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Transfer and Expression of the Catabolic Plasmid pBRC60 in Wild Bacterial Recipients in a Freshwater Ecosystem

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3-Chlorobenzoate (3Cba)-degrading bacteria were isolated from the waters and sediments of flowthrough mesocosms dosed with various concentrations of 3Cba and inoculated with a 3Cba-degrading *Alcaligenes* sp., strain BR60. Bacteria capable of 3Cba degradation which were distinct from BR60 were isolated. They carried pBRC60, a plasmid introduced with *Alcaligenes* sp. strain BR60 that carries a transposable element (Tn*5271*) encoding 3Cba degradation. The isolates expressed these genes in different ways. The majority of pBRC60 recipients were motile, yellow-pigmented, gram-negative rods related to the group III pseudomonads and to BR60 by substrate utilization pattern. They were capable of complete 3Cba degradation at both millimolar and micromolar concentrations. Two isolates, *Pseudomonas fluorescens* PR24B(pBRC60) and *Pseudomonas* sp. strain PR120(pBRC60), are more distantly related to BR60 and both produced chlorocatechol when exposed to 3Cba at millimolar concentrations in the presence of yeast extract. These species showed poor growth in liquid 3Cba minimal medium but could degrade 3Cba in continuous cultures dosed with micromolar levels of the chemical. Laboratory matings confirm that pBRC60 can transfer from BR60 to species in both the beta and gamma subgroups of the proteobacteria and that 3Cba gene expression is variable between species. Selection pressures acting on pBRC60 recipients are discussed.

Bacterial genetic elements encoding the degradation of aromatic and chloroaromatic hydrocarbons are frequently carried on conjugative plasmids and are potentially mobile in natural bacterial communities. On the basis of our knowledge of the spread of plasmid-borne antibiotic and mercury resistance elements (1, 13, 26, 27), catabolic plasmids may be expected to show broad distributions. Interspecific transfer of degradative elements is suggested by the broad distribution of plasmids encoding toluene and xylene degradation (the TOL plasmids) that show strong homology (5). Similarly, the chlorobenzoate catabolic plasmids pAC27, pWR1, and pJP4, isolated on three different continents, show extensive homology (6, 14).

Direct studies on the survival, expression, and host range of a degradative element in natural systems are rare. The wide spread of antibiotic and other resistance elements in bacterial communities can be explained by the extreme selection pressure acting against those bacteria that lack the resistance element. However, the degree of selective pressure exerted by an additional carbon substrate on a degradative element is unknown. Pertsova et al. (19) studied the fate of chlorobenzoate degradative plasmids in small soil samples and found that chlorobenzoate-degrading isolates differed from the species originally inoculated, indicating a transfer of the genetic information. Unfortunately, this study involved the use of artificially high levels of nutrients (16 mM phosphate) and is of limited value in predicting the behavior of degradative elements in the nutrient-limited natural environment. Similarly, most studies on plasmid behavior come from work performed with sterilized systems or systems in which introduced donors and recipients are isolated from the natural system via dialysis bags or chambers.

We were interested in the behavior of a degradative element in a natural system experiencing normal levels of nutrients, predators, competitors, and environmentally relevant concentrations of a chemical contaminant. *Alcaligenes* sp. strain BR60, carrying a 3-chlorobenzoate (3Cba) catabolic plasmid (pBRC60, formerly designated pBR60 [32]) was introduced to large flowthrough freshwater mesocosms where it could interact with natural microfauna continually refreshed from an adjacent lake. The mesocosms were exposed to different levels of 3Cba. In our previous work, we documented the correlation between the frequency of the 3Cba genes in the mesocosms and the rate of degradation of 3Cba (11). We also showed that the original inoculum largely died out in these release experiments, while levels of pBRC60 remained high. In this work we describe those organisms isolated from mesocosm waters and sediments that carry pBRC60 and that we believe were responsible for 3Cba degradation in the mesocosms. They include species from both inside and outside the known host range of plasmid pBRC60.

MATERIALS AND METHODS

Bacterial strains. *Alcaligenes* sp. strain BR60 was isolated from the sediments of Bloody Run Creek, which drains leachate from a chemical landfill site in the Niagara region (33). This species can utilize 3Cba as its sole source of carbon and energy due to the presence of a conjugative plasmid, pBRC60, carrying a 17-kb transposon (Tn*5271*) specifying 3Cba degradation (32). Freeze-dried samples of the following were purchased from the American Type Culture Collection: *Pseudomonas acidovorans* (ATCC 15668), *P. alcaligenes* (ATCC 14909), *P. cepacia* (ATCC 25416), *P. fluorescens* (ATCC 13525), *P. pseudoflava* (ATCC 33668), *P. palleronii* (ATCC 17724), *P. putida* biotype A (ATCC 12633), and *Chromobacterium violaceum* (ATCC 12472). *Alcaligenes* sp. strain H850 was provided by General Electric Co. in Schenectedy, N.Y. *Acinetobacter calcoaceticus* DON2, an aniline-degrading isolate from the Don

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River, was described by Wyndham (31). *Moraxella* sp. strain G. was provided by Josef Zeyer of Geneva, Switzerland (34). *P. putida* PRS2015(pAC27) was provided by A. M. Chakrabarty (6).

Media. Strains were maintained or purified or both on nutrient agar, 0.1% TYE agar (0.1% tryptone, 0.1% yeast extract, 1.8% agar), or CPS agar (0.5 g of Casitone, 0.5 g of peptone, 0.5 g of soluble starch, 1 g of glycerol, 0.04 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of K_2HPO_4, 10 g of agar in 1 liter of water). Fluorescent pigments were determined by streaking on King B agar (16). Chlorobenzoate-utilizing strains were maintained on 3Cba agar (medium A [31], 3 mM 3Cba, 1.8% agar) or 3Cba/ye (3Cba agar, 0.005% yeast extract). 3Cba+ strains were grown in 3Cba or 3Cba/ye broth (as above, without agar).

Mesocosm design and strain isolation. Fifty-liter lake water flowthrough mesocosms containing a 5-cm layer of static sediment were set up at Queen's University Biological Station as described by Fulthorpe and Wyndham (11). These mesocosms were continuously diluted with fresh lake water from adjacent Lake Opinicon over a period of 2 months, 26 June to 5 September 1988. BR60 was introduced at 10^5 cells per ml to 12 of these mesocosms on 26 June, and each one received a specific concentration of 3Cba, between 0 and 25 μM, in the incoming water. The maintenance of the degradative plasmid pBRC60 in these mesocosms is described by Fulthorpe and Wyndham (11).

In this work we focus on those organisms exhibiting the 3Cba+ phenotype isolated from the 3Cba-exposed mesocosms. Throughout the summer, isolations of bacteria were made from colonies appearing on 3Cba agar. Colonies were transferred to 3Cba liquid media. Those cultures showing turbidity after approximately 1 week were maintained and purified until the organism responsible for 3Cba metabolism was isolated.

Isolate characterization. Bacteria isolated from the mesocosms were subjected to rapid plasmid screens and preliminary identification, using BIOLOG GN Microplates from BIOLOG Inc., Hayward, Calif. Colonies were streaked onto 0.1% TYE or CPS agar and grown for 18 h or less at 30°C. Growth was rolled onto a sterile cotton swab, suspended in 1 ml of medium A, and centrifuged at 5,000 rpm for 3 min in an Eppendorf centrifuge to give a loose pellet. A 100-μl portion of this pellet was resuspended in 20 ml of sterile saline (0.85%), vortexed and pipetted into BIOLOG GN plates, and incubated for 24 h. The binary results from the 95-substrate utilization tests in the BIOLOG GN plates were used to generate a matrix of Jaccard similarity coefficients, using a BASIC program (available on request). This matrix was used to cluster the isolates by using the single-linkage CLUSTER procedure of SYSTAT (SYSTAT Inc., Evanston, Ill.).

The remaining cell suspension was recentrifuged at 8,000 rpm for 5 min, and the supernatant was replaced with 4 volumes (50 to 200 μl) of lysis buffer (50 mM Tris, 10 mM EDTA, 2 mg of lysozyme per ml, 25% sucrose, 100 μg of RNase per ml, pH 8); the cells were resuspended and left at room temperature for 2 h. Cell lysis buffer suspensions were diluted with an equal volume of xylene cyanol–30% glycerol loading buffer and pipetted into the wells of a 0.7% agarose gel which had been preloaded with 3 μl of 10% ultrapure sodium dodecyl sulfate (SDS). Cell lysis occurred immediately in most cases. The gel was then transferred to an electrophoresis tray and run in 1× Tris-acetate-EDTA electrophoresis buffer at 20 V overnight or at 40 V for 4 h.

Gels were blotted onto Hybond (Amersham) nylon membranes (22) and stored at −20°C. A DNA probe specific to the pBRC60 plasmid and the resident transposon Tn*5271* carrying the 3Cba degradative genes was prepared from *Eco*RI restriction enzyme fragments E1, E5, and E14, and hybridization of this probe to the blots was carried out under stringent conditions, as described previously (32).

Plasmid extractions. 3Cba-degrading strains were cultured in 300 ml of 3Cba/ye broth; non-3Cba-degrading strains were cultured in 0.1% tryptone-yeast extract broth. Cells were harvested from a log-phase culture by centrifuging at 7,000 rpm for 10 min in a Sorvall RC2-B centrifuge. Pellets were resuspended in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 8) containing 2 mg of lysozyme per ml and left on ice for 10 min. The cell suspension was pipetted slowly into 15 ml of lysis buffer (50 mM Tris, 10 mM EDTA [pH 12.5], 3% SDS) in glass corex tubes. Cells were lysed with a minimum of disturbance for 10 min. A 7-ml amount of 3 M sodium acetate (pH 4.5) was added, and the tubes were slowly inverted to mix and placed on ice for 2 h with intermittent inversions. The chromosomal DNA was removed by centrifuging at 10,000 rpm, and the supernatant was extracted twice with 5 ml of Tris-buffered phenol (pH 8)–chloroform. The extracted precipitate was made 0.3 M NaCl, and 0.6 volume of isopropanol was added. Plasmid precipitation took place overnight at −20°C. Plasmids were centrifuged at 11,000 rpm and washed in 70% ethanol before dissolution in 100 to 400 μl of TE. The plasmid preparation were treated with RNase A and digested with *Eco*RI (Pharmacia) prior to electrophoresis.

Mating experiments. To test the host range of the pBRC60 replicon, a Tn*5* (kanamycin resistance [Km^r]) labeled plasmid was constructed with pBRC40, the deletion derivative of pBRC60 that lacks the inherently unstable catabolic transposon Tn*5271* (32). A suicide plasmid donor of Tn*5*, pGS9 (24), was conjugated from *Escherichia coli* HB101 into *Alcaligenes* sp. strain BR40(pBRC40), and pBRC40::Tn*5* transposition derivatives were selected by mating the pool of Km^r transposon recipients with a plasmid-free chloramphenicol-resistant (Cm^r) tryptophan auxotroph (Trp^-) derivative of *Alcaligenes* sp. strain BR60(BR6024). BR6024 was isolated by selection for spontaneous resistance to 80 μg of chloramphenicol per ml followed by mutagenesis with 200 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co.) per ml for 35 min at 30°C. Following mutagenesis, Trp^- auxotrophs were isolated by the method of Carhart and Hegeman (3). BR6024 recipients of pBRC40::Tn*5* were selected on medium A–12 mM succinate–50 μg of chloramphenicol–0.1 mM tryptophan–50 μg of kanamycin per ml. Overnight nutrient broth cultures of BR6024 (Cm^r Trp^- pBRC40::Tn*5*) and recipients were filter mated for 12 h on nutrient agar; transconjugants were enumerated on medium A agar supplemented with 1 g of sodium succinate, 1 g of sodium acetate, 1 g of glycerol (SAG), and 50 mg of kanamycin per liter. Donors were enumerated on the same medium supplemented with 1 mM tryptophan, and recipients were enumerated on SAG.

Continuous culture. In a 1.2-liter continuous culture vessel, cells were diluted at 0.01 to 0.04 h^{-1} with sterile artificial lake medium (FL) amended with 10 μM 3Cba. FL medium composition was based on the nitrogen and dissolved inorganic carbon levels found in Lake Opinicon and on inorganic ion concentrations typical for hardwater lakes (29). It contained 1.25 mM $CaCO_3$, 650 μM $MgCl_2$, 300 μM $MgSO_4$, 450 μM $NaHCO_3$, 10 μM $NaNO_3$, 5 μM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 1 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.2 μM $ZnSO_4$, 0.01 μM $CoCl_2$, and 0.01 μM Na_2MoO_4. The final pH was 8.2.

When supplemented with 10 μM 3Cba, this medium was capable of supporting approximately 10^6 cells of BR60 per ml.

Populations of total culturable cells in the continuous cultures were enumerating with CPS agar. Culturable cells using 3Cba were enumerated with 3Cba agar dyed with bromothymol blue (50 mg/liter) to indicate the release of HCl following dehalogenation of 3Cba metabolites. Rates of losses of the 3Cba+ phenotype were calculated as follows: loss rate = $1 - (N+/N)^{1/g}$, where g is the number of generations (hours cultured/generation time), $N+$ is the number of 3Cba+ cells, and N is the total number of cells.

Total cell counts were also determined in some cases by direct counting of acridine orange-stained cells with a Zeiss Photomicroscope 3 with epifluorescence illumination.

Kinetic parameters. The rate of uptake of 3Cba and the subsequent production of CO_2 by pBRC60 hosts were determined by using L-[U-^{14}C]3Cba (specific activity, 20.3 mCi/mmol; Sigma Radiochemicals). Measurements were carried out on batch cultures of FL medium amended with 50 μM 3Cba or on samples taken from 10 μM 3Cba; FL medium continuous cultures were grown at a dilution rate of 0.01 h^{-1}. All continuous cultures were inoculated with a batch culture of the appropriate species grown in 3Cba or 3Cba/ye and left to equilibrate for at least three volume changes (12 days) before uptake and carbon dioxide production measurements were made. Uptake rates were determined as described previously (10). The evolution of $^{14}CO_2$ was measured from 3-ml subsamples in 15-ml serum tubes sealed with rubber stoppers. Labeled 3Cba was added at five concentrations between 0.1 and 10 μM, and the serum tubes were incubated for 1 h. Cultures were acidified, and the tubes were recapped with an ethanolamine-soaked filter paper strip suspended from the top of the tube. Samples were boiled briefly, cooled, and left overnight. Filter paper strips were removed and immersed in scintillation cocktail {85% toluene containing 4 g of POPOP [1,4-bis(5-phenyloxazolyl)benzene]–15% methanol or PCS solubilizer from Amersham Corp.} and counted in a Beckman scintillation counter. Triplicate controls inoculated with 25 μl of [^{14}C]$NaHCO_3$ (specific activity, 10 mCi/mmol; Sigma Radiochemicals) were used to determine the efficiency of capture of CO_2. Values of maximum uptake rates (V_{max}) and half-saturation constants (K_m) were determined from $1/v = K_m/V_{max} \times 1/s + 1/V_{max}$, where s is the initial substrate concentration and v is the sum of substrate uptake and respiration rates.

Chemical concentrations. The concentrations of chlorobenzoate and chlorocatechols in culture samples were determined by using high-pressure liquid chromatography with a Waters 501 solvent pump, Nova-Pak C_{18} reverse-phase column, and Lambda-Max model 481 LC spectrophotometer set at 254 nm (33). The solvent used was a 1:1 mixture of methanol–1% acetic acid.

RESULTS

Cluster analysis of isolates. All isolates showing some degree of growth in liquid 3Cba medium were metabolically fingerprinted with the BIOLOG GN plates. Type species of typical freshwater bacteria were also fingerprinted. Of the 27 3Cba-degrading isolates that were probe positive, 16 had BIOLOG fingerprints indistinguishable from that of BR60. Only one of these isolates was included in the analysis. The cluster analysis grouped the isolates and reference strains into five groups: (1) BR60-like species; (2) the PR117 group of yellow-pigmented rods; (3) a fluorescent pseudomonad group; (4) the *Moraxella-Acinetobacter* group; and (5) remaining species distantly related to the other four major groups and to each other (Fig. 1). Two fingerprints for PR117 are included in the analysis to show the distance between replicates of the same strain. PR12 is a BR60 colony reisolated from the experimental mesocosms. These replicate fingerprints indicate that a cluster distance less than about 2.7 (similarity coefficient, 0.27) represents the practical limit of resolutions of strains. Only isolates found to carry pBRC60 (indicated by asterisks) retained the 3Cba+ phenotype on subculturing from the initial isolation. One isolate carrying a plasmid homologous to pBRC60 (strain PR63) showed no 3Cba metabolism. The remaining isolates lost the 3Cba+ phenotype during isolation. The only 3Cba-metabolizing strain isolated from mesocosms not originally inoculated with BR60 was indistinguishable from BR60, indicating that these "control" mesocosms had become contaminated with this strain through either aerosols or the common lake water supply. 3Cba-metabolizing strains differing from BR60 were only isolated from those mesocosms that had been inoculated with large numbers (10^5 cells per ml) of BR60.

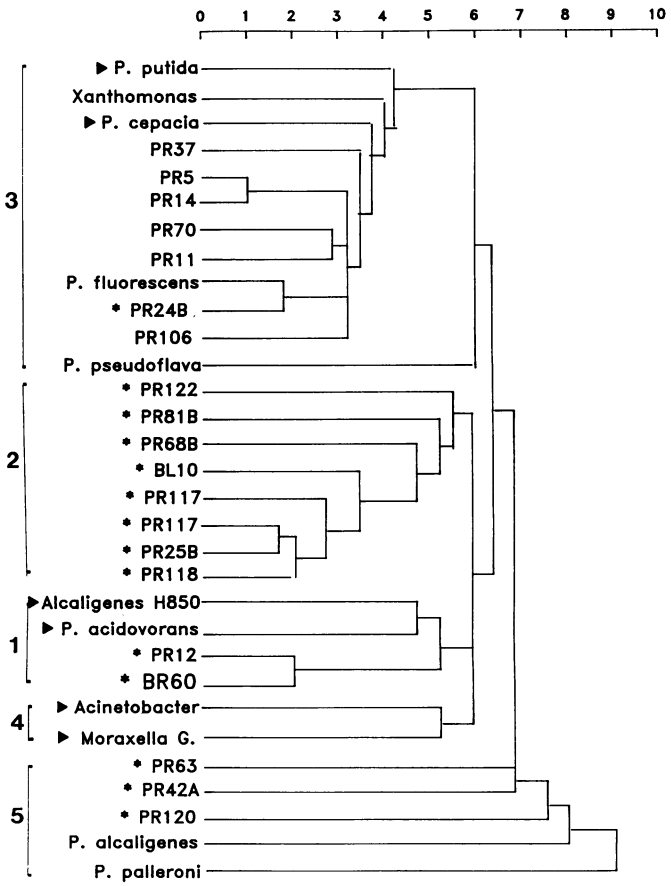

FIG. 1. Dendogram of strains isolated from mesocosms, using 3Cba and key type strains. Distance was calculated from Jaccard similarity coefficients based on reactions in BIOLOG GN plates. The scale shown is proportional to the dissimilarity coefficient, equal to (1 − similarity) × 10. Asterisks indicate those strains carrying pBRC60. Arrows indicate those strains that receive pBRC60 via conjugation during filter mating with BR60 overnight.

Group 1, BR60 donor and related recipients. The BIOLOG metabolic fingerprint and other diagnostic tests place the

original inoculum, *Alcaligenes* sp. strain BR60, in a group with *P. acidovorans* (an rRNA group III pseudomonad) and *Alcaligenes* sp. strain H850. It is identified by the BIOLOG software as being *Comomonas testosteroni* (also an rRNA group III pseudomonad). The *Alcaligenes* sp. and *Comomonas testosteroni* are members of the nonsaccharolytic group in Pickett's schema for identifying gram-negative nonfermentative bacilli (20), and the only key difference seems to be that of flagellar type. When motile, BR60 exhibits variable forms of flagellation, but multitrichous polar flagellation, characteristic of *C. testosteroni*, is common. In many cases BR60 is nonmotile and forms chains, as do *Alcaligenes* species, but unlike the latter, it cannot grow at 37°C or higher temperatures. Since both *Comomonas* and *Alcaligenes* are found in the beta rRNA homology group of the proteobacteria (25), we can only be sure that BR60 belongs in this group and is most closely allied with these two genera.

Group 2, PR117 recipients. Eight of the 3Cba+, probe-positive isolates, PR122, PR81B, PR68B, BL10, PR117, PR25B, PR118, and PR23A1 (not shown in Fig. 1), were morphologically similar and isolated from microcosms receiving 20, 10, 10, 10, 20, 0.73, 20, and 0.12 μM 3Cba, respectively. All of these isolates were motile, aerobic, slightly curved, gram-negative, narrow rods bearing multiple polar or peritrichous flagella (preparations gave variable results). Colonies developed a nonfluorescent yellow pigment on CPS and King B agar and, to a lesser degree, on nutrient agar. Cells accumulated poly-β-hydroxybutyric acid as determined by the formation of Sudan black crystals. BIOLOG GN plates showed that organic acid and amino acid utilization dominated, with limited use of carbohydrates. All of the strains give the same profile, with some variation in the use of *m*-inositol, maltose, and mannose. PR117 was selected as the type strain for this group in further studies. The BIOLOG system could not identify these isolates, giving PR117 a "poor" identification of *A. faecalis* with a similarity of 0.49. All of these isolates are tentatively identified as members of the group III pseudomonads, but they are not any of the three known yellow-pigmented group III pseudomonads (*P. pseudoflava* ATCC 33668 and *P. palleronii* ATCC 11724 in Fig. 1 or *P. flava*, which is known to utilize arabinose, cellobiose, fructose, galactose, maltose, mannitol, mannose, rhamnose, and sucrose).

PR117 isolates are similar to the host strain BR60 in their BIOLOG fingerprint, and they are streptomycin and ampicillin resistant, as is BR60. However, PR117 grows slower on general and selective (3Cba) liquid or solid medium (Fig. 2).

Group 3, fluorescent pseudomonads. Most strains in group 3 lost the 3Cba+ phenotype upon isolation and oxidized 3Cba to chlorocatechol, as indicated by the accumulation of purple oxidation products of this metabolite in the media. Many of the chlorocatechol-accumulating strains harbored large plasmids, bearing no homology to pBRC60. Only strain PR24B, isolated from a mesocosm receiving only 0.12 μM 3Cba, retained full 3Cba metabolism, and it was found to carry pBRC60. It is a motile, aerobic, gram-negative, oval rod with polar monotrichous flagella. On King B agar, colonies accumulate a fluorescent yellow-green pigment. The BIOLOG GN plate identifies it as *P. fluorescens* biovar IV. PR24B grows rapidly on general media, but slowly on 3Cba minimal media. In minimal liquid media containing 3 mM 3Cba, PR24B exhibits either no growth at all or the accumulation of a small amount of chlorocatechol. In liquid culture amended with 0.005% yeast extract, PR24B first

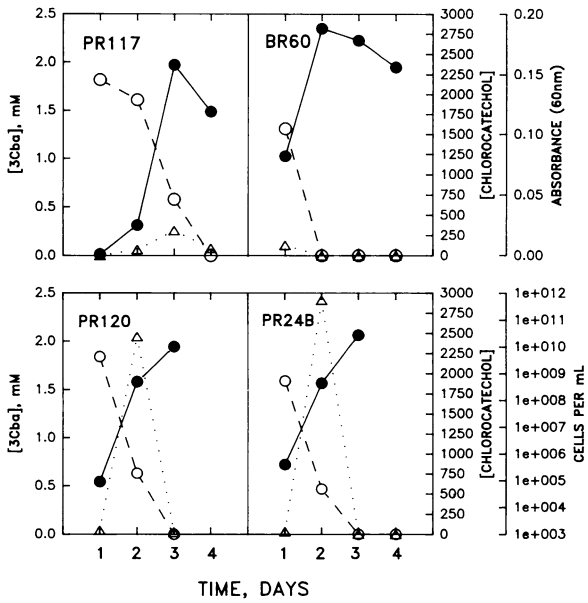

FIG. 2. Growth of pBRC60-bearing strains in 3Cba. Symbols: ○, 3Cba concentration (millimolar); ●, biomass of cells in A_{600} or cells per milliter; △, concentration of chlorocatechol in relative units of A_{256} from high-pressure liquid chromatography analysis.

metabolizes 3Cba to chlorocatechol, which accumulates in the medium, and then metabolizes chlorocatechol to yield a high biomass (Fig. 2).

Group 4, *Moraxella-Acinetobacter*. *Moraxella* or *Acinetobacter* strains were capable of acquiring and maintaining the pBRC60 replicon (pBRC40::Tn*5*) in laboratory matings. However, no natural recipients were found in this group.

Group 5, distantly related recipients. Group 5 is not a cluster, but an arbitrarily defined group of strains distantly related to each other and to other groups. PR42A gives a unique substrate utilization pattern, but is indistinguishable from PR117 in its appearance, colony morphology, and growth on general and 3Cba media. The BIOLOG system gives PR42A a poor identification of *C. acidovorans* with a similarity of 0.45. We assume that it is a group III pseudomonad and that it is closely related to PR117 in spite of the substrate utilization differences that place it in a separate group in the cluster analysis. The group III pseudomonads as a whole are not clustered together by their BIOLOG fingerprints (note positions of *P. pseudoflava* and *P. palleronii*).

Strain PR120 was isolated from a mesocosm receiving 20 μM 3Cba. It is a motile, aerobic, gram-negative, oval rod with polar monotrichous flagella. According to its BIOLOG GN profile, it is similar to *Psychrobacter immobilis* (similarity, 0.458). However, the cell shape and motility of PR120 indicate that it is not a *Psychrobacter* species. PR120 grows very poorly on general media, forming small, translucent, colorless colonies after 2 to 3 days. By contrast, it grows well on 3Cba agar or 3Cba agar amended with 0.005% yeast extract. In 3Cba liquid cultures PR120 accumulates chlorocatechol and then metabolizes this intermediate in a manner similar to *P. fluorescens* PR24B (Fig. 2).

PR63 was isolated from a mesocosm receiving 10 μM 3Cba. It is also a motile, aerobic, gram-negative rod with polar monotrichous flagella. On first isolation, it exhibited

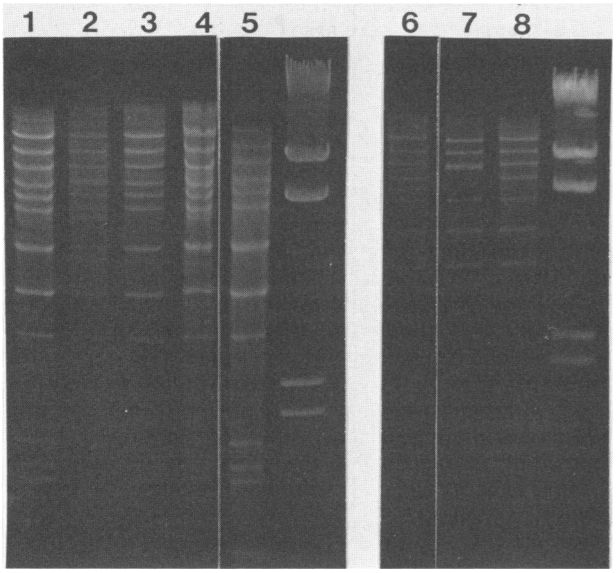

FIG. 3. *Eco*RI digestion of plasmids extracted from (lane 1) BR60, (lane 2) PR24B, (lane 3) PR117, (lane 4) PR120, (lane 5) PR42A, (lane 6) BR60, (lane 7) PR63, and (lane 8) BR40.

scant but positive growth on 3Cba agar. This property was difficult to reestablish; nevertheless, the organism hybridized to a pBRC60 probe (fragments E1, E5, and E14). The BIOLOG GN profile suggests that it is *P. delafieldii*; it has a similarity value of only 0.368 to this species, but does share the characteristics of the group III pseudomonads.

Plasmids in natural recipients. Figure 3 shows the plasmids isolated from our natural recipients. PR24B, PR42A, PR117, and PR120 all carry pBRC60. PR63 is carrying a smaller derivative of pBRC60 lacking Tn*5271*. It is shown beside pBRC40, the plasmid extracted from BR40, a strain identical to BR60 that has lost the 3Cba+ phenotype along with the deletion of Tn*5271* from its plasmid. The stability of the 3Cba+ phenotype was determined for PR117, PR24B, and PR120 by several transfers in general medium (TYE or nutrient broth). For each strain the loss rate was approximately 7% per generation under these conditions.

Competition experiments in cultures of BR60 and a natural plasmid recipient. During 110 days of continuous culture of BR60 with 10 μM 3Cba, the total free-living population fell from 10^7 to 10^6 cells per ml as phosphorus was removed from the medium and wall growth occurred. During this time BR60 maintained the ability to grow on 3Cba, showing only a small loss in the proportion of 3Cba+ cells (Fig. 4). When it was clear that BR60 was stable in this system after 110 days, an introduction of 3Cba-utilizing PR117 was made at 10^4 cells per ml. PR117 was not detectible after 3 days; therefore, a second introduction was made at 10^5 cells per ml. Thereafter, BR60 decreased from 10^6 to 10^5 cells per ml. Over the subsequent weeks, numbers of 3Cba+ BR60 dropped to 10% of the total BR60 population. PR117 stabilized at 10^5 cells per ml (equal to the total population of BR60). Estimates of total PR117 populations from CPS plates gave slightly lower counts than those made on 3Cba plates, since BR60 tended to inhibit PR117 on general media. BR60 rapidly outgrew PR117 on nutrient or King B agar, but inhibition was minimized on CPS plates. The two species were distinguished by their pigmentation on general media (PR117 is yellow) and by the slower rate of growth and

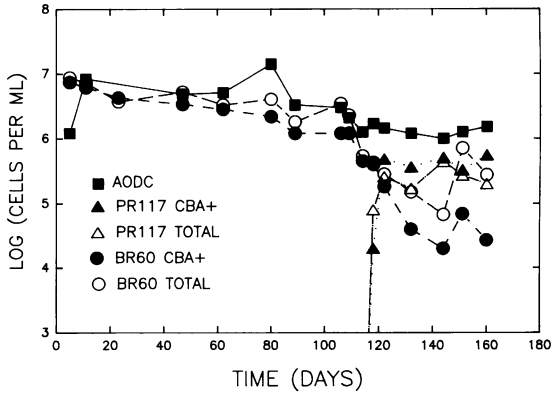

FIG. 4. Continuous coculture of BR60 in FL media supplemented with 10 μM 3Cba. PR117 was introduced at day 110; see text.

smaller colony size of PR117 on chlorobenzoate plates. Plating on general media also revealed that the culture maintained a small population of contaminating yeast cells (10^4 per ml) after the establishment of PR117.

Kinetics of 3Cba utilization in BR60 and natural pBRC60 recipients. Preliminary experiments on the uptake of 3Cba by BR60 and pBRC60 recipients were performed on overnight batch cultures of the strains in FL media amended with 50 μM 3Cba. The V_{max} values for PR81b, BL10, and PR42A (3.4×10^{-16}, 1.5×10^{-16}, and 1.2×10^{-16} mol/cell per h, respectively) compared favorably with those for BR60 and *P. putida* PRS2015(pAC27), a 3Cba degrader carrying different chlorobenzoate genes (6.2×10^{-17} and 3.6×10^{-16} mol/cell per h, respectively). To determine the kinetic parameters for uptake in a more realistic situation, BR60, PR117, PR120, PR24B, and PR42A were each continuously cultured with artificial lake water amended with 10 μM 3Cba. All of the strains except PR42A proved to be capable of contributing to the heterotrophic potential measured in the original mesocosms, in which the highest V_{max} seen was approximately 50 nM/h (11). The results (Table 1) show that, in these monocultures, BR60 outperforms the three other pBRC60 hosts with respect to overall V_{max}, stability of the chlorobenzoate genes, and affinity for 3Cba (a K_m for PR24B could not be obtained since the uptake rate did not vary with substrate concentration for this species). However, the specific maximum uptake rates indicate that PR117 and PR24B may have higher activities per cell. PR117 and PR24B 3Cba+ cells were present at low frequencies, and the resulting cell specific uptake rates appear to be higher than for BR60 and PR120. In addition, the culture of PR117 became contaminated with yeast cells at approximately 100 cells per ml, as did the continuous coculture of BR60(PR117). A pure culture of PR42A lost viability after three volume changes. These results suggest that these isolates survive poorly in pure culture. PR117, and the related strain PR42A, may require alternative nutrients for growth in continuous cultures which may be provided by the contaminating yeast cells.

Laboratory host range of pBRC60. With BR6024 as a donor, only a few species could be tested for the host range of the pBRC60 replicon. Counterselection against the BR6024 donor necessitated the use of minimal media supplemented with simple carbon sources, so fastidious strains could not be tested. In addition, a number of potential

TABLE 1. Kinetic parameters of chlorobenzoate-using strains in continuous culture with 10 μM 3Cba

Strain	V_{max} (nM/h)	Total cells per liter	3CBA+ cells per liter	V_{max} (mol/ cell per h)	K_m (μM)	Loss of 3Cba+ phenotype (% per generation)
BR60	771	6.5×10^9	6.5×10^9	1.2×10^{-16}	0.77	0
PR117	267	6.4×10^9	8.0×10^6	3.3×10^{-14}	2.37	79
PR120	100	1.3×10^9	5.3×10^8	1.9×10^{-16}	1.30	6
PR24B	66	1.0×10^9	1.0×10^5	6.6×10^{-13}	<0.5	23

recipients were naturally resistant to kanamycin. Results for those strains tested that were not naturally resistant to kanamycin are shown in Table 2. Transfers into *P. putida* and *Moraxella* sp. were confirmed by electrophoresis of crude cell lysates; that into *Acinetobacter* sp. was confirmed by hybridization of a Southern blot with a pBRC60-specific probe. Transfer to *P. alcaligenes* (ATCC 14909) could not be detected because of the poor growth of this strain on minimal media.

DISCUSSION

3Cba-degrading bacteria differing from *Alcaligenes* sp. strain BR60 in their taxonomic characteristics have been isolated from mesocosm waters and sediments inoculated with BR60. They all carry the catabolic plasmid pBRC60. There are two alternate explanations for the presence of this particular plasmid in these strains. The first is that the plasmid is indigenous to the microflora of Lake Opinicon, and the selective pressure of 3Cba in the mesocosms increased the plasmid-bearing population. The second explanation is that pBRC60 transferred from BR60 into PR117, PR24B, and PR120 via conjugation. There are two facts that speak against the first explanation. Lake Opinicon sediment microflora sampled prior to the introduction of BR60, plated on 3Cba/ye and probed with a Tn*5271*-specific DNA probe, failed to show organisms with DNA homologous to Tn*5271*. Similar probing of colonies from the sediments of Bloody Run Creek in the Niagara region, where BR60 was isolated, indicated large populations of probe-positive organisms in spite of a lack of recent 3Cba inputs at the time of sampling (10). These facts suggest that pBRC60-bearing bacteria are localized in the Niagara region, where there has been strong selection for 3Cba degradation.

The second explanation, that pBRC60 transferred to wild recipients from BR60, is the more parsimonious. Isolated 3Cba-degrading probe-positive strains carry pBRC60 with no changes in restriction pattern. pBRC60 is a conjugative plasmid, and laboratory matings indicate that a broad range of bacteria act as recipients of a Tn*5*-marked pBRC60 replicon. Under laboratory conditions this plasmid transfers into species in both the beta and gamma subgroups of the proteobacteria (25), although not consistently into either one. Recent studies in this laboratory also confirm that transfer to *E. coli* JM109 is detectible when strong counterselection is applied (17a). The plasmid transfers at very high frequencies into some strains (10^{-1} per donor into conspecifics and to *P. acidovorans*). Finally, alternate pBRC60 carriers were only isolated from those 3Cba-exposed mesocosms to which BR60 had been introduced in inoculum densities of $>10^5$ ml^{-1}.

TABLE 2. Results of filter mating between BR6025 (pBRC40::Tn*5*) and various potential recipients

Species	ATCC no.	Ratio, donor cells/ recipients	Rate of transconjugant cells per donor[a]
Acinetobacter sp. strain DON2		9	6.2×10^{-5}
Moraxella sp. strain G.		19	4.5×10^{-4}
P. acidovorans	15668	0.2	5.4×10^{-2}
P. cepacia	25416	82	2.2×10^{-3}
P. putida biotype A	12633	44	5.5×10^{-4}
Chromobacterium violaceum	12472	200	ND
P. fluorescens	13525	100	ND
P. palleronii	17724	2,000	ND
P. pseudoflava	33668	6.5	ND

[a] ND, Transconjugants not detected.

Plasmid transfer has been demonstrated in natural aquatic environments. Bale et al. (1) demonstrated transfer of a natural large plasmid between *P. aeruginosa* strains on filters applied to the surface of nonsterile stones in a river bed. Rates of transfer equalled 5×10^{-6} transconjugants per donor at 11°C. O'Morchoe et al. (18) documented transfer of R68.45 and pFP5 between *P. aeruginosa* strains in test chambers suspended in 20 to 28°C lake water at rates ranging from 10^{-6} to 10^{-4} transconjugants per donor even though donor and recipient densities were low (10^3 to 10^4 cells per ml). In our study, assuming conservatively that each pBRC60-bearing isolate was from a unique colony on its original isolation plate, 500 transconjugants per ml of sediments or 50 per ml of water must have been present in the mesocosms. Given that 10^5 cells of BR60 per ml were originally introduced to the mesocosm waters, and assuming that the transconjugants did not increase in number, we calculate a comparable conjugation rate of 5×10^{-4} transconjugants per donor. The mesocosm environment was conducive to plasmid transfer: temperatures were above 20°C, and the sediment layer and natural lake water provided an abundance of solid surfaces known to enhance conjugative transfer rates (13). *Alcaligenes* sp. strain BR60 died off in these mesocosms, but O'Morchoe et al. (18) and Gealt et al. (12) documented plasmid transfer from donors that did not actively grow in the test media. Genthner et al. (13) have demonstrated that a large proportion of freshwater gram-negative bacteria are "recipient active" for a broad-host-range plasmid.

In previous work we showed that the frequency of pBRC60 probe-positive bacteria in lake mesocosms dosed with high levels of 3Cba was greater than the introduced population of BR60 in the mesocosms (11). At that time we hypothesized that this might be due to (i) a loss of culturability of BR60 on 3Cba plates or (ii) the existence of other pBRC60-bearing bacteria in the aquatic system. While we have demonstrated the latter, the former cannot be completely discounted. Changes in culturability have been observed in a number of organisms after exposure to the

nutrient limitations of natural aquatic systems (21). In this study we cultured BR60 for 100 days in a low-nutrient artificial lake water to mimic conditions in the original mesocosms. We failed to obtain a state of nonculturability on chlorobenzoate or on general solid media. BR60 survived well in FL media until challenged by the presence of PR117 and contaminating yeast cells, at which time its population dropped to 10% of its former value and 3Cba metabolism was abandoned by the majority of the population. After PR117 was introduced to the BR60 cultures, culturable counts of the two did not sum to the acridine orange direct counts. Based on our observation that PR117 exhibited a loss of culturability following prolonged continuous culture in FL medium in the absence of BR60, we believe that the discrepancy between total plate counts and acridine orange direct counts in the coculture was largely due to nonculturable PR117 cells.

Previous work indicated that 3Cba degradation continued in experimental mesocosms after BR60 died off and could be predicted from the total population of probe-positive organisms (11). We suggest the bacteria isolated in this study were responsible for 3Cba degradation in situ. The continuous culture work shows that most of the organisms carrying Tn*5271* were capable of 3Cba metabolism at substrate concentrations of 10 μM, even those that exhibited poor 3Cba degradation at millimolar levels. While they exhibited comparable or higher levels of specific maximum activity than did BR60, all wild recipients showed a lower affinity for 3Cba and/or greater losses of the 3Cba+ phenotype than did BR60 under these conditions. These results were surprising since, a priori, we expected isolated pBRC60 recipients to be better than BR60 at sequestering small amounts of 3Cba within the natural environment. In hindsight, we speculate that ecological factors, not mimicked in our continuous cultures, selected for the recipients isolated in this study. We suggest that pBRC60 was found predominantly in these species not because the 3Cba genes were most efficiently expressed in them, but because the acquired 3Cba+ phenotype enhanced preexisting levels of fitness superior to that of BR60. Scheuerman et al. (23) demonstrated the importance of protozoan predation and competition for available carbon, nitrogen, and phosphorus in the survival and growth of bacteria in lake water. Klein and Alexander (17) documented the seasonal occurrence of chemical inhibitors in lake water. The alternate pBRC60 hosts might be superior to BR60 in their ability to utilize other carbon substrates typical of Lake Opinicon waters, their abilities to sequester nitrogen and phosphorus in a competitive environment, or their resistance to predation and chemical inhibition. The relatively low affinity of PR117, PR24B, and PR120 for 3Cba leads to more interesting questions about the factors that actually determined their fitness in the mesocosms. Henis and Alexander (15) have proposed that the apparent K_s, μ_{max}, length of lag phase, and resistance to stress of bacteria in lake water receiving low levels of organic nutrients are important parameters in predicting the behavior of those bacteria. Our results indicate that the 3Cba kinetic parameters of the pBRC60 carriers and their resistance to the low-nutrient stress of the continuous monocultures were of limited value in predicting the behavior of these species in situ. de Taxis du Poët et al. (8) have shown that plasmid stabilities and expression rates differ in immobilized cells from those in free-living cells, so studies on the behavior of the strains within biofilms might be particularly illuminating.

The alternate hypothesis that the particular pBRC60-bearing isolates characterized in this work maintained the plasmid but did not express the 3Cba genes in situ, and that our techniques failed to isolate those bacteria actually degrading 3Cba, must also be acknowledged. Recent studies have reported the stability of plasmids after introduction to soil or aquatic systems (2, 7, 9) or in continuous cultures (4, 30). Host bacteria experience long-term growth disadvantages only when plasmids carry constitutive genes or large pieces of heterologous DNA (28, 30). Thus, the persistence of the parent plasmid independent of 3Cba selective pressure is feasible. However, in this study we have documented the persistence of a transposable element that under laboratory conditions is deleted at a rate of 0.07 per generation via a recombinational event. Slow growth rates in the natural environment may mean that losses of Tn*5271* are infrequent relative to losses in rich medium batch cultures.

The majority of pBRC60 recipients isolated from mesocosms were in the PR117 group. The identification of this group remains unclear. From their BIOLOG fingerprint, they appear to be extremely close to BR60, but they are not closely related to the other yellow-pigmented polar flagellated group III pseudomonads. It is tempting to conclude that pBRC60 transferred most frequently from BR60 into taxonomically closely related organisms and was found less frequently in more distantly related species such as *P. fluorescens* or *Pseudomonas* sp. strain PR120. However, culture techniques optimized isolation of strains with properties similar to those of BR60, that is, reasonable growth on 3Cba agar plates and in 3Cba liquid culture. These properties were shared by the PR117 group but not by the other two organisms. PR120 and PR24B, more distantly related to BR60, exhibited dysfunctional expression of the 3Cba genes at high nutrient concentrations. That PR24B and PR120 were isolated under less than optimal conditions for their growth suggests that other recipients of pBRC60, nonculturable under the conditions we used, were present in the mesocosms.

A number of factors would contribute to a failure to detect the full range of bacteria harboring pBRC60. A poor or complete lack of expression of 3Cba genes in alternate hosts exposed to high 3Cba concentrations is an important problem. As an example, *P. putida* (ATCC 12633) will express the Tn*5* kanamycin resistance gene when carried by pBR40::Tn*5*; however, this species does not appear to express the 3Cba genes on pBRC60. Repeated attempts to transfer pBRC60 into *P. putida* yielded no transconjugants when chlorobenzoate was the selective agent, in spite of the fact that *P. putida* is the normal host of another 3Cba catabolic plasmid (pAC25 [6]). We have no idea how many more common bacteria fail to express these genes under laboratory conditions. Other potential hosts may not have been detected because of their requirements for growth factors in the isolation media. No supplement such as yeast extract was used in the isolation of 3Cba degraders because this encouraged abundant growth of oligotrophs, which were difficult to separate from true 3Cba degraders. Some recipients of pBRC60 may simply have been nonculturable. Finally, Tn*5271* is lost from pBRC60 at a rate of 7% per generation. This instability makes it difficult to maintain the 3Cba+ phenotype through successive culture purification steps.

Overall, this study shows that a catabolic plasmid, introduced to a freshwater system in one bacterial strain, can transfer via conjugation to other proteobacteria and can be actively expressed at low (micromolar) substrate concentrations. The proliferation of the plasmid in chlorobenzoate-dosed mesocosms was mediated through alternate hosts that

were, we believe, better adapted a priori to conditions in the mesocosms than the original host. The ability of catabolic plasmids to transfer to and be expressed in indigenous bacteria is clearly beneficial from the point of view of remediating contaminated waters. Our observations are encouraging in that regard.

At the same time, the data presented here emphasize gaps in our knowledge about plasmid behavior after introduction. Prediction of host range is complicated by our limited catalog of nonpathogenic bacteria and problems of culturability and counterselection inherent in the methods used to study conjugation. Under laboratory conditions, none of the pBRC60-carrying recipients isolated in this study were efficient 3Cba degraders relative to the originally introduced strain. We need better methods to assess the fitness of particular plasmid-host associations in the natural environment.

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