15).

One ecosystem, the rhizosphere, looks promising as a site for bioremediation of contaminated soils. Unlike bulk (plant-free) soil, which may be considered an oligotrophic environment (24), the rhizosphere is rich in nutrients, including carbon, and as such provides an ecological niche for those microbes adapted to its unique environment. The persistence of recalcitrant compounds has been shown to decrease in the rhizosphere (14, 16), and the microbial degradation of a trichlorinated hydrocarbon, trichloroethylene, occurs faster in the rhizosphere (23) than in bulk soil. Expanding the degradative capabilities of rhizosphere-competent bacteria may be a useful strategy for generating strains for bioremediation purposes. Fluorescent pseudomonads which colonize the roots of plants can be suitable hosts for the introduction of heterologous genes into the rhizosphere ecosystem.

Bioremediation of polychlorinated biphenyl (PCB)-contaminated soils by inoculation of PCB-degrading bacteria has been suggested (19) as a useful approach to reduce the level of these compounds in soil. Bacteria capable of degrading PCBs have been isolated from a range of sites, and the pathways and encoding genes (*bph*) have been well studied. The genes encoding the degradative pathway are organized in an operon structure, $bphA_1$ to $bphA_4$, *bphB*, *bphC*, and *bphD*. Genes $bphA_1$ to $bphA_4$ encode a multicomponent dioxygenase enzyme complex that converts biphenyl (BP) to a dihydrodiol, which is transformed by the *bphB* gene product, a dihydrodiol dehydrogenase, to 2,3-dihydroxybiphenyl. Another dioxygenase enzyme, the *bphC* gene product, cleaves 2,3-dihydroxybiphenyl to yield a colored *meta* cleavage product. This yellow compound is converted subsequently to benzoate and a pentanoic acid derivative by the product of the *bphD* gene (5, 10, 11). One species, *Pseudomonas* sp. strain LB400, has the capability to degrade PCB congeners with up to six chlorine atoms per molecule (3). The *bph* genes were cloned from LB400 and inserted into a transposable genetic module, Tn*PCB*, which can be stably inserted into the genome of a range of recipient bacteria with the plasmid pDDPCB (6). The advantages of this system are that no transposase is present within the element and retransposition does not occur, allowing stable integration into the target genome (13).

The objectives of this work were to construct a rhizosphere pseudomonad with the potential for PCB degradation. *Pseudomonas fluorescens* F113, which is an important biocontrol strain for the sugar beet, was chosen as a host strain. The stability of

* Corresponding author. Phone: 353 21 276871. Fax: 353 21 275934.

TABLE 1. Bacterial strains and plasmids[a]

Species	Strain	Relevant phenotype or genotype	Reference
P. fluorescens	F113	Wild type	9
	F113Rif	Spontaneous rifampin-resistant mutant; rhizosphere competent	This work
	F113Km	Tn*5*-induced kanamycin-resistant mutant of F113; rhizosphere competent	This work
	F113pcb	BP^+ $BphC^+$ derivative of F113Km	This work
	F113(pDD530)	*bph* operon on cosmid pRK7813 in F113Km	This work
	F113pcblac3.1	F113Rifpcb::Tn*5 Sm/bphA'lacZ* Rif^r $BphC^+$ BP^+ Lac^+ Sm^r	This work
	M114pcb	$BphC^+$ BP^+	
Pseudomonas sp.	LB400	$BphC^+$ BP^+, original source of *bph* operon.	3
E. coli	Sm10λ*pir* (pDDPCB)	Delivery system of *bph* operon; $bphC^+$ BP^+	6
	Sm10λ*pir* (pUT Tn5Sm/*bphA'lacZ*)	Delivery system of Tn*5Sm/bphA'lacZ*; lac^+ Sm^r	This work
	Sm10λ*pir* (pUTKm)	Delivery system of mini Tn*5*	13

[a] Abbreviations: BP^+, ability to utilize BP as a sole carbon source; $BphC^+$, formation of the yellow *meta* cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, from 2,3-diOHBP; Rif^r, rifampin resistant; Lac^+, production of β-galactosidase; Sm^r, streptomycin resistant.

the trait and the expression of the new phenotype were investigated in vitro and in situ.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains used are shown in Table 1. All derivatives of *P. fluorescens* F113 used in this study, including a spontaneous rifampin-resistant mutant and a Tn*5* mutant, were shown to be rhizosphere competent, i.e., able to colonize and persist in the sugar beet rhizosphere in nonsterile soil microcosms. Rhizosphere competence was shown by inoculating seeds with test strains and reisolating the bacteria from roots after 14 days in nonsterile soil microcosms (passaging). Strains isolated were reinoculated onto seeds and passaged once or twice more. All *Pseudomonas* strains were maintained on SA agar (18) and cultured in Luria-Bertani (LB) broth (17) or SA broth at 28°C for 18 h. *Escherichia coli* was cultured in LB agar supplemented with 100 μg of ampicillin per ml. Minimal medium (MM) was K_2HPO_4 (1 g/liter), KH_2PO_4 (0.5 g/liter), $MgSO_4 \cdot 7H_2O$ (0.1 g/liter), NaCl (0.1 g/liter), and $(NH_4)_2SO_4$ (1 g/liter). PAS salts (2) were added at 1 ml/liter postautoclaving. Agar (1.5%) was added when solid medium was required. Carbon sources were added from stock solutions at the appropriate concentration. For MM plus BP (MM+BP), 0.05% (wt/vol) yeast extract was added. BP crystals (Sigma) were added to lids of petri dishes containing solid medium, and plates were sealed with Parafilm M (American National Can). BP was added to liquid medium at 10% (wt/vol). Antibiotics were supplemented as required.

Growth rate experiments were carried out in LB, SA broth, or MM plus an appropriate carbon source by standard methods.

Construction of a strain capable of growth on BP. Broth and filter matings were used to construct the modified pseudomonad strains, F113pcb and M114pcb. *E. coli* donors, Sm10λ*pir* containing pUT vectors, were grown at 37°C with aeration in LB supplemented with 100 μg of ampicillin per ml to select for plasmid retention. Recipient *Pseudomonas* strains were grown in SA.

In filter matings, 0.5-ml volumes of donor and recipient were centrifuged together in an Eppendorf tube for 1 min. Cells, resuspended in 100 μl of broth, were placed on a sterile 0.45-μm-pore-size cellulose acetate filter on an LB plate. Following incubation for 18 h at 28°C, bacterial cells were removed from filters by vortexing in 1 ml of Ringer's solution (Oxoid). Serial dilutions were made in Ringer's solution, and 100-μl aliquots were plated onto MM+BP or SA agar plus appropriate antibiotics to counterselect against *E. coli* donors. As controls, 100-μl aliquots of donor and recipient were plated separately onto selective media. All plates were incubated for 3 to 4 weeks at 28°C for BP plus transconjugants or until colonies arose. Aliquots (100 μl) of appropriate dilutions were also plated onto media selective for the *Pseudomonas* recipient to calculate the frequency of transconjugants per recipient. For broth matings, 500-μl volumes of donor and recipient were added to 10 ml of LB and incubated for 18 h at 28°C. Following incubation, dilutions were made and treated as described for filter matings. Bacterial colonies that arose on MM+BP agar were purified by streaking for single colonies three times on selective media. Following purification, BP^+ transconjugants grew on MM+BP agar within 2 to 3 days at 28°C.

2,3-Dihydroxybiphenyl 1,2-dioxygenase (BphC) assay. To measure BphC activity, a modification of the method of Eltis et al. (7) was used. Bacteria were grown in liquid media, and 1-ml aliquots were washed in Ringer's solution and resuspended in 1 ml of 50 mM Tris (pH 8.0). 2,3-Dihydroxybiphenyl (2,3-diOHBP; Wako Chemicals Gmbh, Neuss, Germany) was added to a final concentration of 250 μM, and formation of the *meta* cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, was measured at 434 nm with a Beckman DU640 spectrophotometer, which automatically calculates the rate of formation of the yellow *meta* cleavage product per minute. BphC activity was expressed as micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid per minute per milligram of protein. Protein determination was done with the Bio-Rad protein determination kit. For a plate assay, 5 μl of 0.1% 2,3-diOHBP solution (in 10% [vol/vol] acetone) was added to bacterial colonies on solid media. Yellow color appeared within 5 s.

Resting cell assay: removal of 4-chlorobiphenyl. For the resting cell assay, the method described by Bopp (3) was used. 4-Chlorobiphenyl was added to cells at a final concentration of 200 ppm. Controls of F113 wild type were also prepared. Reactions were stopped at time zero and following incubation at 28°C for 24 h by the addition of perchloric acid. The remaining 4-chlorobiphenyl was extracted with hexane (12) and measured by gas chromatography (Perkin-Elmer Sigma 3 gas chromatograph with a flame ionization detector) with a Supelco PTE-5 column (film thickness, 5 mm; inside diameter, 0.53 mm). Samples were chromatographed for 50 min with a temperature program beginning at 100°C, rising to 280°C at a rate of 50°C/min, and then held at 280°C. The carrier gas was helium, with a flow rate of 5.9 ml/min.

Stability of ability to utilize BP as a sole carbon source. To determine the stability of the BP^+ phenotype, bacteria were grown in SA broth for 40 generations. Serial dilutions were made, and appropriate dilutions were plated in triplicate onto SA and MM+BP agar. The stability of the *bphC* gene was assayed by growing bacteria in nonselective media as described before and plating dilutions on SA or LB agar. Following incubation, plates were sprayed with a 0.1% (wt/vol) solution of 2,3-diOHBP with an atomizer. The percentage of yellow colonies was calculated.

Characterization of essential biocontrol traits. Siderophore production was demonstrated by growing F113 and the GMM F113pcb on SA agar plates and observing the production of a yellow fluorescent pigment. The presence of the antifungal metabolite 2,4-diacetylphloroglucinol was shown by high-performance liquid chromatography (HPLC) analysis or *Bacillus* bioassay (9). HCN production was measured as described by Voisard et al. (21).

Inoculation of sugar beet seeds. *Pseudomonas* strains used for the inoculation of sugar beet seeds (cv. Rex) were grown in SA medium. Cells were washed in Ringer's solution. The bacterial suspension (ca. 10^8/ml) was added to seeds and mixed for 2 min. Prior to sowing, bacteria were washed from three sets of seeds into Ringer's solution and serially diluted onto selective media to enumerate the number of bacteria applied to the seed.

Rhizosphere microcosms. The nonsterile microcosms used were similar to those described by Fenton et al. (9). Soil from Ovens, County Cork, is a sandy loam soil (pH 6.9) in which sugar beet had been grown previously. Soil was collected from the upper 5 cm of the soil profile and sieved through a 0.5-cm-mesh screen prior to use. Water content was kept at 23% (wt/wt) with tap water. One hundred fifty grams of soil was put in 7.5-cm diameter plastic pots, and nine seeds were sown per pot at a depth of 1.5 cm. The pots were placed in a randomized complete block within a propagator tray and incubated in an environmental chamber at 12°C with a 12-h day-night cycle. Soil moisture was monitored by weighing the pots every 3 days and adjusted with tap water as required.

Extraction and enumeration of bacteria from the sugar beet rhizosphere. After appropriate time intervals, seeds or seedling roots were removed from the soil as described by Fenton et al. (9). Excess soil was removed, and roots were removed from seedlings and placed in 10 ml of Ringer's solution. Plant material was vortexed for 2 min to remove bacteria. Washings were serially diluted into Ringer's solution, and five 20-μl aliquots of each dilution were spotted onto selective media. The plates were incubated for 2 days at 28°C.

Competition between a marked wild-type F113 and the genetically modified microorganism (GMM) for colonization of sugar beet roots. The genetically modified strain F113pcb and a marked wild-type F113 were grown separately in SA broth for 18 h at 28°C with aeration. The cells were washed in Ringer's solution, and equal numbers of the bacteria were added together to the sugar beet seeds prior to sowing. Bacterial numbers of each strain coinoculated onto seeds were enumerated as described before. Serial dilutions of seed washings were plated onto media selective for each strain to enumerate the number of bacteria applied to the seed. Seeds were sown as usual, and bacteria were isolated as described before, serial dilutions of washings being plated onto medium selective for each strain.

β-Galactosidase expression on the seed coat and rhizosphere of sugar beet. To detect the expression of the *bph* genes on the seed or in the sugar beet rhizosphere, sugar beet seeds were inoculated with F113pcblac3.1. In this strain, the *lacZ* reporter gene is under the control of the multiple *bph* promoters (see Fig. 2). To decrease the lactose-positive background due to organisms present on the seeds and in the soil, 20 g of seeds was sterilized with methanol (for 30 s) and then 14% (vol/vol) sodium hypochlorite (for 5 min) and washed six times in 100 ml of Ringer's solution prior to inoculation with F113pcblac3.1. The soil was sterilized by autoclaving on 3 consecutive days at 15 lb/in^2 for 20 min. As a control, the seeds were inoculated with F113Rif and also with Ringer's solution alone. The seeds were sown and incubated as described before. They were removed after appropriate time intervals and placed into 1 ml of Ringer's solution. Bacteria were removed by vortexing for 2 min. β-Galactosidase activity of 0.1 ml of the retained washing was determined by the standard method (17). Bacteria on seeds were enumerated by plating serial dilutions of the same retained washing onto selective media plus X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Expression of β-galactosidase was normalized as β-galactosidase units per 10^8 CFU after incubation for 1 h at 37°C.

RESULTS

Construction of rhizosphere-competent pseudomonads with the potential to degrade PCBs. The plasmid pDDPCB has been developed for the stable introduction of *bph* genes from *Pseudomonas* sp. strain LB400 into a range of bacteria (6). Two *P. fluorescens* strains, F113 and M114, isolated from the rhizosphere of sugar beet, were chosen as potential candidates for constructing BP-degrading GMMs. Rhizosphere-competent marked derivatives of F113 and M114 were used as recipients. The delivery system was shown to function in wild-type rhizosphere bacteria initially by use of the kanamycin-resistant pUTKm vector (13). Kanamycin-resistant transconjugants were isolated at a frequency ranging from 10^{-5} to 10^{-2} transconjugants per recipient.

F113 and M114 transconjugants, with the potential to degrade PCBs, were selected by their ability to utilize BP as a sole carbon source on MM+BP. Transconjugants that could utilize BP as a sole carbon source arose at lower frequencies than those of kanamycin-resistant transconjugants. F113 transconjugants that could utilize BP arose at approximately 10^{-8} per recipient, and M114 transconjugants were isolated only following the enrichments of mating mixtures. BP-utilizing transconjugants were purified on selective media prior to further characterization. On subculture, transconjugants grew when inoculated on MM+BP within 2 to 3 days at 28°C.

To test for the presence of an active *bphC* gene product, transconjugant colonies were sprayed with 2,3-diOHBP. The yellow *meta* cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, was observed when colonies were grown on MM+BP, MM plus succinate, LB, or SA agar, suggesting constitutive expression of the *bph* operon in the strain. This result was also observed with the donor strain LB400.

Characterization of *P. fluorescens* F113pcb. (i) Growth rate of GMMs in complex and defined media. A purified transconjugant, F113pcb, which could utilize BP as a sole carbon source, was selected for further characterization. F113pcb was compared with its parent, F113Km, and with the source of the *bph* genes, *Pseudomonas* sp. strain LB400. To investigate any major effect of the introduced degradative pathway on the metabolism of F113, the growth of F113pcb was compared with that of its parent F113Km in different media. No difference in growth in LB, SA broth, or MM plus succinate (data not shown) was observed. The generation time of the modified strain was unaffected by the insertion of the *bph* module. The generation times for both parental and modified strains in LB (30 min), SA broth (60 min), and MM plus succinate (2.2 h) were similar. The ability of F113pcb to grow in MM plus succinate as well as that of the wild-type strain suggests that no major metabolic changes were caused by the insertion of the module. The colony morphology of the transconjugant was also similar to that of its parent.

(ii) Secondary metabolites. F113 is capable of promoting biological control of damping-off disease of sugar beet seedlings (9). This is due to the production of an antifungal metabolite 2,4-diacetylphloroglucinol. The production of 2,4-diacetylphloroglucinol was monitored by HPLC and also in a bioassay. Other traits implicated in biocontrol processes, such as HCN and fluorescent siderophore production, were also evaluated. No difference in the production of these metabolites was observed between F113pcb and the wild-type strain F113 (data not shown).

(iii) Expanded metabolic activities and growth on BP. When F113pcb and LB400 were grown in MM plus 15 mM succinate, both bacteria grew at similar rates (Fig. 1a and b). The ability of F113pcb to utilize BP as a sole carbon source is shown in Fig. 1c (MM+BP). Growth of F113pcb was compared with that of LB400 in MM+BP (Fig. 1d), and the growth rates were similar. The expression of the *bph* pathway in the new host was confirmed in resting cell assays. Removal of 4-chlorobiphenyl was observed in F113pcb cells grown in BP and succinate but not in F113 cells (data not shown).

Regulation of the *bph* operon in F113pcb. (i) BphC enzyme activity. To investigate the expression and regulation of the *bph* operon in F113pcb, the activity of one enzyme in the pathway 2,3-dihydroxybiphenyl 1,2-dioxygenase, i.e., BphC, the product of the *bphC* gene, was measured. BphC-specific enzyme activity in F113pcb and LB400 was monitored at different phases of growth in batch culture in MM plus 15 mM succinate and MM+BP (Fig. 1).

The patterns of BphC activity at different phases of growth in batch culture were similar for F113pcb and LB400. Maximum BphC-specific activity was found in the late exponential phase, and BphC activity decreased when cells were not growing actively. In succinate-grown cultures of both LB400 and F113pcb, BphC enzyme levels were similar to those from cells grown in MM+BP. This suggests that the expression of the *bph* operon is constitutive.

(ii) Transcriptional regulation of the *bph* promoter. To investigate promoter activity of the *bph* operon, strains bearing transcriptional fusions were constructed. The transposon Tn*5Sm*/*bphA'lacZ* contains within its inverted repeats a β-galactosidase reporter gene (*lacZ*) under the control of the *bph* promoters (Fig. 2). By use of pUT Tn5Sm/*bphA'lacZ*, Lac$^+$ insertions into F113pcb were constructed by conjugation. Following purification, Lac$^+$ transconjugants with β-galactosidase activity were cycled three times through the sugar beet rhizosphere system to ensure that the insertion of the transposon had not effected rhizosphere competence. One transconjugant, F113pcblac3.1, was chosen for transcriptional studies. β-Galactosidase measurements in F113pcblac3.1 showed that β-galactosidase activity reflected BphC enzyme activity throughout growth in batch culture. Again, constitutive expression of the *bph* operon in succinate-grown cells as measured by β-galactosidase activity was found (data not shown).

Stability of BP$^+$ phenotype. The stability of the chromosomally inserted *bph* module was investigated in F113pcb transconjugants by investigating any loss of the ability to utilize

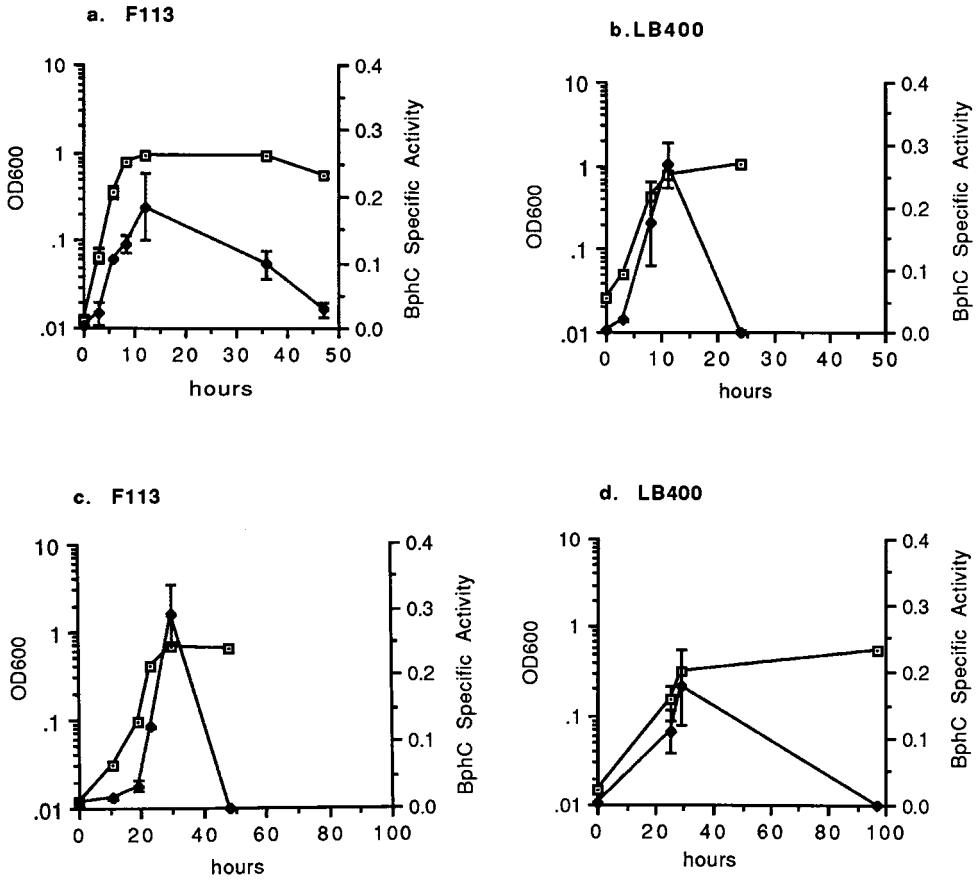

FIG. 1. Growth of F113pcb and LB400 in MM plus 15 mM succinate (a and b) and in MM+BP (c and d) at 28°C. BphC-specific activity units are micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid produced per minute per milligram of protein. Results are means of triplicate experiments. Symbols: ⊡, growth; ◆, BphC specific activity. OD600, optical density at 600 nm.

BP as a sole carbon source and the retention of the *bphC* gene product following growth in nonselective media. The ability to grow on BP and the BphC$^+$ phenotype was stable in F113pcb after growth for 40 generations in nonselective media. This is in contrast to the parent strain, LB400, or to F113(pDD530), where the *bph* module is plasmid borne. In LB400, 0.1% of cells lost the BphC$^+$ phenotype after 10 generations in nonselective media, and in strain F113pDD530, 99% of cells lost the trait after 10 generations.

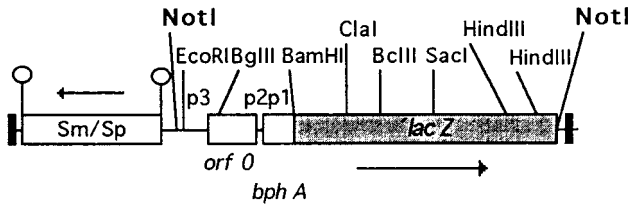

FIG. 2. Construction of Tn*5Sm/bphA'lacZ*. The entire *bph* operon, cloned in pDD5301 (6), was cut with *Mlu*I. Blunt ends were created by S1 nuclease digestion, allowing ligation to any of the three reading frames of the *lacZ* gene from pUJ9 (4). Following S1 treatment, the fragment was cut with *Eco*RI, and the resulting 1,927-bp fragment containing the *bph* promoters P_1, P_2, and P_3 was isolated and ligated to *Eco*RI- and *Sma*I-cut pUJ8 (4). Ampicillin-resistant Lac$^+$ transformants were selected to ensure selection of the correct frame. A *Not*I fragment spanning the entire fusion was cloned into pUTSm, the promoter proximal to ΩSm, resulting in Tn*5Sm/bphA'lacZ*. Further details are to be published elsewhere.

The introduced *bph* trait was found to be stable in F113pcb under rhizosphere conditions in nonsterile soil microcosms. Following colonization of sugar beet seedling roots, 100% of F113pcb colonies isolated from roots over a period of 25 days were BphC$^+$ and could utilize BP as a sole carbon source.

Evaluation of genetically modified F113pcb in nonsterile soil microcosms. (i) Colonization of sugar beet seedling roots. To assess whether the insertion of the *bph* module had any effect on the colonization ability of the strain, colonization of sugar beet seedling roots by F113pcb and a marked strain F113Rif in nonsterile soil microcosms was performed. Prior to use, rifampin-resistant mutants of F113 were shown to behave like wild-type F113 by investigating growth rates in defined and complex media. F113Rif was also shown to colonize efficiently the sugar beet rhizosphere.

F113Rif and F113pcb were each inoculated onto sugar beet seeds, and colonization was monitored during a 25-day period. F113pcb could colonize sugar beet seedlings as effectively as the wild type (Fig. 3). Both the kinetics of colonization of roots by F113pcb and the numbers of bacteria colonizing the roots were not significantly different from those of F113Rif. This indicates that the ability of the strain to establish and maintain colonization of sugar beet seedling roots was not effected by the insertion of the *bph* module into the genome. The engineered strain F113pcb also persisted for a length of time equal to that of the wild-type strain on roots.

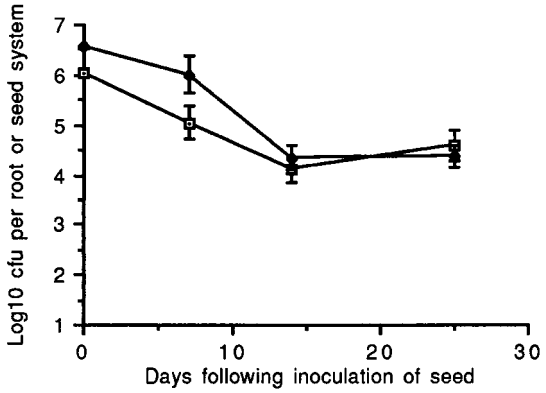

FIG. 3. Colonization of sugar beet seedling roots by GMM F113pcb (◆) and F113Rif (⊡) in nonsterile soil microcosms at 12°C. Results are geometric means of triplicate samples. Error bars represent standard errors of means.

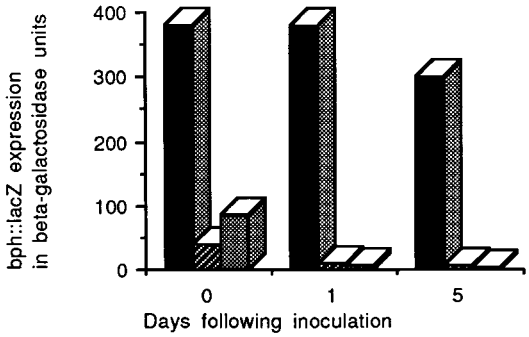

FIG. 5. *bph*::*lacZ* reporter gene activity measured as β-galactosidase activity from sugar beet seeds or roots from soil microcosms. β-Galactosidase activity is normalized after 60 min as β-galactosidase units per 10^8 CFU. Results are the average measurements of three seeds or root systems. Symbols: ■, F113pcb lac 3.1; ▨, F113Rif; ▩, no inoculum.

(ii) Competition of F113pcb with a marked F113 on sugar beet seedling roots. The GMM F113pcb retained colonization competence in nonsterile soil microcosms. The behavior of F113pcb in direct competition with a rifampin-marked F113 was also investigated. Sugar beet seeds were coinoculated with F113Rif and F113pcb and sown in nonsterile soil. The bacterial numbers of each strain on roots were monitored over a 25-day period. Both the GMM and F113Rif behaved in similar manners in their colonization ability. The relative number of the coinoculated bacteria to each other remained constant over the time course of the experiment (Fig. 4), indicating that the wild type and the GMM were equally competitive in the rhizosphere of sugar beet under the experimental conditions employed.

Evaluation and assessment of *bph* gene expression under in situ conditions. Evidence has been presented that F113pcb can utilize BP as a sole carbon source and that the expression of the *bph* operon is constitutive in in vitro studies. To investigate whether the *bph* genes present on the module are expressed in the sugar beet rhizosphere, F113pcblac3.1 and F113Rif were inoculated onto seeds and β-galactosidase activity was measured from seed washings at the time of sowing and after 5 days in soil microcosms. Uninoculated seeds were also included as a control. Seeds and soil were sterilized as described in Materials and Methods to decrease any interference from indigenous β-galactosidase-producing microorganisms. Figure 5 shows that β-galactosidase activity was detected in bacteria on the seeds at the time of sowing and for at least 5 days following sowing, by which time some seeds had germinated. In control microcosms, where seeds were not inoculated with bacteria or were inoculated with F113Rif, there was no significant β-galactosidase activity. These data indicate that the *bph* genes are being transcribed under the in situ conditions used and that, presumably, the gene products are present under these conditions.

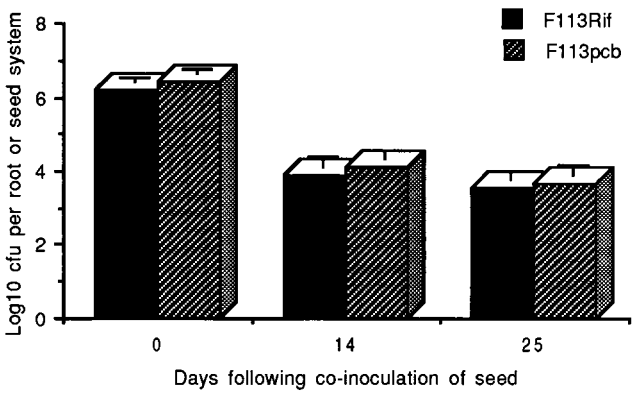

FIG. 4. Coinoculation of sugar beet seeds by GMM F113pcb and F113Rif to investigate potential competition. Colonization of seedling roots by each strain was determined by selective platings. Results are geometric means of triplicate samples. Error bars represent standard errors of means.

DISCUSSION

The goal of this work was to confer the potential to degrade PCBs to pseudomonads adapted for rhizosphere environments by use of Tn*PCB*. In this study, two rhizosphere pseudomonads were constructed, F113pcb and M114pcb, in which the *bph* pathway was expressed. F113pcb was studied further and found to have retained rhizosphere competence and to behave in a manner similar to that of the wild-type strain in in vitro and in vivo studies. In the laboratory, F113pcb utilized BP as a sole carbon source and produced 2,3-dihydroxybiphenyl 1,2-dioxygenase in a manner similar to that of the donor strain, LB400. Transcriptional studies and direct measurements of the BphC gene product indicated constitutive expression of the *bph* operon in F113pcb. The constitutive expression of *bphC* as measured in our assays is not surprising since Erickson and Mondello (8) showed that in LB400, two of the three *bph* promoters are active in succinate-grown cells. Important biocontrol-linked traits such as siderophore, HCN, and 2,4-diacetylphloroglucinol production were unaffected by the insertion of the new trait. The novel trait was found to be stable in vitro and when bacteria were introduced into the sugar beet rhizosphere in nonsterile microcosms.

Experiments in this laboratory have indicated that the *bph* element in F113pcb is stable and that lateral transfer of the element to a homologous recipient occurs at nondetectable frequencies in the rhizosphere (3a). Other methods to stably insert novel traits into the chromosome of rhizosphere pseudomonads have necessitated homologous recombination between flanking chromosomal sequences (22). The advantage of the plasmid pDDPCB is that the element can be transferred into the chromosome of many unrelated rhizosphere-competent strains. Once inserted, the element remains stable, and lateral transfer is not observed at a detectable frequency.

Our results have indicated that the insertion of the Tn*PCB*

element does not necessarily result in a decrease in rhizosphere fitness of a strain. Insertion of an element into a nonessential region of the bacterial chromosome can be selected by passing putative transconjugants through the rhizosphere, where only competent strains will be selected to survive and colonize the root.

F113pcb transconjugants were more stable with respect to the *bph* genes as monitored by the ability to utilize BP or the presence of the *bphC* gene product than either strain LB400, where the genes are chromosomally located, or F113 transconjugants, where the module was plasmid encoded. This is in agreement with the data of Dowling et al. (6), in which B13FR1pcb was more stable than a strain with the element located on a plasmid. No loss of the BP^+ phenotype was observed from F113pcb in the absence of selection, even after 25 days in nonsterile soil microcosms, suggesting that no strong selection for the elimination of the *bph* module exists in this microcosm.

F113pcb retained efficient colonization abilities on sugar beet seedling roots in nonsterile soil microcosms. Both the kinetics and efficiency of colonization of sugar beet seedling roots did not differ from those of the wild type. The insertion of the *bph* operon did not affect the ability of F113pcb to either colonize roots or to be maintained on plant roots.

To test the competitiveness of the GMM in nonsterile rhizosphere microcosms, F113pcb and a marked F113 were inoculated together onto sugar beet seeds prior to sowing. F113pcb was not less competitive than F113. Both strains behaved in a similar manner with respect to kinetics and efficiency of colonization. The proportion of the GMM to the wild type remained constant throughout the experiment. In competition studies in the wheat rhizosphere between wild-type *P. fluorescens* and mutants containing Tn*5* or Tn*5tox*, the GMMs were found to be less competitive than the parent strain (20). However, the authors do not consider that this decrease in numbers of modified bacteria was ecologically significant. The decrease in competitiveness was more acute in the rhizosphere and rhizoplane than in bulk soil or under in vitro conditions (20). In contrast, in our studies, no difference in competition was found between F113pcb and its parent. It would be interesting to see how the GMM behaves over a longer time period, during which subtle differences in fitness may be manifested. Of interest also would be the survival of F113pcb in bulk soil after the plants have been removed and the ability of the strain to persist and reinfect sugar beet roots.

One of the major problems for in situ bioremediation of pollutants including PCBs is the general decline of the populations of bacteria when introduced into a competitive environment (1). To counteract this, PCB-degrading strains must be repeatedly inoculated into a site. Alternatively, selective pressure must be exerted to maintain adequate populations of PCB degraders in the soil by amendment of soil with BP (1) or the use of a second carbon source such as surfactants (15).

In the case of a rhizosphere-adapted GMM, reinoculation or amendment with other carbon sources may not be necessary since the modified strain is likely to be maintained in the rhizosphere at the same numbers as those of the adapted parent strain.

The efficacy of such a rhizosphere strain at degrading PCBs in contaminated soils remains to be tested. We have used an indirect method to investigate the presence of gene products in the rhizosphere. The expression of the introduced *bph* operon in situ was investigated by measuring β-galactosidase activity in bacteria recovered from seeds. Seeds were inoculated with F113pcblac3.1, where β-galactosidase activity is under the control of *bph* promoters. The results indicate that the transcription of the *bph* operon is not repressed on the seed coat and following germination of the seed in sterile soil. Interference by indigenous Lac^+ organisms was eliminated in the experiment by sterilizing seeds and soil prior to inoculation and sowing. These results indicate that the *bph* operon is expressed during the colonization process.

Measurements of β-galactosidase activity throughout the colonization process would be of interest in determining whether expression of the operon is repressed at a late stage of plant development. However, more sensitive methods than those used currently may need to be employed if the numbers of bacteria decline during the colonization process. The units of transcriptional activity (i.e., β-galactosidase activity per 10^8 bacteria) are largely qualitative. Viable counts may not represent the total number of bacteria present on the root. Some bacteria may be viable but nonculturable, whereas others may not be metabolically active on the root.

The stable introduction of degradative genes into an organism of choice may allow us to improve the catabolic abilities of strains for degradation of pollutants. By choosing host strains adapted to specific ecosystems, e.g., the plant rhizosphere, there is potential to enhance the efficiency of bioremediation in different environments. The colonization of the plant root by the modified bacterium may provide an effective biofilm for the remediation of pollutants in soil.

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REFERENCES

1. **Barriault, D., and M. Sylvestre.** 1993. Factors affecting PCB degradation by an implanted strain in soil microcosms. Can. J. Microbiol. **39:**594–602.
2. **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.
3. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.

3a. **Brazil, G. M.** Unpublished data.

4. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
5. **Dowling, D. N., and F. O'Gara.** 1994. Genetic manipulation of ecologically adapted Pseudomonas strains for PCB degradation. Curr. Top. Mol. Genet. Life Sci. Adv. **2:**1–8.
6. **Dowling, D. N., R. Pipke, and D. F. Dwyer.** 1993. A DNA module encoding *bph* genes for the degradation of polychlorinated biphenyls (PCBs). FEMS Microbiol. Lett. **113:**149–154.
7. **Eltis, L. D., B. Hofmann, H. J. Hecht, H. Luensdorf, and K. N. Timmis.** 1993. Purification and crystallisation of 2,3-dihydroxybiphenyl 1,2-dioxygenase. J. Biol. Chem. **268:**2727–2732.
8. **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.
9. **Fenton, A. M., P. M. Stephens, J. Crowley, M. O'Callaghan, and F. O'Gara.** 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Environ. Microbiol. **58:**3873–3878.
10. **Furukawa, K.** 1982. Microbial degradation of polychlorinated biphenyls, p. 33–57. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Inc., Boca Raton, Fla.
11. **Furakawa, K., N. Hayase, and K. Taira.** 1990. Biphenyl/polychlorinated biphenyl catabolic gene (*bph* operon): organization, function, and molecular relationship in various pseudomonads, p. 111–120. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

12. **Goerlitz, D. F., and L. M. Law.** 1974. Determination of chlorinated insecticides in suspended sediment and bottom material. J. Assoc. Off. Anal. Chem. **57:**176–181.
13. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance foreign genes in gram-negative bacteria. J. Bacteriol. **172:**5557–5567.
14. **Hsu, T. S., and K. Bartha.** 1979. Accelerated mineralization of two organophosphate insecticides in the rhizosphere. Appl. Environ. Microbiol. **37:**36–41.
15. **Lajoie, C. A., G. J. Zylystra, M. F. DeFlaun, and P. F. Strom.** 1993. Development of field application vectors for bioremediation of soils contaminated with polychlorinated biphenyls. Appl. Environ. Microbiol. **59:**1735–1741.
16. **Lappin, H. M., M. P. Greaves, and J. H. Slater.** 1985. Degradation of the herbicide mecoprop by a synergistic microbial community. Appl. Environ. Microbiol. **49:**429–433.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Scher, F. M., and R. Baker.** 1982. Effect of *Pseudomonas putida* and synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. Phytopathology **72:**1567–1573.
19. **Unterman, R., D. L. Bedard, M. Brennan, L. H. Bopp, F. J. Mondello, R. E. Brooks, D. P. Mobley, D. P. McDermott, C. C. Schwartz, and D. K. Dietrich.** 1988. Biological approaches for PCB degradation, p. 253–269. *In* G. S. Omenn (ed.), Environmental biotechnology: reducing risks from environmental chemicals through biotechnology. Plenum Press, London.
20. **van Elsas, J. D., L. S. van Overbeek, A. M. Feldman, A. M. Dullemans, and O. de Leeuw.** 1991. Survival of genetically engineered *Pseudomonas fluorescens* in soil in competition with the parent strain. FEMS Microb. Ecol. **85:**53–64.
21. **Voisard, C., C. Keel, D. Haas, and G. Defago.** 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. **8:**351–358.
22. **Waalwijk, C., A. Dullemans, and C. Maat.** 1991. Construction of bioinsecticidal rhizosphere isolate of *Pseudomonas fluorescens*. FEMS Microbiol. Lett. **77:**257–269.
23. **Walton, B. T., and T. A. Anderson.** 1990. Microbial degradation of trichloroethylene in the rhizosphere: potential applications for biological remediation of waste sites. Appl. Environ. Microbiol. **56:**1012–1016.
24. **Williams, S. T.** 1985. Oligotrophy in soil: fact or fiction, p. 81–110. *In* M. Fletcher and G. Floodgate (ed.), Bacteria in the natural environment: the effect of nutrient conditions. Academic Press Ltd., London.