APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3910–3913 Vol. 62, No. 10
0099-2240/96/$04.00+0

Genetic Exchange in Soil between Introduced Chlorobenzoate Degraders and Indigenous Biphenyl Degraders

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Received 5 March 1996/Accepted 13 March 1996

Pseudomonas aeruginosa **JB2, a chlorobenzoate degrader, was inoculated into soil having indigenous biphenyl degraders but no identifiable 2-chlorobenzoate (2CBa) or 2,5-dichlorobenzoate (2,5DCBa) degraders. The absence of any indigenous chlorobenzoate degraders was noted by the failure to obtain enrichment cultures with the addition of 2CBa, 3CBa, or 2,5DCBa and by the failure of soil DNA to hybridize to the *tfdC* gene, which encodes ortho fission of chlorocatechols. In contrast, DNA extracted from inoculated soils hybridized to this probe. Bacteria able to utilize both biphenyl and 2CBa as growth substrates were absent in uninoculated soil, but their presence increased with time in the inoculated soils. This increase was related kinetically to the growth of biphenyl degraders. *Pseudomonas* sp. strain AW, a dominant biphenyl degrader, was selected as a possible parental strain. Eight of nine recombinant strains, chosen at random, had high phenotypic similarity (90% or more) to the inoculant; the other, strain JB2-M, had 78% similarity. Two hybrid strains, *P. aeruginosa* JB2-3 and *Pseudomonas* sp. JB2-M, were the most effective of all strains, including strain AW, in metabolizing polychlorinated biphenyls (Aroclor 1242). Repetitive extragenic palindromic-PCR analysis of putative parental strains JB2 and AW and the two recombinant strains JB2-3 and JB2-M showed similar fragments among the recombinants and JB2 but not AW. These results indicate that the *bph* genes were transferred to the chlorobenzoate-degrading inoculant from indigenous biphenyl degraders.**

Xenobiotic compounds which are readily biodegradable frequently require only a single organism for biodegradation, while persistent compounds such as polychlorinated biphenyls (PCBs) require more than one organism. PCBs are cometabolized by biphenyl-utilizing bacteria to chlorobenzoates in culture (2, 16, 17) and in the environment (8). Furukawa and Chakrabarty (15) were the first to suggest combining the genes of chlorobenzoate degraders with those of biphenyl degraders for complete mineralization of PCBs. Single strains able to utilize chlorobiphenyls have recently been constructed by genetic complementation of the catabolic pathways from parental strains containing the *bph* (biphenyl) and *clc* (chlorocatechol) genes (1, 19, 22, 32, 33). Presently, no strains have been constructed which can utilize environmentally relevant PCBs.

An alternative strategy to development of recombinants in the laboratory is to produce them in situ. Although biphenyl degraders are common in the environment (4, 16, 20), chlorobenzoate degraders appear not to be (11, 12, 20, 24, 35). Thus, inoculation of soil with the "missing genes" may allow a recombinant strain to arise that could mineralize PCBs. Fulthorpe et al. (12, 13) showed that genes for 3-chlorobenzoate (3CBa) degradation persisted in an aquatic community as a result of genetic exchange, even though the inoculated 3CBa degrader did not survive.

In a previous study (24), we showed that mineralization was enhanced by inoculation with chlorobenzoate degraders and that the frequency of isolates able to utilize both 2-chlorobenzoate (2CBa) and biphenyl increased with time. The study reported herein was undertaken to determine the nature of genetic exchange between the chlorobenzoate-degrading inoculants and indigenous biphenyl utilizers.

Altamont soil was amended with 4,000 mg of biphenyl (Aldrich Chemical Company, Milwaukee, Wis.) kg^{-1}, 100 mg of Aroclor 1242 (Foxboro Analytical Laboratories, North Haven, Conn.) kg^{-1}, and 300 mg of NH_4NO_3 kg^{-1} and was periodically sampled for 2CBa degraders and biphenyl degraders according to previously described procedures (6, 24). Inoculation of soils was carried out with *Pseudomonas aeruginosa* JB2 (23) obtained from late exponential growth in a mineral salts (MS) medium (9) containing 2,5-dichlorobenzoate (2,5DCBa) (500 $mg \cdot liter^{-1}$, Na salt; Lancaster Inc., Windham, N.H.). Washed cell suspensions were prepared in sterile 50 mM phosphate buffer (pH 7.0) at a density of 10^8 $cells \cdot ml^{-1}$, and 18 ml was added to 100 g of soil to give about 50% saturation of the pore space volume. Sterile 50 mM phosphate buffer was added to uninoculated microcosms.

Periodically, a measured amount of soil (about 1 g) was removed for dilution plate counts by using Noble agar (Difco) containing either 2-chlorobenzoate (Sigma Chemical Company, St. Louis, Mo.) or biphenyl as the sole growth substrate. Biphenyl crystals were added to the bottom lid of the inverted petri plate for growth on MS agar plates. Colonies from 2CBa plates were transferred to biphenyl plates to determine the frequency of isolates able to grow on both substrates. Colony formation was not evident from dilutions greater than 10^{-3} on agar plates containing neither 2CBa nor biphenyl.

Recombinants capable of utilizing both 2CBa and biphenyl were first isolated on 2CBa agar from soil at 15 days (Fig. 1). Nine such isolates were randomly selected for further analysis. All recombinant strains grew on 2,5DCBa, 3CBa, 2CBa, and biphenyl. All but one of the nine showed strong phenotypic similarity (90% or higher) to *P. aeruginosa* JB2 when compared by Biolog GN microplates (Biolog Inc., Hayward, Calif.). The exception was JB2-M (similarity, 78%). A dominant biphenyl utilizer, unable to metabolize chlorobenzoates, was obtained from dilution plates and identified as *Pseudomonas* sp. strain AW. Strains JB2, AW, and all nine recombinant strains were catalase and oxidase positive, did not ferment glucose, showed no change in litmus milk, and, except for strain AW, were

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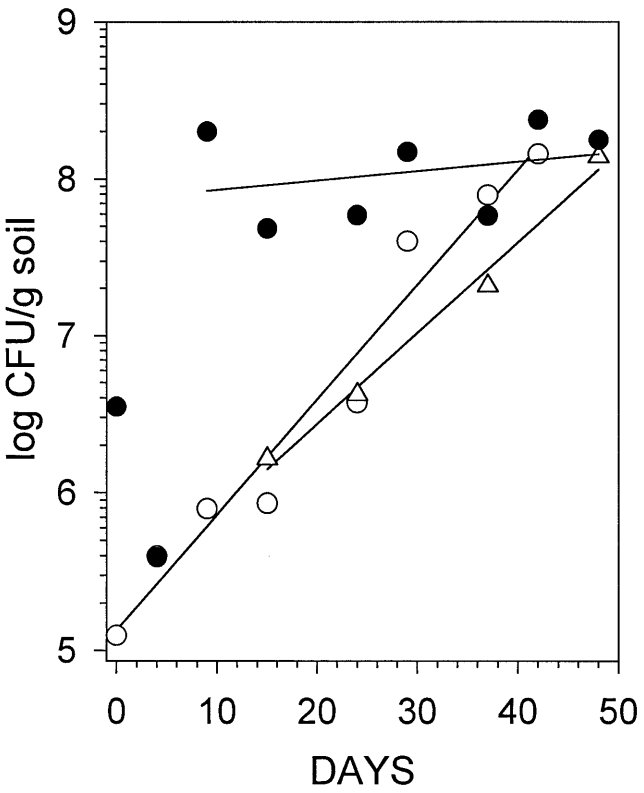

FIG. 1. Population densities of 2CBa utilizers (inoculant) (●), indigenous biphenyl utilizers (○), and recombinant strains (△) in soil containing biphenyl (1 $g \cdot kg^{-1}$) and Aroclor 1242 (100 $mg \cdot kg^{-1}$). CFU were determined from MS agar plates containing 2CBa or biphenyl. Recombinants were determined from at least 50 CFU per plate (on 2CBa agar), which were transferred to biphenyl agar. Biphenyl and 2CBa utilizers unable to utilize the other respective substrate were determined by subtraction from the regression line ($r^2 = 0.98$) of the recombinants.

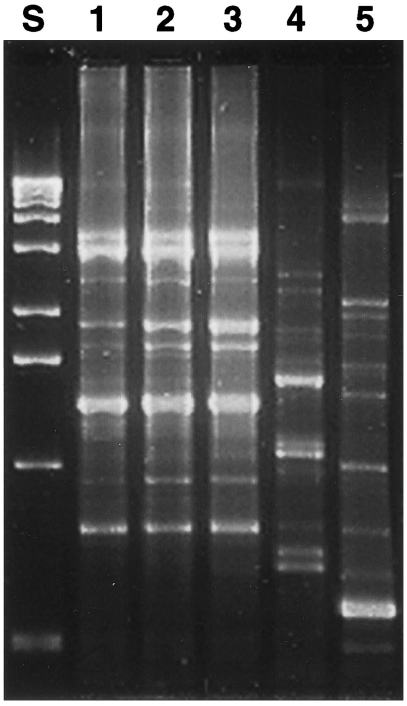

FIG. 2. REP-PCR fingerprint patterns of *P. aeruginosa* JB2 (lane 1), strain JB2-3 (lane 2), strain JB2-M (lane 3), strain AW (lane 4), and *E. coli* HB101 (lane 5). DNA standard ladder (1 kb) is shown in lane S.

resistant to kanamycin, chloramphenicol, and tetracycline at concentrations of 100 $mg \cdot liter^{-1}$. All strains except AW and JB2-M were fluorescent on King's A and King's B agar and grew on tryptic soy agar with 1,10-phenanthroline (considered indicative of *P. aeruginosa*) (27).

Recombinant strains were unstable, and retention of the ability to utilize both biphenyl and 2,5DCBa required alternate transfers between both substrates; if cultured on one substrate continuously (either biphenyl or 2,5DCBa), the recombinants lost the other catabolic pathway. However, no plasmid DNA was isolated from the recombinants. Recombinant strains JB2-3 and JB2-M were also found to be unstable when added to Altamont soil in another experiment conducted under similar conditions (38). First-order reversion constants of 0.04 day^{-1} ($r^2 = 0.87$) and 0.13 day^{-1} ($r^2 = 0.89$) were reported with strains JB2-3 and JB2-M, respectively.

Cultures, extracted and analyzed by gas chromatography (29) after 3 weeks, showed marked differences in the ability to cometabolize Aroclor 1242 (100 $mg \cdot liter^{-1}$) when grown with biphenyl (500 $mg \cdot liter^{-1}$). Two strains would not grow in the presence of PCB, three strains transformed less than 2%, two strains transformed between 20 and 28%, and the most effective strains, JB2-M and JB2-3, transformed 56 and 37%, respectively, of the PCBs. These levels were significantly greater than that of the indigenous strain AW (27%). Neither strain AW nor the recombinants could grow on Aroclor 1242 as the sole carbon source. The greater cometabolism of PCBs by recombinant strains JB2-M and JB2-3 than by strain AW may be explained by the elimination of inhibitory intermediates produced in PCB transformation. Sondossi et al. (39) found that chlorobenzoates could be fortuitously transformed by biphenyl-induced dioxygenases, leading to formation of chlorocatechols and muconic semialdehydes, which in turn inhibit 2,3-dihydroxybiphenyl-1,2-dioxygenase (1).

Strains JB2-M, JB2-3, and AW were investigated in more detail to assess their genetic relatedness. For extraction of DNA, parental and recombinant strains were grown in 50 ml of MS medium containing either biphenyl or 2CBa. Cells were pelleted (6,000 × *g* for 10 min) and resuspended in 8 ml of TE buffer (50 mM Tris, 20 mM EDTA, pH 8.5), and 0.5 ml 20% sodium dodecyl sulfate (SDS) was added and the mixture was heated at 65°C for 15 min. The mixture was cooled to 50°C and incubated for 2 h after the addition of 1 ml of pronase E (5 mg/ml; Sigma) and 0.2 ml of proteinase K (20 $mg \cdot liter^{-1}$; United States Biochemical Corp., Cleveland, Ohio). Three phenol-chloroform extractions were followed by one chloroform-isoamyl alcohol extraction (31). DNA was collected by ethanol precipitation and digested with *Bam*HI and *Hin*dIII (New England Biolabs, Beverly, Mass.). Repetitive extragenic palindromic (REP)-PCR determinations were obtained from cell pellets which had been washed once with TEN buffer (50 mM Tris-HCl, 20 mM EDTA, 100 mM NaCl) containing 0.5% sarcosine and then resuspended in 600 ml of TEN buffer containing SDS (1.25%) and proteinase K (0.63 $mg \cdot liter^{-1}$) and incubated at 37°C for at least 1 h. Chromosomal DNA was extracted from the lysed cells (3), and REP-PCR determinations were performed (25) by using 100 ng of template DNA per reaction.

REP-PCR banding patterns indicated that the two recombinant strains JB2-M and JB2-3 were very similar to the inoculant strain JB2 (Fig. 2). The origin of the indigenous parental

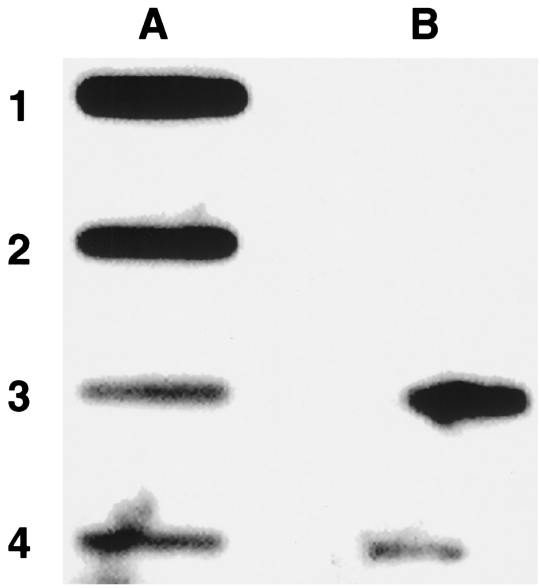

FIG. 3. Hybridization of the *tfdC* gene encoding catechol 1,2-dioxygenase II (26) to DNA from parental strain *P. aeruginosa* JB2, recombinant strains JB2-3 and JB2-M, and from soil. (A) Plasmid pTCB45 carrying the *clc* operon (42) (row 1), *P. aeruginosa* JB2 (row 2), JB2-3 (row 3), and JB2-M (row 4). (B) soil (row 1), soil treated with 2CBa (2-chlorobenzoate) (row 2), soil treated with 2CBa and inoculated with strain JB2 (row 3), and *E. coli* HB101 (row 4).

strain could not be resolved, as the likely candidate, *Pseudomonas* sp. strain AW, showed no similarity in banding patterns. The dominant band sizes, ranging from 210 to 5,000 bp, were also similar to those of amplified fragments from *P. aeruginosa* PAO1C (7). The PCR pattern of *Escherichia coli* HB101 (Fig. 2, lane 5) was also similar to that reported elsewhere (7), indicating that similar PCR conditions were maintained throughout the experiments. Our data indicate that the inoculant strain, by virtue of its genetic similarity to the recombinants, would be the recipient of *bph* genes donated from indigenous biphenyl utilizers. However, recombinants were selected on 2CBa agar and then transferred to biphenyl agar. Selection on biphenyl agar may have resulted in recombinants that were more similar to strain AW, or another indigenous biphenyl utilizer, and less similar to strain JB2.

As enrichment culture from uninoculated soil failed to yield any indigenous bacteria able to utilize 2CBa, 2,5DCBa, or 3CBa after 21 days, *P. aeruginosa* JB2 was assumed to be the parental strain contributing the CBa genes. To verify that nonculturable indigenous chlorobenzoate degraders were not present, DNA was extracted from soils inoculated with strain JB2 and from uninoculated soils and probed with the *tfdC* gene (26), which encodes catechol 1,2-dioxygenase II. DNA was extracted from the Altamont soil (36) and purified by passage through Sephadex columns (31). Digestion with restriction enzymes was not done because of shearing during extraction. Hybridizations were performed under high stringency conditions (>70% of base pairings) with random-primed dioxigenin-labeled DNA by using the Genius 1 Nonradioactive DNA Labeling and Detection kit (Boehringer Mannheim, Indianapolis, Ind.) and were detected by chemiluminescence (Lumi-Phos 500) (28) with the Biodot SF slot blot system (Bio-Rad, Hercules, Calif.). DNA from strain JB2, from recombinant strains JB2-3 and JB2-M, and from inoculated soil hybridized to the probe, while DNA from both uninoculated soils did not (Fig. 3). Genomic DNA of *E. coli* HB101, supposedly a negative control, has been previously reported to hybridize to a related probe (32), due to similar conserved sequences.

Genetic exchange can be described as a second-order reaction dependent on the concentration (cell $\cdot$ g^{-1}) of chlorobenzoate degraders (A) and biphenyl degraders (B) to produce a concentration of cells having both traits (C)

$$dC/dt = KAB \quad (1)$$

where K is the rate constant (cell^{-1} $\cdot$ g^{-1} $\cdot$ day^{-1}). This model is based on recombinants being formed by genetic exchange without their subsequent growth. Because recombinant strains JB2-3 and JB2-M were unstable when grown continuously on biphenyl in pure culture and in soil (38), it is unlikely that they would grow on biphenyl while retaining the ability to utilize 2CBa. As a relatively constant density of chlorobenzoate degraders of about 10^8 cell $\cdot$ g^{-1} was observed after 10 days, the increase in recombinants between 10 and 40 days was dependent upon an increase in biphenyl degraders (equation 1; Fig. 1). For example, the rate of genetic exchange would be 100 times greater at 40 days than at 10 days. An approximation of the rate constant can be obtained from the exponential growth phase of biphenyl utilizers:

$$dB/dt = \mu B - KAB \quad (2)$$

where μ is the growth rate constant (day^{-1}, in common log notation). Dividing both sides by B gives

$$d\log B/dt = \mu - KA \quad (3)$$

The slope ($d\log B/dt$) of 0.07 day^{-1}, as shown in Fig. 1, is the net difference between the growth rate μ and KA. Although we cannot determine the growth rate, it must be greater than the slope and it is not likely to be greater than what was found (1.1 day^{-1}) in an earlier study with Altamont soil (10). For $A \cong 10^8$ cell $\cdot$ g^{-1} and $0.08 < \mu < 1.1$, the rate constant K is between $1.0 \cdot 10^{-10}$ and $1.4 \cdot 10^{-9}$ cell^{-1} day^{-1}. Thus, the rate of recombination at 40 days would be between $1.0 \cdot 10^6$ and $1.4 \cdot 10^7$ cells $\cdot$ g^{-1} $\cdot$ day^{-1}.

Although the dominant biphenyl-degrading strain AW would be stochastically the most likely parental donor, REP-PCR analysis showed no evidence of similar conserved sequences. Nevertheless, the amplified sequence that was used may not necessarily be related to the *bph* genes transferred to strain JB2. The presence of nonculturable biphenyl degraders, genetically more similar to strain JB2 and having a higher K value than strain AW, cannot be precluded early in the experiment (15 days) when they would not be as greatly outnumbered by strain AW than they would be near the end (45 days).

The occurrence of genetic exchange in the environment has been well documented with respect to biodegradation. Fulthorpe and Wyndam (12) observed an increase in cells having homology for the *cba* region of pBR60, which encodes for 3CBa metabolism, despite a decline of the 3CBa-degrading inoculum. That genetic exchange of degradative genes should occur is not surprising as many of them are found on plasmids (37). Although we could find no evidence of plasmids in the recombinant strains or in the recipient, integration into the host chromosome may have occurred by a transposon. Transposons have been reported with genes coding for degradation of biphenyl (40), chlorohydroxyquinone (18), chlorobenzoate (14), trichlorobenzene (42), 3,4-dichlorobenzoate (34), naphthalene (41), and for the inoculant used in this study (21).

The recombinant strains isolated from this study were found to be unstable; if cultured continuously on either substrate (i.e., biphenyl or 2CBa), they would lose the ability to grow on the other. Instability of catabolic genes may be caused by

plasmid or transposon loss under conditions which do not select for that genetic element. There are examples of unstable chlorobenzoate (5, 23) and biphenyl genes (15, 30). However, the high rate of genetic exchange, estimated from the kinetic data, may render concern about stability of recombinants moot. While this instability presents difficulties in maintaining stable cultures or inoculants for bioremediation, it may be desirable in minimizing perceived risks of persistent recombinants in the environment.

This work was supported by grants from the National Science Foundation and the Kearney Foundation of Soil Science.

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