Distribution of the Catabolic Transposon Tn5271 in a Groundwater Bioremediation System

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Received 11 June 1993/Accepted 2 November 1993

The distribution of Tn5271-related DNA sequences in samples of groundwater and a groundwater bioremediation system at the Hyde Park (Niagara Falls, N.Y.) chemical landfill site was investigated. PCR amplification of target sequences within the cba genes of Tn5271 revealed similar sequences in the groundwater community and in samples from the sequencing batch reactors treating that groundwater. Cell dilution combined with PCR amplification indicated that cba sequences were carried in about 1/10 culturable bacteria from the treatment system. Characterization of isolates involved in chlorobenzoate and toluene biodegradation in the treatment system indicated that two phenotypic clusters, Alcaligenes faecalis type 2 and CDC group IVC-2, contained all of the Tn5271 probe-positive isolates from the community. These two groups differed phenotypically from recipient groups isolated following horizontal transfer of pBRC60 (Tn5271) in pristine freshwater microcosms. A genetic rearrangement in Tn5271 attributable to the intramolecular transposition of the flanking element IS1071R was detected in an isolate from the treatment system. Comparison of the structure of the intramolecular transposition derivative from groundwater isolate OCC13(pBRC13) with a laboratory-derived intramolecular transposition derivative of pBRC60 revealed similarities. The rearrangement was shown to increase the stability of the plasmid under starvation conditions.

Class I (insertion element [IS] family) and II (Tn3 family) transposable elements are most often associated with the worldwide spread of antibiotic resistance determinants in bacteria following the widespread use of antibiotics (8, 9). However, strong selection for the assimilation of unusual carbon sources has also resulted in the mobilization of catabolic genes by these types of transposable elements. Class II (Tn3 family) elements are represented by toluene-catabolic transposon Tn4653 and the defective naphthalene element Tn4655 of Pseudomonas putida (27, 28). Class I elements are also represented, including the composite transposon Tn5280, which carries genes for chlorobenzoate dioxygenase on P. putida plasmid pPS1 (29). The 2,4,5-T-catabolic genes (ehg) of P. cepacia are bracketed by copies of IS931 to form a putative composite transposon (10). Biphenyl-degradative genes have recently been localized to a transposable 59-kb DNA element designated Tn4371 in Alcaligenes eutrophus A5 (25). We have reported the structure of an unusual composite transposon, Tn5271, found on Alcaligenes sp. strain BR60 plasmid pBRC60 (17). This transposon is flanked by copies of a class II element, designated IS1071, that carries a tnpA transposase gene but lacks resolution functions. The internal region of Tn5271 carries cba genes that code for 3-chlorobenzoate-3,4-dioxygenase (18).

All of the transposable elements described above were discovered in bacteria which had been subjected to intense selection in the laboratory for contaminant biodegradation. In every case, the starting materials for enrichment of these isolates were soils, sediments, or freshwater. The importance of these well-characterized transposons or related elements in situ, during bioremediation of groundwaters, industrial wastewaters, or contaminated soils, is largely unknown. There is no information on the frequency of transposition, the nature of transposition products, or the phenotypic consequences of transposition of elements like these in contaminated environments.

Naturally occurring and genetically engineered bacteria have been applied in laboratory scale studies of strain survival in activated-sludge systems (3, 15, 16). Instability in the plasmid content or chlorobenzoate-catabolic activities of these strains was not observed. However, there is some direct evidence and a great deal of inferential evidence that genetic rearrangements are important during genetic adaptation. Kröckel and Focht have noted the rearrangement of DNA in plasmid pKFL2 of P. putida R5-3 during the adaptation of laboratory cocultures of R5-3 and P. alcaligenes C-0 to chlorobenzene degradation (14). The chlorocatechol ortho ring fission genes of chlorobenzoate-degrading P. putida P111, which are located on the chromosome, have been observed to be deleted from the chromosome and to be transposed to the indigenous 75-kb plasmid pPB111 of this strain (1). The nature of the element carrying these genes is unknown. An estimate based on the amount of DNA acquired by plasmid pPB111 indicates a size of 55 kb. Following the introduction of Alcaligenes sp. strain BR60(pBRC60 [Tn5271]) to freshwater microcosms exposed to 4-chloroaniline (not a substrate for strain BR60), plasmid transfer and selection of Tn5271 in alternate hosts was detected (7). One isolate from these microcosms, Actidium rax delafeldii T6-11, was found to have one copy of IS1071, the insertion sequence flanking Tn5271, on a large plasmid.
unrelated to pBRC60 but had lost the cba genes associated with Tn5271. While several possible origins for the IS on this plasmid were discussed, the most likely origin involved IS1071-mediated cointegrate formation between pBRC60 and an existing plasmid in strain T6-11, followed by resolution of the cointegrate and loss of pBRC60. No phenotypic change could be assigned to the putative transposition event (7). In this study, we determined the distribution of Tn5271-like sequences in samples of groundwater from the Hyde Park landfill site and in a full-scale groundwater remediation system. We also characterized a representative Tn5271-like element from this site and found that a specific genetic rearrangement may increase the stability of Tn5271 under certain conditions.

MATERIALS AND METHODS

Bacterial strains and sources of Hyde Park samples. Alcaligenes sp. strain BR60(pBRC60 [Tn5271] Cba++) was the source of the DNA probes and primer sequences used in this study (17, 18, 31). Alcaligenes sp. strain BR6024(pBRC54 [Tn5271::IS1071(i)], Trp-- Cm++ Cba++) was isolated following a series of seven transfers of BR604(pBRC60 [Tn5271], Trp-- Cm++ Cba++) under nonselective conditions in TYE broth (19, 20). BR60 and BR6024 were routinely maintained on medium A agar (30) containing 4 mM 3-chlorobenzoate, with a supplement of 0.1 mM tryptophan and 50 μg of chloramphenicol ml⁻¹ for maintenance of BR6024. Escherichia coli JM109(pBR2H) and JM109(pBRH4) were maintained on TYE agar supplemented with 50 μg of ampicillin (Sigma Chemical Co.) ml⁻¹ (17). The reference strains used in the cluster analysis were Acinetobacter sp. strain DON2 (30), Alcaligenes sp. strain H850 (General Electric Co., Schenectady, N.Y.), and Comamonas acidovorans NR4 (5). In addition, cultures representing four pBRC60 recipient groups isolated following the transfer of pBRC60 from Alcaligenes sp. strain BR60 to a natural freshwater community were used (6, 7). These included the following isolates: PR117(pBRC60), a group 2 recipient; P. fluorescens PR24B (pBRC60), a group 3 recipient; T3-5(pBRC60), a group 5 recipient; and T6-11(pT611::IS1071), an A. delafeldii group 6 transposition derivative (7).

Hyde Park (Niagara Falls, N.Y.) chemical landfill site sampling was coordinated by the Occidental Chemical Corporation Technology Center, Grand Island, N.Y. The site covers an area of 6.1 hectares and contains an estimated 73,000 metric tons of chemical waste, including halogenated organics. The site has been capped with clay, and a series of three perimeter trenches collect groundwater leachate. Samples of groundwater were collected from wells A and B, which pump the perimeter trench.

Since April 1990, Occidental Chemical Corporation has operated a groundwater bioremediation system on site (32). The treatment system employs the following steps: removal of nonaqueous-phase liquids for incineration, adjustment of the aqueous phase pH to 7.5, venting and aeration to remove volatile organics to vapor phase absorbers and to precipitate iron hydroxides, removal of suspended solids, sand filtration, pretreatment sacrificial carbon absorption of polychlorinated biphenyls and dioxins, addition of urea and phosphoric acid supplements to bring the C/N/P ratio to 150:10:2, three sequencing batch reactors (SBR) in series for biological treatment in 350,000-liter working volumes with a 24-h cycle time, chlorination, sand filtration, posttreatment carbon absorption, and release of the treated groundwater aqueous phase to the municipal sewage system.

Samples from two of the SBR (SBR1 and SBR2), along with samples from wells A and B, were shipped to Ottawa, Ontario, Canada, by courier and divided into three equal portions. One portion was supplemented with sterile glycerol to a final concentration of 10% and frozen at −70°C as a reference sample. Another was frozen at −70°C for subsequent DNA extraction, while the final portion was maintained at 4°C for enrichments.

Continuous culture of SBR samples. A 50-ml SBR2 sample was transferred to a 1.2-liter continuous fermentor (Pegasus Industrial Specialties) and cultured on an artificial contaminant mixture (HPFG medium) containing nutrients and the major organic and inorganic contaminants found in the Hyde Park groundwater. HPFG medium contained 2.5 mM Na₂HPO₄, 2.4 mM sodium benzoate, 1.25 mM (NH₄)₂SO₄, 1 mM phenol, 0.74 mM 2-chlorobenzoate, 0.48 mM ZnSO₄, 7H₂O, 0.25 mM 4-chlorobenzoate, 0.25 mM MgSO₄ 7H₂O, 0.22 mM 3-chlorobenzoate, 75 μM EDTA, 25 μM FeSO₄ 7H₂O, 25 μM toluene, 17 μM CuSO₄ 5H₂O, 6 μM Pb (CH₃COO) 3H₂O, 6 μM ZnSO₄ 4H₂O, 1 μM MnO₄, 2H₂O, and 0.05% yeast extract, pH 7.2. The phenols and toluene were added to the sterile medium after autoclaving. The culture was diluted with this medium at a rate of 0.04 h⁻¹ and maintained for 48 days. The concentrations of benzoate, chlorobenzoates, and phenols in the culture were determined by high-pressure liquid chromatography as described previously (6, 7). All aromatics were detected by UV A254, and concentrations were determined relative to standards.

Isolation and characterization of culturable bacteria. SBR 2 continuous-culture samples were sonicated in an ice bath with a Sonic Inc. Vibracell microprobe at an intensity determined to disperse clumped cells and yield an optimum dilution plate count (50% output, 50% duty, 2 min). The suspended cells were diluted in sterile water and spread on Casitone-peptone-starch (CPS) agar for determination of total viable counts (7). Selection for growth on various defined carbon sources was carried out on minimal medium A agar (30) supplemented with 4 mM 2-, 3-, or 4-chlorobenzoate or in the presence of toluene-saturated air. Isolates from these plates were designated by the letters OCC followed by the isolate number. They were stored at −70°C in 10% glycerol.

Isolates were characterized by Gram staining, oxidase reaction, catalase reaction, and production of fluorescent pigments by established procedures (13, 24). Selected isolates were grown on CPS agar for 18 h at 32°C and inoculated into GN Microplates (Biolog Inc., Hayward, Calif.) for rapid identification in accordance with the manufacturer’s instructions. Microplate substrate utilization profiles were used to generate a matrix of Jaccard similarity indices for all strains, including reference strains and isolates from studies of pBRC60 transfer in a freshwater community (7). A single-linkage cluster analysis was performed on these indices (6).

DNA isolation and restriction mapping. DNA for amplification was isolated from groundwater, SBR, and continuous-culture samples by boiling a 1-ml sample in the presence of 40 mg of insoluble polyvinylpyrrolidone (IPVP; Sigma Chemical Co.) for 10 min. The boiled samples were centrifuged briefly in an Eppendorf microcentrifuge, and 10 μl of the supernatant was taken as template DNA for PCR amplification.

Plasmids were isolated from all Alcaligenes sp. strain BR60 derivatives and all OCC isolates by using a modified Crosa and Falkow procedure as previously described (31). Plasmids pBRC60 (Tn5271), pBRC54 [Tn5271::IS1071(i)],

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and pBRCl3 [Tn5271::IS1071(i)] were mapped in the region of Tn5271 with restriction enzymes NheI, HindIII, EcoRI, BamHI, PstI, and SalI (Bethesda Research Laboratories) by using the digestion buffers and conditions recommended by the manufacturer. The exact placement of restriction sites within the Tn5271 element was based on the original Tn5271 map published elsewhere (17). The placement of restriction enzyme sites outside the border of Tn5271 was based on hybridizations of labeled DNA fragments to restriction enzyme digests of plasmids pBRC54 and pBRC13, as described below.

E. coli JM109(pBRH2) was used as the source of Tn5271 fragment H2 (8.8 kb), and E. coli JM109(pBRH4) was used as the source of internal IS1071 probe H4 (17). Plasmid DNA was isolated from E. coli JM109 by alkaline lysis followed by Sepharose 4B column chromatography (31).

PCRs. Reaction mixtures in a total volume of 100 µl contained 10 µl of 10× reaction buffer supplied with Taq polymerase (Promega Biotech), a 0.2 µM concentration of each deoxyadnucleotide triphosphate, 1.0 µM concentration of each primer, 10 µl of template DNA, and 2.5 U of Taq DNA polymerase (Promega Biotech). Primers (Oligonucleotide Synthesis Laboratory, Queen’s University, Kingston, Ontario, Canada) were synthesized by using sequence information from the cba catabolic genes of Tn5271 (unpublished data). Primer sequences were 17 bp long and located 1,125 bp apart in 1.7-kb EcoRI fragment E13 within the cba catabolic-gene region of Tn5271. Primer E13.4 had the sequence 5′-CAAATCGAGACGTCTC; primer E13.9 had the sequence 5′-CGGACGATACCCAGT. The template DNA was denatured for 5 min at 95°C and cooled rapidly on ice, the PCR reagents were added, and the mixture was placed in a Titertek 96-well microtiter plate in an MJ Research Inc. Programmable Thermal Controller. Amplification was carried out for 30 cycles with denaturation for 2 min at 94°C, annealing for 1 min at 54°C, and synthesis for 1 min at 72°C. The final cycle was followed by 10 min of incubation at 72°C and then a 4°C hold. PCR amplification products were either blotted onto nylon membranes (Micron Separations Inc.) with a Bio-Rad Bio-Dot filter manifold or resolved by 1.0% agarose gel electrophoresis.

To quantitate the number of cells in the SBR2 continuous culture that contained DNA homologous to the Tn5271 catabolic genes, sonicated samples (see above) were diluted to extinction in a 10-fold dilution series with minimal medium A in microtiter plates. The dilutions were supplemented with 0.1% yeast extract and 0.1% tryptone to a final volume of 200 µl and incubated for 4 days at 32°C. Growth in the microtiter plates was then scored as positive or negative relative to an uninoculated control well. The well contents were then diluted to 1 ml, and the cells were treated for DNA extraction and PCR amplification as described above. PCR amplification products were denatured by boiling for 5 min, filtered onto nylon membranes in a Bio-Rad Bio-Dot manifold, fixed to the membrane with exposure to UV light, and hybridized with the H2 fragment of Tn5271 as described below.

Preparation of DNA probes and hybridizations. HindIII fragment H2 of Tn5271, which spans the 3-chlorobenzoate-catabolic genes and part of the flanking IS1071L sequence, and HindIII fragment H4, which is located within IS1071, were purified from agarose gels of HindIII restriction digests of pBRH2 and pBRH4 by using glass milk (Bio 101, Inc.) and labeled with digoxigenin-11-dUTP by using the protocol of the manufacturer (Boehringer Mannheim Canada). Additional DNA probes used to prepare restriction maps of plasmids pBRC13, pBRC54, and pBRC60 were cut from agarose gels of large-plasmid digests, purified with glass milk, and labeled as described above.

DNA transferred to nylon membranes (Micron Separations Inc.) by dot blotting (see above) or by capillary transfer from agarose gels was hybridized with digoxigenin-labeled probes at high stringency as recommended (21; Boehringer Mannheim Canada). Colony hybridizations were done with digoxigenin-labeled probes. Colonies were streaked onto nylon membranes and placed on a 1-ml pool of 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate, 2H2O, pH 7.0)–0.5% sodium dodecyl sulfate for 10 min. Membranes were transferred to glass petri dishes, cells were lysed, and DNA was fixed to the membranes by microwave treatment for 30 s at full output in a Kenmore 650-W microwave oven. The membranes were then washed extensively with 0.1× SSC–0.1% sodium dodecyl sulfate and hybridized as recommended by the manufacturer (Boehringer Mannheim Canada).

Tests for stability of the native plasmid and a transposition derivative. To place the two plasmid derivatives, pBRC60 (Tn5271) and pBRC54 [Tn5271::IS1071(i)], into isogenic hosts for stability experiments, standard filter matings were carried out. Alcaligenes sp. strain BR6024(pBRC60 [Tn5271 Trp+ Cm]) was mated with plasmid-free Centers for Disease Control and Prevention (CDC) group IVC-2 isolate OCC23 (this study). Alcaligenes sp. strain BR6024(pBRC54 [Tn5271:: IS1071(i)] Trp+ Cm) was mated separately with the same recipient strain. Selection of transconjugants was done on 3-chlorobenzoate minimal medium.

OCC23 strains carrying pBRC60 (Tn5271) or pBRC54 [Tn5271::IS1071(i)] were grown to the mid-exponential phase in 20 ml of medium A supplemented with 4 mM 3-chlorobenzoate. The cells were centrifuged and resuspended in 1 liter of deionized and distilled water containing 10 mg of yeast extract and 50 µM 3-chlorobenzoate (starvation medium). Flasks were then incubated at 22°C for 14 days with brief shaking once per day. Samples were taken daily for plate counts on CPS agar and 3-chlorobenzoate agar. At the end of this starvation period, 3×108 Cba+ cells of each strain were mixed together in replicate 1-liter starvation medium flasks and incubated at 22°C for 14 days. Transfers of 100 ml of starvation culture to 1 liter of fresh starvation medium were repeated every 14 days for a total of 56 days. At weekly intervals, plate counts were made on CPS agar and 3-chlorobenzoate agar. Colonies on 3-chlorobenzoate agar were selected at random and screened for plasmid content by using the large-plasmid procedure described above, and the presence of pBRC60 or pBRC54 was determined by restriction digestion. The presence of pBRC60 or pBRC54 restriction fragment sizes (the latter having a unique 7.9-kb HindIII fragment) was determined for plasmids isolated from 10 colonies at each sampling time.

RESULTS

Detection of Tn5271 in groundwater and SBR. The target sequence within the cba operon of Tn5271 was detected in groundwater and SBR samples as shown in Fig. 1. Probe-positive amplifications were detected down to a cell dilution of 10−7 for an SBR2 sample (Fig. 1a). Cell growth was detected in the microtiter plate corresponding to this sample to a dilution of 10−8 (data not shown), indicating that approximately 1 in 10 cultivable bacteria carried sequences similar to the Tn5271 cba genes. We attribute the increase in signal intensity with dilution to a reduction in competition.
FIG. 1. Detection of Tn5271 catabolic gene sequences in groundwater and SBR samples. (a) Dilution dot blot of a sample of SBR2 organisms grown in continuous culture on HPFG medium. Dilutions of the original sample in microtiter plate wells are indicated. Growth was detected to a dilution of 10⁻⁴ in the wells. Hybridization was carried out with HindIII fragment H2 of Tn5271. (b) PCR amplification products from groundwater and SBR samples. Lanes: 1 and 9, lambda HindIII-XbaI size markers (sizes are shown in kilobases on the left); 2, groundwater well A, no IPVP; 3, SBR1, no IPVP; 4, groundwater well A with IPVP; 5, SBR1 with IPVP; 6, SBR2 with IPVP; 7, Alcaligenes sp. strain BR60 control; 8, plasmid pBRH2 control; 10, plasmid pBRC60 control. Negative controls (no template DNA) contained no amplification products (data not shown).

for the tryptone-yeast extract enrichment medium in the microtiter plate dilution wells. A screening of samples from Hyde Park by direct extraction of DNA and PCR amplification showed the presence of Tn5271 cba genes in groundwater well A and SBR samples SBR1 and SBR2 (Fig. 1b). In each case, the 1.1-kb cba target sequence was amplified. Similarity of the 1.1-kb amplification product to Tn5271 was confirmed by hybridization with the H2 Tn5271 probe (data not shown). In the absence of IPVP during DNA extraction, no amplification products were formed from samples of the SBR. In all cases, a positive amplification of the target sequence was observed, non-specific amplification products were formed. Most non-specific amplifications occurred in the SBR1 and SBR2 samples, yielding DNA smaller than 1.1 kb. These products did not hybridize to the H2 probe of Tn5271 (data not shown).

Characterization of the dominant culturable bacteria in the SBR2 community. The SBR2 community, when transferred into a continuous culture on HPFG medium, attained viable cell densities of between 3 × 10⁷ and 9 × 10⁸ CFU ml⁻¹ on CPS agar and between 1 × 10⁷ and 5 × 10⁸ CFU ml⁻¹ on 3-chlorobenzoate agar. Counts on the other chlorobenzoate isomers were in the same range. There was considerable daily variation in the counts due to the tendency of the culture to form a biofilm and grow in macroscopic clumps of cells. The appearance of the culture resembled the clumped appearance of microbial cells in the original SBR samples. The SBR2 culture degraded the mixture of aromatic contaminants in the feed. After 1 week in continuous culture, there were no detectable chlorooaromatics, phenols, or benzoates in the culture supernatant at a detection limit of 10 μM (high-pressure liquid chromatography data not shown). Toluene concentrations could not be determined because the culture was aerated, and losses due to evaporation were not accounted for.

The culture collection from the SBR2 community was screened to eliminate isolates with identical characteristics in the phenotype tests. This narrowed the collection to 40 isolates, which were grouped by single-linkage cluster anal-
olog system as *Alcaligenes faecalis* type II (Biolog similarity, 0.694). The next group, consisting of nine isolates, included the fluorescent pseudomonads, with isolate OCC16 identified as *P. putida* subgroup B (Biolog similarity, 0.781). The only other identifiable group consisted of isolates similar to OCC13, identified by the Biolog system as CDC group IVC-2 (Biolog similarity, 0.660), with five representatives. Outliers giving species identifications with Biolog similarities of >0.5 were isolates OCC34 (*Acinetobacter* genospecies 10), OCC31 (*Klebsiella pneumoniae* subgroup A), and OCC39 (*P. pickettii*). The remaining isolates were not clustered closely with reference strains and were generally poorly identified on the basis of Biolog test results.

All isolates were probed for the presence of sequences similar to that of Tn5271 by using the H2 fragment for colony hybridizations (Fig. 2, column Pr). All of the SBR2 isolates showing positive hybridization (14 of 40) belonged to either the *A. faecalis* type II group or CDC group IVC-2. All of these probe-positive isolates degraded 3-chlorobenzoate, except one, isolate OCC23 in CDC group IVC-2 (Fig. 2, column Pr). Of the three *A. faecalis* isolates and the four CDC group IVC-2 isolates probing positive for Tn5271 and growing on 3-chlorobenzoate that were subsequently tested for growth on 4-chlorobenzoate, all grew immediately upon subculturing or mutated on plates to form colonies that grew on 4-chlorobenzoate (Fig. 2, column 4). No isolates that grew on all three chlorobenzoate isomers were recovered in this screening. Isolate OCC39 (*P. pickettii*), and isolates OCC22 and OCC27 (unidentified) grew on 2-chlorobenzoate alone (Fig. 2, column 2) but did not hybridize to the Tn5271 probe. The tolune-degrading fluorescent pseudomonads (Fig. 2, column T) did not hybridize with the Tn5271 probe.

Cluster analysis showed that the industrial isolates differed phenotypically from the isolates that were the dominant recipients of pBRC60 in freshwater microcosms (reference strains numbered 2, 3, 5, and 6 in the last column of Fig. 2) (6, 7). In all cases, the freshwater isolates were separated in the cluster analysis by a dissimilarity coefficient of between 6 and 7 from the SBR2 isolates that probed positive to Tn5271 (Fig. 2).

**Characterization of putative intramolecular transposition derivatives.** Plasmid extraction of Tn5271 probe-positive isolate OCC13 (CDC group IVC-2) from the SBR2 community revealed a plasmid of approximately 90 kb. Restriction digestion with HindIII revealed all of the fragment sizes found in a pBRC60 HindIII digest plus an additional 8.7-kb fragment (data not shown). The plasmid was designated pBRC13. Digestion with EcoRI and Sall revealed novel fragment sizes of 1.9 and 2.6 kb, respectively. In addition, restriction digestion with enzymes that do not cut within the IS071 element, followed by hybridization of the digest fragments to the H4 fragment of pBRC60 (from within the IS element), revealed the presence of three copies of IS071 on the plasmid, as opposed to the usual two copies on pBRC60 (data not shown). Plasmid pBRC13 was mapped in the vicinity of Tn5271 by using several restriction enzymes combined with probe hybridizations. The results are shown in Fig. 3c.

This structure was closely related to a novel pBRC60 (Tn5271) derivative in *Alcaligenes* sp. strain BR6024 that had been isolated several months previously in unrelated experimental work (19, 20). This strain had been recovered following a series of transfers on a complex medium without selection for the *cba* genes. Characterization of the plasmids in surviving chlorobenzoate-degrading strains from this experiment showed the presence of a plasmid designated pBRC54 that contained a novel 7.9-kb HindIII digestion fragment. Digestion with EcoRI and Sall revealed novel 2.9- and 1.65-kb digestion fragments in comparison with pBRC60 digests. Plasmid pBRC54 was mapped in the vicinity of Tn5271 by using several restriction enzymes combined with probe hybridizations, and the results are shown in Fig. 3b. Both plasmids pBRC54 and pBRC13 exhibited rearrangements that involved insertion of a new copy of IS071 in an inverted orientation relative to the two copies found flanking Tn5271 in pBRC60. In addition, the DNA between the IS071R element and the new copy of IS071 was inverted relative to pBRC60 in both derivatives.

**Stability of the native plasmid and a putative intramolecular transposition derivative.** The stability of pBRC60 (Tn5271) and its putative intramolecular transposition derivative pBRC54 [Tn5271::IS071(i)] was tested by conjugating these plasmids into isogenic hosts. Isolate OCC23 was used as the host for these plasmids because it is representative of an important group of isolates from the SBR (CDC group IVC-2, Fig. 2) and because it lacks large plasmids. This isolate was probe positive for the H2 fragment of Tn5271; however, it failed to grow on chlorobenzoates (Fig. 2). Total genomic DNA isolation and NheI digestion, followed by H2 probing, revealed a 3.2-kb sequence identical in size to the NheI fragment of IS071 but no hybridizing fragment of 10.6 kb indicative of the Tn5271 internal region (data not shown). This isolate therefore contains IS071 in its genome but lacks the *cba* catabolic genes of Tn5271.

OCC23 transconjugants carrying pBRC60 or pBRC54 were isolated on 3-chlorobenzoate minimal medium at frequencies of $1.1 \times 10^{-1}$ and $2.4 \times 10^{-1}$ per recipient, respectively. Restriction digests with the H2 probe showed that the transconjugants both carried their respective plasmids unchanged in structure. Both transconjugants were unstable, like the parent strains, with loss of the *cba* genes occurring at frequencies of approximately $10^{-3}$ cell$^{-1}$ generation$^{-1}$ (data not shown).

The growth of both OCC23(pBRC60) and OCC23 (pBRC54) on starvation medium reached maximum densities of between $7 \times 10^9$ and $9 \times 10^9$ CFU ml$^{-1}$ after 2 days, after which cell numbers declined to between $3 \times 10^8$ and $5 \times 10^8$. FIG. 3. Restriction enzyme digest maps of Tn5271 and putative intramolecular transposition derivatives. The locations of IS071 are indicated by stippled 3.2-kb regions showing the orientations of transcription of the *trpA* (class II transposase) gene of IS071. IS071L is the left element and IS071R is the right element of pBRC60(Tn5271). Abbreviations and symbols: B, BamHI; E, EcoRI; H, HindIII; N, NheI; P, PstI; S, Sall; cba, 3-chlorobenzoate-catabolic genes; open box, PCR amplification target sequence; curved arrow, putative intramolecular transpositions and target sites for generation of pBRC54 and pBRC13. (a) Tn5271 as found on pBRC60(Tn5271) (17). (b) Putative intramolecular transposition derivative pBRC54 [Tn5271::IS071(i)] in Alcaligenes sp. strain BR6024. (c) Putative intramolecular transposition derivative pBRC13 [Tn5271::IS071(i)] in CDC group IVC-2 isolate OCC13.
TABLE 1. Relative stability of pBR60 and intramolecular transposition derivative pBR54 during starvation

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a Minimal 3-chlorobenzoate agar.
b Total count.
c Number of isolates (out of 10), sampled randomly from 3-chlorobenzoate plates, that had plasmid structures like pBR60 or pBR54. Determination of structure was based on HindIII digests of plasmid DNA.

CFU ml^-1 over the next 5 to 7 days. Continued incubation resulted in a very slow decline in numbers for several weeks; however, numbers did not decline below 10^4 CFU ml^-1. When OCC23(pBR60) and OCC23(pBR54) were mixed in equal proportions and starved, the intramolecular transposition derivative was the more stable of the two in replicate experiments (Table 1). While the numbers of CFU per milliliter on 3-chlorobenzoate agar and CPS agar remained more or less constant at the indicated sampling times over 56 days, plasmid content in 3-chlorobenzoate degraders isolated at random from the starvation medium shifted toward the pBR54 structure after 21 to 28 days.

DISCUSSION

Catabolic transposon Tn5271 was originally found on an IncP plasmid (pBR60) in Alcaligenes sp. strain BR60 (17, 31). This isolate was recovered from sediment surface samples from Bloody Run Creek, a small tributary of the Niagara River in the vicinity of the Hyde Park landfill. Very little is known concerning the distribution of this catabolic genotype in contaminated sediments or freshwater. A preliminary study employing colony hybridizations with a pBR60 probe indicated that elevated numbers of probe-positive colonies could be recovered from samples from Bloody Run Creek relative to samples from an uncontaminated control creek (5). However, because a whole-plasmid probe was used, the results could be interpreted as an increased incidence of IncP plasmids similar to pBR60 in the samples.

This study determined the distribution of Tn5271-like sequences in an operating treatment system developed for bioremediation of groundwater contaminants. We found that PCR amplification products from samples of the groundwater and two of the SBR at Hyde Park were the expected length (1.1 kb) and hybridized under stringent conditions to cba gene probes. The DNA primers used for amplification were located entirely within the coding region for the cba catabolic genes; therefore, we rule out the possibility that these were nonspecific amplification products unrelated to the cba catabolic genes. Nonspecific amplification products were observed in all of the samples, especially SBR1 and SBR2, and we attribute these to false priming sites in the complex mixture of DNA recovered from the bioremediation reactors.

The cell dilution-PCR amplification experiments revealed that an SBR2 community actively degrading a mixture of chlorinated and nonchlorinated aromatic contaminants contained Tn5271-like sequences in approximately 1 of 10 cultivable bacteria. This result indicates that the metabolic pathway encoded by the cba genes of Tn5271 is important in the overall conversion of chlorobenzoates to biomass, C1^-ion, CO2, and H2O in the bioremediation system at Hyde Park. The cba-encoded catabolic pathway proceeds through protocatechuate or chloroprotocatechuate intermediates that are subsequently degraded by meta-ring fission enzymes (18). This pathway is quite different from the chlorocatechol ortho-ring fission (clc) pathway expressed in most fluorescent pseudomonads and A. eutrophus strains (4). The significance of this difference in terms of the overall operation of the bioremediation system is unknown. It is conceivable that clc- and cba-like genetic systems coexist in the groundwater and SBR at the site, in which case it would be interesting to know how the substrate is partitioned and how the host bacteria that express these alternative pathways occupy the same niche. We do not know the operating conditions that select for the growth of bacteria which carry alternative metabolic pathways for the same contaminants.

The phenotypic characterization of isolates from the SBR2 continuous culture revealed some important properties of the community as a whole. For example, isolates capable of degrading 2-chlorobenzoate did not degrade other isomers. These findings support previous work showing that isolates capable of degrading ortho-substituted aromatics like 2-chlorobenzoate usually cannot degrade meta- or para-substituted substrates (26). An exception to this was recently reported in P. aeruginosa JB2, which apparently carries two alternative chlorobenzoate-1,2-dioxygenases (11). In our cluster analysis of the SBR2 community, 2-chlorobenzoate degraders were phenotypically diverse and distinct from the two clusters of 3- and 4-chlorobenzoate degraders.

The data for the structure of the SBR2 community also support the general finding that metabolism of chloro-substituted aromatics and that of methyl-substituted aromatics are mutually exclusive unless specific criteria for substituent positioning are met (12, 22). In our study, all 3- and 4-chlorobenzoate degraders were found to belong to the A. faecalis type II or CDC group 1VC-2 cluster, clearly differentiated from the toluene-degrading fluorescent Pseudomonas cluster. Interestingly, in a study of chlorobenzoate degradation by natural and genetically engineered strains in a laboratory...
scale activated-sludge unit, McClure et al. (15) isolated an indigenous 3-chlorobenzoate-degrading organism that was an effective survivor in their system. The isolate, designated AS2, was identified as a CDC group IVC-2 strain. It contained a plasmid, pQM300, which conferred 3-chlorobenzoate degradation on a *P. putida* transconjugant. The plasmid lacked homology to the *tfdC* and *tfdD* genes of pJP4 encoding the essential steps in chlorocatechol metabolism. The relationship of this CDC group IVC-2 isolate and its plasmid to the cluster of similar isolates recovered from the Occidental SBR would be worth investigating.

The study reported here assigns a phenotype (improved stability) to an intramolecular rearrangement of a catabolic transposon and shows that similar rearrangements can be detected in an operating bioremediation system. The Tn5271 element is inherently unstable because of the direct repeats of *IS1071*. Homologous recombination results in deletion of the internal region of Tn5271, so that in the absence of selection for growth on 3-chlorobenzoate the *cfa* genes are deleted at a frequency of $1.6 \times 10^{-3}$ cell$^{-1}$ generation$^{-1}$ (17). Typically, after seven transfers in a medium lacking chlorobenzoate, less than 1% of the cells retain the 3-chlorobenzoate growth phenotype (20). A screen of these surviving 3-chlorobenzoate degraders has revealed a number of chromosome rearrangements of Tn5271 (20) and also strains showing pBCR60 rearrangements (19). One of the latter strains was found to carry an intramolecular transposition-inversion of one of the *IS1071* elements flanking Tn5271 (Fig. 3b). Intramolecular transposition and inversion of the DNA between the transposing element and the target site is a property of class II (Tn3 family) transposons (23). Inversion results from the replicative nature of class II transposition and the specific strand breakage and ligation events that occur in the intramolecular transposition intermediate. Intramolecular transposition-inversion by class II elements requires the activity of the transposase (tnpA) gene product but not the resolvase (res) gene product. *IS1071* contains only the tnpA gene and would therefore be expected to undergo this type of rearrangement to generate multiple copies of the element (17).

The reason for improved stability of the pBCR54 *cfa* genes relative to pBCR60 is unknown. As far as we can detect, these plasmids are identical except for the intramolecular transposition-inversion in pBCR54. One hypothesis is that the additional copy of *IS1071* on pBCR54 provides an additional region of homology for the formation of a secondary structure. An inverted repeat of *IS1071* only 4.2 kb away from Tn5271 may disrupt the recombination complex and lower the frequency of deletion of the catabolic genes. This would explain why a plasmid with the structure of pBCR54 was isolated after many transfers of *Alcaligenes* sp. strain BR60 in the absence of selection for chlorobenzoate degradation (19, 20). Alternatively, there may be some function encoded within the intramolecular transposition target site, or within the inverted region, that is disrupted or altered as a result of transposition. We have recently mapped the plasmid replication origin (*oriV*) of pBCR60 just to the right side of Tn5271, near the target site for intramolecular transposition (2). The function of *oriV* may be affected by the rearrangements in pBCR54 and pBCR13 relative to pBCR60, altering the stability of the plasmid. We are currently investigating the precise location of *oriV* in both pBCR60 and the intramolecular transposition derivatives described here.

Direct comparisons between the stability of pBCR13 from CDC group IVC-2 strain OCC13 and the stability of pBCR54, which it resembles, could not be done. The reason is that pBCR13 was an independent isolate from the SBR2 community and therefore most probably contains many nucleotide sequence differences from either pBCR54 or pBCR60. Differences were not detected at the restriction enzyme level, except in the region of the *IS1071* inversion; however, comparison of the stabilities of these plasmids would not be useful without knowledge of all of the differences that exist between them. If our hypothesis concerning the stability of the intramolecular transposition derivative is correct, then we expect that this plasmid structure would dominate in the treatment facility. We are screening some of the other probe-positive isolates from the OCC collection to assess this possibility.

Attempts are being made to introduce bacterial strains and consortia with specific catabolic activities for bioremediation in various environments. Regulations dealing with microbial introductions are being developed in Canada, the United States, Europe, and elsewhere. The consensus of these regulations seems to be that any genetic modifications of these organisms should be stable, preferably involving irreversible integration of genes into chromosomal DNA. However, in the bioremediation field, this approach is contrary to what we can observe in organisms that have successfully adapted to organic contaminants. Plasmid- and transposon-mediated gene transfer and rearrangements are known to be important for microbial adaptation to complex contaminant mixtures. To require that these adaptive processes not occur seems counterproductive.

**ACKNOWLEDGMENTS**

We appreciate the assistance of Wei-Chi Ying and James Duffy of Occidental Chemical Corporation in providing samples from the Hyde Park site. Maysun Salih provided valuable technical assistance.

This research was funded by a Natural Sciences and Engineering Research Council of Canada Strategic Research Grant to R.C.W.

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