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Selection of *clc*, *cba*, and *fcb* Chlorobenzoate-Catabolic Genotypes from Groundwater and Surface Waters Adjacent to the Hyde Park, Niagara Falls, Chemical Landfill

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The frequency of isolation of three nonhomologous chlorobenzoate catabolic genotypes (*clc*, *cba*, and *fcb*) was determined for 464 isolates from freshwater sediments and groundwater in the vicinity of the Hyde Park industrial landfill site in the Niagara watershed. Samples were collected from both contaminated and noncontaminated sites during spring, summer, and fall and enriched at 4, 22, or 32°C with micromolar to millimolar concentrations of chlorobenzoates and 3-chlorobiphenyl (M. C. Peel and R. C. Wyndham, Microb. Ecol: 33:59–68, 1997). Hybridization at moderate stringency to restriction-digested genomic DNA with DNA probes revealed the chlorocatechol 1,2-dioxygenase operon (*clcABD*), the 3-chlorobenzoate 3,4-(4,5)-dioxygenase operon (*cbaABC*), and the 4-chlorobenzoate dehalogenase (*fcbB*) gene in isolates enriched from all contaminated sites in the vicinity of the industrial landfill. Nevertheless, the known genes were found in less than 10% of the isolates from the contaminated sites, indicating a high level of genetic diversity in the microbial community. The known genotypes were not enriched from the noncontaminated control sites nearby. The *clc*, *cba*, and *fcb* isolates were distributed across five phenotypically distinct groups based on Biolog carbon source utilization, with the breadth of the host range decreasing in the order *clc* > *cba* > *fcb*. Restriction fragment length polymorphism (RFLP) patterns showed that the *cba* genes were conserved in all isolates whereas the *clc* and *fcb* genes exhibited variation in RFLP patterns. These observations are consistent with the recent spread of the *cba* genes by horizontal transfer as part of transposon Tn*5271* in response to contaminant exposure at Hyde Park. Consistent with this hypothesis, IS*1071*, the flanking element in Tn*5271*, was found in all isolates that carried the *cba* genes. Interestingly, IS*1071* was also found in a high proportion of isolates from Hyde Park carrying the *clc* and *fcb* genes, as well as in type strains carrying the *clcABD* operon and the biphenyl (*bph*) catabolic genes.

Metabolic redundancy in bacteria is a common feature of microbial ecosystems. For example, multiple biochemical pathways encoded by genetically divergent operons may function in the degradation of single, relatively simple organic compounds. Among the aerobic members of the class *Proteobacteria* alone, toluene degradation is initiated by a range of dioxygenase (*tod*) and monooxygenase (*xyl*, *tmo*, *tbm*, *tbu*, and *tou*) catabolic operons (2, 5, 15, 33, 45). Two or more of these operons may be expressed in a single strain (16). Similarly, two nonhomologous phenol degradation operons occur in aerobic members of the *Proteobacteria*: the multicomponent phenol hydroxylases represented by the *dmp* operon (30, 31) and the single-component phenol hydroxylases represented by the *pheA* or *tbuD* genes (20, 22). For chlorobenzoic acid (CBA) degradation, there are at least three distinct biochemical pathways found in isolates collected from around the world (Fig. 1) (9, 10, 27, 29, 37, 42). The *clc*, *cba*, and *fcb* operons are nonhomologous and are found at a variety of different loci including chromosomes, plasmids, and transposons in different bacteria (8, 25, 32, 41, 46). A fourth pathway via gentisate has recently been characterized at the biochemical level in *Alcaligenes* sp. strain L6 (21).

While redundancy of metabolic pathways in microbial ecosystems is recognized, few studies have been designed to show whether pollutants in natural environments select a single, dominant genotype or multiple, redundant genotypes. In addition, we know little about how genotypes found at polluted sites differ from those that occur at pristine sites. These were questions we addressed in this study of the selection of *clc*, *cba*, and *fcb* genotypes at the Hyde Park chemical landfill site. In recent studies of the genotypes responsible for 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in 2,4-D-amended soils, it was found that selection of divergent families of 2,4-D-α-ketoglutarate dioxygenase (*tfdA*) genes occurred (12, 17, 24). *tfdA* gene families I and III, representative of the *Sphingomonas* and broad-host-range pJP4-like operons, respectively, were most commonly selected following long-term 2,4-D exposure. In other work, the distribution of the nonhomologous phenol hydroxylase genes was studied in isolates from freshwater and marine samples (31, 36). Genes homologous to the multicomponent phenol hydroxylase *dmp* operon were most often detected in isolates from both of these environments. The single-component phenol hydroxylase operon *pheBA*, which is part of a composite transposon structure, occurred rarely. This property allowed Peters et al. (36) to infer that *pheBA* had transferred horizontally between bacteria in river water following the introduction of this genotype for bioremediation of phenol contaminants.

In recent studies of enrichments from pristine soils, it was found that the ability to degrade 3-chlorobenzoate (3-CBA) was widespread and that the predominant pathway was via chlorocatechol intermediates (12, 13, 23). Nevertheless, genomic DNA of isolates from these pristine soils failed to

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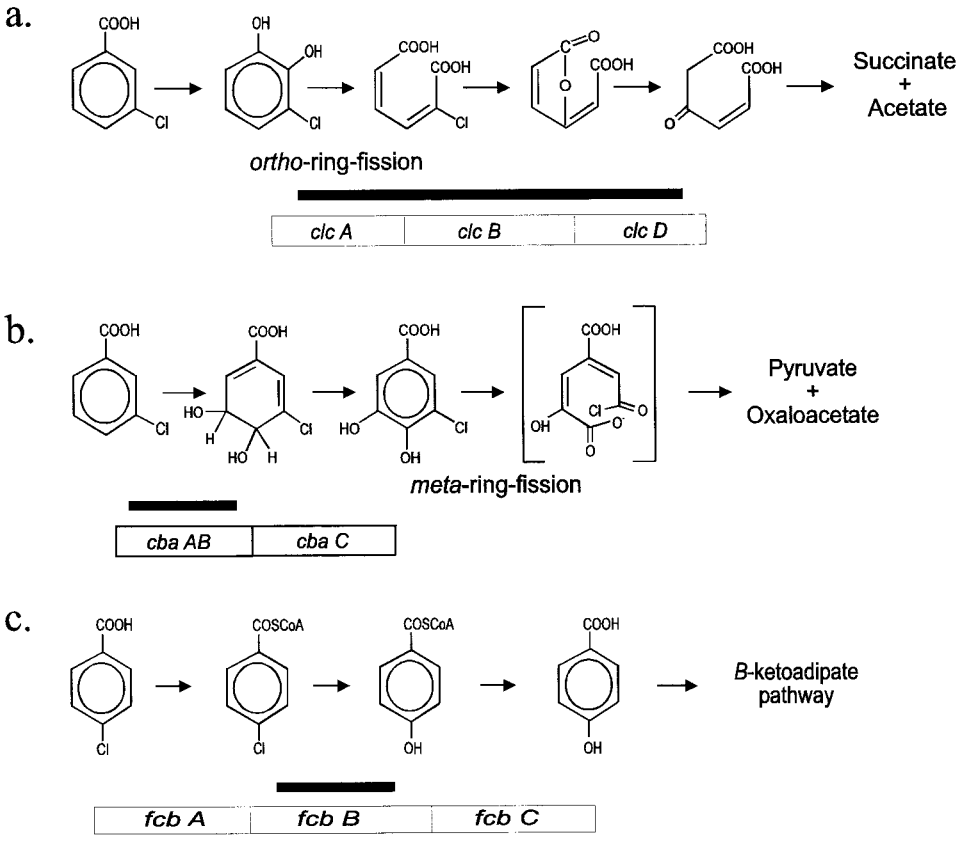

FIG. 1. Representative metabolic pathways encoded by the nonhomologous CBA-degradative operons *clcABD*, *cbaABC*, and *fcbBAC*. The genes that make up these operons are indicated in open boxes below the metabolic steps they encode. Schematic representations of the locations of the three DNA probes used in this study, with respect to the operons, are shown as black bars. (a) Chlorocatechol *ortho*-ring-fission pathway. Following initial attack by a nonspecific dioxygenase such as the benzoate (*benABC*) or toluate (*xylXYZ*) dioxygenase and dihydrodiol dehydrogenase to form chlorocatechols, the *clcABD* gene products catalyze *ortho*-ring fission to open the aromatic ring and continue metabolism. (b) CBA 3,4-(4,5)-dioxygenase pathway. The *cbaABC* genes encode dioxygenase, reductase, and dehydrogenase enzymes that initiate 3-CBA and 3,4-DCBA (not shown) degradation. Protocatechuic acid is also formed in this pathway. The host's protocatechuate 4,5-dioxygenase (*meta*-ring-fission) enzymes then complete the pathway. (c) 4-CBA dehalogenase pathway. The *fcbA* gene encodes a 4-CBA-CoA ligase; *fcbB* encodes the dehalogenase; *fcbC* encodes a 4-hydroxybenzoate-CoA thioesterase. Note that the gene order in the control strain *Pseudomonas* sp. strain CBS-3 is *fcbBAC*.

hybridize with DNA probes derived from two of the known CBA catabolic operons (*clc* and *cba*). Studies using gene probes representative of the range of known CBA metabolic pathways have not been done on samples from environments chronically contaminated by CBA. This is surprising given the central role of CBA degradation in the aerobic metabolism of polychlorinated biphenyls. In a previous study, we determined the 3-CBA, 4-CBA, and 3,4-dichlorobenzoate (3,4-DCBA) degradation potentials of contaminated and noncontaminated freshwater sediment samples taken from three streams at the Hyde Park, Niagara Falls, chemical landfill site (34, 35). The previous study showed that contaminants leaching from the site, which include CBA congeners, chlorobiphenyls (CBPs), chlorinated hydrocarbons, and phenols, have significantly increased the CBA degradation potential relative to that of a noncontaminated control stream nearby. The previous study also revealed seasonal and year-to-year variation in degradation potentials. In the study reported here, we used the same sites to determine the incidence of the *clcABD*, *cbaABC*, and *fcbB* catabolic genotypes among isolates enriched from samples of both contaminated and noncontaminated sources at Hyde Park. Genetic variation at the *clc*, *cba*, and *fcb* loci; the incidence of IS*1071*, which flanks the *cba* operon; and phenotypic relationships between the hosts of these genes were determined.

MATERIALS AND METHODS

Isolation of chlorobenzoate-degrading bacteria. Enrichment cultures were previously described (35). Briefly, samples were taken from the sediment-water interface of several creeks and groundwater seeps in the Niagara watershed, including Bloody Run Creek and Devil's Hole Creek (both contaminated by Hyde Park leachate) and Fish Creek (a noncontaminated control). Samples from groundwater wells within the perimeter trench of the Hyde Park landfill site and from the sequencing batch reactors used for aerobic bioremediation of the groundwater were provided by the owners (Occidental Chemical Co., Niagara Falls, N.Y.). The Hyde Park site and the content of chlorinated aromatics including CBPs and chlorobenzoates in the groundwater leachate have been described previously (34, 35, 49). Subsamples of 950 μl of suspended sediment were enriched in 1-ml batch cultures initially containing 10 μM substrate, followed by sequential transfers to 100 μM, 600 μM, 1.2 mM, and 1.5 mM 3-CBA, 4-CBA, 3,4-DCBA, or 3-CBP in minimal medium A (35). Each enrichment was shaken for 14 days at 4°C, 10 days at 22°C, or 8 days at 32°C, followed by transfer of 500 μl to the next highest substrate concentration. The 1.5 mM enrichments were serially diluted; spread onto minimal medium A plates containing 1.5 mM appropriate CBA congener (3-CBA for the 3-CBP enrichments) plus 1.6% agar (Difco Bacto Agar); and incubated for 8, 10, and 14 days at 32, 22, or 4°C, respectively. All enrichment culture dilutions were also plated onto medium A without a carbon source to distinguish false-positive growth on agar impurities from growth on CBA. Isolated colonies were transferred on selection plates to ensure purity and then were confirmed to degrade the CBA congeners by high-pressure liquid chromatography as previously described (35). A 20% glycerol stock suspension of each purified enrichment culture was stored at −70°C and used as the source of inoculum in all subsequent experiments.

Genomic DNA and plasmid isolation. Table 1 lists the bacterial strains and plasmids used in this study. The genotype screening design was based on previous knowledge of the range of CBA congeners degraded by the control strains and

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Plasmid and/or phenotypes[a]	Genotype[b]	Reference(s)
E. coli			
DH1	pDC100 Ap^r	*clcABD*	11
JM109	pBRH4 Ap^r	IS*1071*	25, 27–29
JM109	pBRH2 Ap^r	*cbaABC*	25, 27–29
JM105	pHUG01 Ap^r Sm^r Su^r	*fcbBAC*	4
Pseudomonas sp.			
CBS3	4-CBA^+	*fcbBAC*	9
AC866	pAC27 3-CBA^+ 4-CBA^+	*clcABD*	11
B356	BPH^+ CBP^+	*bph*	14
Burkholderia sp. strain LB400	BPH^+ CBP^+	*bph clc*	14
Alcaligenes sp. strain H850	BPH^+ CBP^+	*bph*	14
Comamonas sp. strain BR60[c]	pBRC60 3-CBA^+ 3,4-$DCBA^+$	*cbaABC*	25, 27–29

[a] Resistance phenotypes: Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Su^r, sulfathiazole resistance; growth phenotypes: 3-CBA^+, 3-CBA; 4-CBA^+, 4-CBA; 3,4-$DCBA^+$, 3,4-DCBA; BPH^+, biphenyl; CBP^+, polychlorinated biphenyls.

[b] Genotypes: *clc*, chlorocatechol *ortho*-ring-fission pathway genes; IS*1071*, insertion sequence flanking Tn*5271*; *cba*, chlorobenzoate 3,4-(4,5)-dioxygenase pathway; *fcb*, 4-CBA dehalogenase pathway; *bph*, biphenyl dioxygenase pathway.

[c] The identification of *Alcaligenes* sp. strain BR60 has been revised to *C. testosteroni* BR60 based on 100% sequence identity of the 16S ribosomal DNA gene to that of the type strain.

their homologues (Table 1) (9, 10, 37, 42). Isolates from all CBA enrichments were screened for the *cbaABC* and *clcABD* genotypes, but only the 4-CBA isolates were screened for the *fcbB* genotype. The 464 CBA-degrading isolates in the collection were pooled in groups of 10 for genotype screening. A single colony from a selection plate of each isolate was resuspended in 5 ml of tryptone-yeast extract broth (1% tryptone, 1% yeast extract, 0.5% NaCl) along with colonies of nine other isolates. The cell suspensions were grown overnight on a shaker-incubator at the original isolation temperature. Genomic DNA was extracted from the pooled cells by using a 10-times-scaled-up version for DNA isolation as described previously (3). Positive hybridization to the pooled genomic DNAs (see below) was followed by isolation of total genomic DNA from each isolate by the same protocol. The methods of extracting pooled isolate genomic DNAs followed by probe hybridization were validated with the control *Comamonas testosteroni* BR60 and by randomly screening for false negatives among the isolates.

Two plasmid isolation methods were used to screen for high-molecular-weight plasmids in all isolates carrying the known genes, a standard alkaline lysis method (6) and a modified alkaline lysis method for high-molecular-weight plasmids (47). Plasmid DNA from isolates and control strains was detected by electrophoresis in 0.65% agarose at 80 V.

Detection of sibling species with ERIC PCR. Genomic DNA was obtained from all isolates that hybridized with the *clc*, *cba*, or *fcb* probes (see below) as described above. PCR was used to amplify genomic DNA fragments from 50 ng of purified genomic DNA for each isolate by using the enterobacterial repetitive intergenic consensus (ERIC) sequence primers and following the ERIC PCR conditions specified previously (44). Vent polymerase and 10× Vent buffer (New England Biolabs Canada, Mississauga, Ontario, Canada), deoxynucleoside triphosphates (Boehringer Mannheim Canada, Montreal, Canada), and primers (University of Ottawa, Biotechnology Research Institute, Ottawa, Canada) were used with a Perkin-Elmer Cetus 480 Thermocycler (Foster City, Calif.). After PCR amplification, the products (10 μl) were separated on 1.5% agarose gels with Tris-acetate-EDTA buffer at 105 V for 8 h, stained in ethidium bromide, and photographed under UV illumination. Genomic DNAs from all *clc*, *cba*, and *fcb* isolates were subjected to ERIC PCR amplification two or three times to ensure a reproducible pattern of fragment sizes. Isolates with similar ERIC PCR fragment sizes were considered to be sibling species. All siblings isolated from different sites and at different times were included in the isolate collection as independent isolates. One sibling of each pair was selected at random for inclusion in the phenotype analysis (see below).

Probe preparation. Plasmid DNA for probe preparation was isolated by alkaline lysis (6). The three probes were prepared from *clcABD* genes carried on a 4.2-kb *Eco*RI fragment of pDC100 (corresponding to the original *Bgl*II fragment E of pAC27) (11), *cbaABC* genes carried on the 1.1- plus 1.4-kb *Eco*RI fragments (pooled) of pBRH2 (27–29), and the 4-CBA coenzyme A (CoA) dehalogenase (*fcbB*) gene carried on a 1.6-kb *Sac*I-to-*Sal*I fragment of pHUG01 (kindly provided by M. Sylvestre [4]). The *fcbB* gene alone, rather than the operon, was used as a probe because of the broad substrate specificity of the ligase (*fcbA*) and thioesterase (*fcbC*) functions (4, 9).

The probe DNA fragments were separated by electrophoresis on 1.0% low-melting-temperature agarose gels (FMC Bioproducts, Rockland, Maine) submerged in Tris-acetate-EDTA buffer and purified with glass milk according to the protocol supplied by the manufacturer (Gene Clean; Bio 101, Inc., Mississauga, Ontario, Canada). All probes were labeled with digoxigenin-11-dUTP, for 16 to 20 h under reaction conditions recommended by the manufacturer (Boehringer Mannheim Canada).

Genomic DNA restriction fragment length polymorphism (RFLP) analysis and hybridization. Restriction endonucleases (New England Biolabs) were selected for treatment of genomic DNAs based on the known sequences (4, 11, 28): *Nde*I for *clcABD* hybridizations and *Eco*RI for all *cbaABC* and *fcbB* hybridizations. Endonuclease reactions were carried out in 20-μl volumes with 1 μg of DNA according to the manufacturer's protocol (New England Biolabs Canada). Following digestion, DNA was separated on a 0.7% agarose gel by electrophoresis at 30 V overnight. The DNA in the gels was denatured, neutralized, and transferred to nylon membranes by capillary action with materials and protocols provided by Boehringer Mannheim Canada. These blots were fixed under UV light for 5 min prior to hybridization with the digoxigenin-labeled probes. The blots were prehybridized at 65°C for 2 h and hybridized at 65°C overnight in 5× sodium chloride-sodium citrate–0.1% (wt/vol) *N*-laurylsarcosine–0.02% (wt/vol) sodium dodecyl sulfate–1% (wt/vol) skim milk powder (Carnation). The membranes were washed and developed by the chemiluminescence protocol described by the manufacturer (Boehringer Mannheim Canada) followed by detection with Kodak XAR-5 film.

All isolates and type strains that hybridized with either the *clc*, *cba*, or *fcb* probes and type strains were screened for the presence of IS*1071*, the insertion sequence known to flank the *cba* operon in *C. testosteroni* BR60 (8, 25). Total genomic DNAs were digested with *Nhe*I (having two recognition sites within the 110-bp inverted repeats of IS*1071* at positions 43 and 3158), yielding a 3.1-kb fragment that was resolved by gel electrophoresis and hybridized with the *Hin*dIII-H4 probe from IS*1071* (25) as described above. Further characterization of selected IS*1071*-containing genomic DNAs was done by digestion with additional restriction enzymes (*Bam*HI, *Hin*dIII, *Bgl*II, *Eco*RI, *Sac*I, *Sal*I, and *Nar*I [New England Biolabs Canada]) followed by hybridization with IS*1071* probes.

Phenotype analyses of isolates. The carbon substrate utilization range of each isolate carrying the *clc*, *cba*, or *fcb* genes and the control strains, which were all gram negative, was determined with 96-well Biolog GN (gram negative) plates (Biolog, Inc., Hayward, Calif.) according to the suggestions by Kidd-Haack et al. (18) for the analysis of environmental isolates. The cluster analysis program of SSPC for MS Windows (version 6.0) was used to show phenotypic similarities among the Hyde Park isolates, the archetypal strains, and the type cultures *C. testosteroni*, *Serratia ficaria*, *Burkholderia pickettii*, and *Pseudomonas tolaasii*, based on carbon substrate utilization range.

RESULTS

Chlorobenzoate-catabolic isolates. A collection of 464 independent isolates was made following CBA congener enrichment and selective plating from the sampling locations at Hyde Park (35). Each isolate was tested for CBA congener degradation by high-pressure liquid chromatography. The different CBA congeners were generally metabolized to undetectable concentrations (<10 μM) within 14 days at 4°C, 10 days at 22°C, or 8 days at 32°C (data not shown). The isolation frequency of CBA-degrading bacteria from the control sampling sites on unpolluted Fish Creek was low in comparison to that for the contaminated sites (33 of 464 isolates). This was a consequence of the low CBA biodegradation potentials found previously for Fish Creek (35) and of the occurrence of false positives, unable to degrade CBA congeners in liquid culture. Direct 3-CBA enrichment yielded no isolates from samples of Fish Creek; however, prior enrichment on 3-CBP followed by plating on 3-CBA yielded 11 isolates. Another 21 isolates from Fish Creek were enriched on 4-CBA, and 1 isolate was enriched on 3,4-DCBA. The remaining 431 isolates were obtained from the contaminated sites, Bloody Run Creek (206 isolates), Devil's Hole Creek (177 isolates), and the ground-

TABLE 2. Frequencies of isolation of CBA-degrading isolates and the genotype distribution by carbon source

Carbon source[a]	No. of isolates at enrichment temp (°C):				No. of isolates carrying the three genotypes[b] (%)		
	32	22	4	Total	*cbaABC*	*clcABD*	*fcbB*
3-CBA	54	51	22	127	3 (2.4)	9 (7.1)	
4-CBA	91	75	48	214	0 (0)	3 (1.4)	4 (1.9)
3,4-DCBA	15	10	6	31	2 (6.5)	0 (0)	
3-CBA (3-CBP)	45	31	16	92	4 (4.3)	3 (3.3)	
Total	205	167	92	464	9 (1.9)	15 (3.2)	4 (0.9)

[a] Abbreviations are as noted for Table 1, with the addition of 3-CBA (3-CBP), isolates from 3-CBA plates of samples previously enriched on 3-CBP.
[b] Genotypes are as described for Table 1. The numbers in parentheses are the percentages of isolates of each genotype relative to the total number of isolates for that carbon source, as listed in column 4. The *fcbB* genotype was only screened for among the 4-CBA isolates. ERIC PCR revealed four sets of two sibling species among the 15 *clcABD* isolates (see Materials and Methods). Each sibling was isolated independently from different sites and different sampling times; therefore, they are included in the total for that genotype. No sibling species were found among the *cbaABC* and *fcbB* isolates.

water and sequencing batch reactor samples from the Hyde Park facility itself (48 isolates). All carbon sources and all temperatures of enrichment yielded isolates from the contaminated sites, with no clear trends in these selection variables between the contaminated sites.

The greatest number of CBA-degrading isolates was obtained by using 4-CBA for enrichment (214 of 464). More isolates from all CBA enrichments were obtained from the 32 and 22°C enrichments (205 of 464 and 167 of 464, respectively) than from the 4°C enrichments (92 of 464). These proportions reflected the yield of fast-growing isolates that retained the CBA-degradative phenotype after subculturing. They do not necessarily reflect the proportions of different populations active in situ.

Genotype screening of isolates. The *clcABD*, *cbaABC*, and *fcbB* genes were detected among the 464 isolates in the collection (Tables 2 and 3). All isolates carrying these genes were enriched from the contaminated sites adjacent to Hyde Park. The known CBA-degradative operons or genes were not detected among the 33 isolates collected from the control site, Fish Creek. The genomic DNA of the vast majority of isolates (436 of 464) failed to hybridize to the known CBA-degradative probes. No single CBA-degradative genotype was numerically dominant in the isolate collection from the Hyde Park sites. The *cbaABC* and *clcABD* genotypes were both found in the 3-CBA and 3-CBA (3-CBP) enrichments. The *cbaABC* genotype was also represented in 2 of 31 isolates from the 3,4-DCBA enrichments. The *clcABD* and *fcbB* genotypes were represented in isolates from 4-CBA enrichments in almost equal proportions. None of the 4-CBA enrichment cultures harbored the *cbaABC* genotype.

Sibling pairs were found only among the 15 *clcABD* isolates by ERIC PCR (data not shown). Each of the four sibling pairs was found in different samples from the same stream or groundwater source. They were independently enriched from different sites along the stream bed, different groundwater wells, or different sequencing batch reactors. They were therefore considered to be independent isolates. Interestingly, in the case of siblings BRC3-2-3A and BRC4-4-3, different enrichment regimens were followed. The former was enriched on 3-CBA directly, while the latter was enriched initially on 3-CBP followed by isolation on 3-CBA agar. All *cbaABC* and *fcbB* isolates were nonclonal. No single isolate or sibling pair showed hybridization to more than one of the CBA-degradative gene probes used in this study.

A large proportion of the *clcABD* isolates (6 of 15 [40%]) was obtained from the 4°C enrichment cultures (data not shown). By contrast, none of the *cbaABC* isolates was collected from enrichment cultures incubated at 4°C. Isolates containing different CBA-degradative genes were found in the same sample in some cases. For example, isolates WellD1-3A, WellD1-3B, and WellD1-3C, enriched from one sample of Hyde Park groundwater on 3-CBA, at 4, 22, and 32°C, respectively, contained the *clcABD* genes in the first two isolates but the *cbaABC* genes in the last (Fig. 2). Another example of the coexistence of different genotypes in the same sample is found

TABLE 3. Numbers of *clc*, *cba*, and *fcb* isolates; their substrate ranges; and genetic properties

Genotype[a]	No. of isolates distributed by sampling site[b]						Substrate range[c]			RFLP pattern(s)[d]	HMW plasmid[e]	IS*1071*[f]
	Total	BRC	DHC	Well	GW	SBR	3-CBA	4-CBA	3,4-DCBA			
clcABD	15	6	0	3	4	2	12	6	0	ii–vi	8	6
cbaABC	9	6	2	1	0	0	8	4	8	i	4	9
fcbB	4	4	0	0	0	0	0	4	0	ii–iv	2	3

[a] Genotypes are as described for Table 1.
[b] Abbreviations for isolate sources: BRC, isolates from samples of the sediment-water interface of Bloody Run Creek (this site was previously shown to be contaminated by Hyde Park leachate [35]); DHC, isolates from the Devil's Hole Creek groundwater seep contaminated by Hyde Park leachate (35); Well, isolates from sampling wells drilled into the unconsolidated wastes at Hyde Park; GW, isolates from contaminated groundwater collected from the perimeter trench around the Hyde Park site; SBR, isolates from the sequencing batch reactors used for treating contaminated groundwater at Hyde Park (49).
[c] Numbers of isolates able to degrade selected CBA congeners. Substrate abbreviations are as described in the footnotes to Tables 1 and 2. Some isolates degraded more than one CBA congener.
[d] RFLP patterns grouped according to the fragment sizes following restriction enzyme digestion as follows. For *clcABD*, *Nde*I restriction fragments that hybridized with the *clcABD* probe were as follows: i, 19.5, 9.0, and 1.6 kb (detected in the control strain AC866 only); ii, 7.4 kb; iii, 9.5 and 5.7 kb; iv, 13.3, 10.3, and 5.7 kb; v, 10.3 and 5.7 kb; vi, 7.1 kb. For *cbaABC*, *Eco*RI restriction fragments that hybridized with the *cbaABC* probe represented one pattern only: i, 1.4- and 1.1-kb fragments (as for the control strain BR60). For *fcbB*, *Eco*RI restriction fragments that hybridized with the *fcbB* probe were as follows: i, 2.0 and 1.6 kb (detected in the control strain CBS3 only); ii, 4.9 kb; iii, 11.2 kb; and iv, 3.4 kb.
[e] Number of isolates containing plasmids greater than 30 kb in size. HMW, high molecular weight.
[f] Number of isolates containing the IS*1071* 3.1-kb *Nhe*I restriction fragment detected by hybridization with the *Hin*dIII fragment H4 probe.

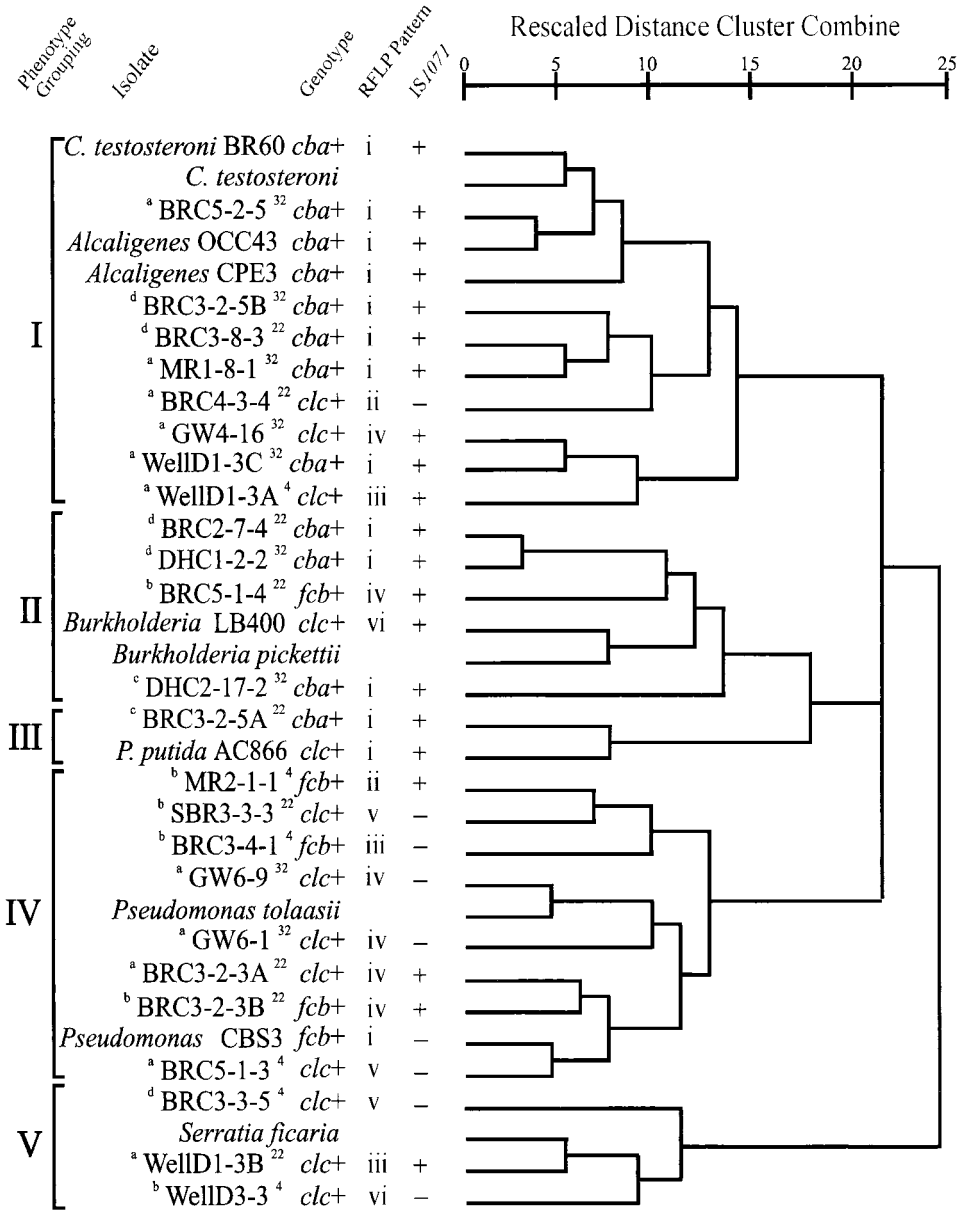

FIG. 2. Phenotype distance cluster dendrogram of the *clcABD*, *cbaABC*, and *fcbB* isolates from the Hyde Park landfill based on carbon source utilization (Biolog GN). Control strains (*clcABD*, *cbaABC*, and *fcbBAC*) and representative β- and γ-*Proteobacteria* members from the Biolog GN database are included for reference. Phenotype grouping, an arbitrary division of isolates into five groups at a rescaled distance of 15. Isolate, the numbers following the source designation (Table 3) in each isolate name give the sampling site, the isolate number, and the sampling trip number (35), respectively. The superscript preceding the isolate name refers to the enrichment carbon source: a, 3-CBA; b, 4-CBA; c, 3,4-DCBA; d, 3-CBA (3-CBP). The superscript after the isolate name gives the enrichment temperature (degrees Celsius). Genotype, as described in the legend to Fig. 1. RFLP pattern, as described in footnote *d* to Table 3. IS*1071*, detection of the insertion sequence in the genome by hybridization to *Nhe*I digests. Rescaled distance cluster combine, dendrogram created from average linkage (between groups) data (SPSS).

with isolates BRC3-2-3A (*clcABD*) and BRC3-2-3B (*fcbB*) from a single sample of Bloody Run Creek (Fig. 2).

RFLP patterns of target genotypes. The RFLP patterns of the *clcABD*-harboring isolates following *Nde*I digestion and hybridization with the *clcABD* probe were diverse, and all were different from the RFLP pattern for the control *Pseudomonas putida* AC866(pAC27). The RFLP types were classified into six groups (Table 3 and Fig. 2). The isolates in RFLP groups iii, iv, and v contained a 5.7-kb fragment in common but showed variation in other fragments. One isolate, BRC4-3-4, had a unique 7.4-kb fragment (group ii). One isolate, WellD3-3, had a single 7.1-kb fragment as found for the control *Burkholderia* sp. strain LB400 (group vi).

All *cbaABC*-containing isolates had the same RFLP pattern as *C. testosteroni* BR60 following *Eco*RI digestion and hybridization with the *cbaABC* probe (Table 3 and Fig. 2). The RFLP patterns of the *fcbB* isolates were diverse, and all were different from the pattern for *Pseudomonas* sp. strain CBS3 (Table 3 and Fig. 2). Two *fcb* isolates, BRC5-1-4 and BRC3-2-3B, shared the same RFLP pattern (group iv).

Chlorobenzoate congener degradation and phenotype cluster analysis. The *clcABD* isolates degraded 3- and/or 4-CBAs

as predicted from previous studies of the specificity of the modified catechol *ortho*-ring-fission pathway (9, 10) (Table 3). Siblings exhibited the same patterns of CBA substrate utilization (data not shown). No isolates in this group metabolized 3,4-DCBA.

Almost all *cbaABC* isolates degraded 3-CBA and 3,4-DCBA (Table 3), with the exception of isolate DHC2-17-2, which degraded 4-CBA and 3,4-DCBA, and isolate BRC2-7-4, which degraded only 3-CBA (data not shown). Three isolates degraded all three isomers. These results are also consistent with the biochemical characterization of the pathway (27, 29). Interestingly, while 4-CBA enrichment did not yield isolates carrying the *cbaABC* genes (Table 2), three of the nine isolates with these genes degraded the 4-CBA substrate (Table 3).

The *fcbB* collection of four isolates degraded only 4-CBA as predicted from the specificity of the dehalogenase pathway in other isolates (4, 9, 38, 50).

Cluster analysis of the isolates carrying the known genes, based on the ability to utilize 95 carbon sources in Biolog GN plates, is presented in Fig. 2. Several strains from the Biolog database representative of the β- and γ-*Proteobacteria* were included in this dendrogram as reference strains. The *clcABD* siblings are represented by only one strain of each pair. Five major phenotypic groups were identified, arbitrarily separated at a rescaled distance of 15. The *cbaABC* isolates clustered in groups I and II, which included the β-*Proteobacteria* genera *Alcaligenes*, *Comamonas*, and *Burkholderia*, with a single *cbaABC*-containing isolate in the fluorescent *Pseudomonas* (γ-*Proteobacteria*) group III along with the control *P. putida* AC866. This isolate was confirmed to be a fluorescent pseudomonad by detection of fluorescence on King's B agar (19).

The *clcABD* isolates were broadly distributed in the β-*Proteobacteria* groups I and II, as well as groups IV and V. Group IV includes *P. tolaasii* and the *fcb* control *Pseudomonas* sp. strain CBS3, while group V contains *S. ficaria*, all of which belong to the γ-*Proteobacteria*. Interestingly, the only group not represented in the *clcABD* isolate collection, group III, contained the *clcABD* control strain *P. putida* AC866. Isolates that were grouped by *clcABD* operon RFLP pattern are widely separated by phenotype. For example, three of four nonsibling isolates in the *clcABD* RFLP group iv are found in phenotype group IV (*Pseudomonas*), but the other member of this RFLP group is found in phenotype group I along with *Alcaligenes* and *Comamonas* control strains. *clcABD* RFLP groups v and vi show similar broad distributions in the phenotype cluster analysis. Hosts of the *fcbB* genotype were primarily found in group IV, with a single isolate from group II. Of additional interest in the group of WellD1-3A, -B, and -C isolates from the same Hyde Park groundwater sample discussed above is the observation that two of these isolates, WellD1-3A and WellD1-3C, are phenotypically very similar (group I [Fig. 2]) but carry different chlorobenzoate-degradative operons (*clcABD* and *cbaABC*, respectively). The third isolate in this group, WellD1-3B, is phenotypically very different (group V [Fig. 2]) and yet carries a *clcABD* operon with the same RFLP pattern as the group I isolate WellD1-3A. Another example of the presence of different CBA-degradative genotypes in similar hosts is the occurrence of the *clcABD* genes in BRC3-2-3A and of the *fcbB* gene in BRC3-2-3B, very similar hosts (although not siblings) within phenotype group IV (Fig. 2). In keeping with previous studies of the genus *Burkholderia* that have emphasized its metabolic versatility, we have found that phenotype group II, which includes this genus, contains isolates with all three CBA-degradative operons (Fig. 2).

Plasmid content and the incidence of IS*1071*. High-molecular-weight plasmids (>30 kb) were detected from isolates representative of all three genotypes (Table 3). There was no strong correlation of plasmid content with the genotype, the RFLP group, the temperature of isolation, or the CBA substrate utilization profile. We note that the three *clcABD* isolates enriched on 3-CBP and transferred to 3-CBA (Table 2) contained high-molecular-weight plasmids that may be involved in either 3-CBP or 3-CBA degradation, or both.

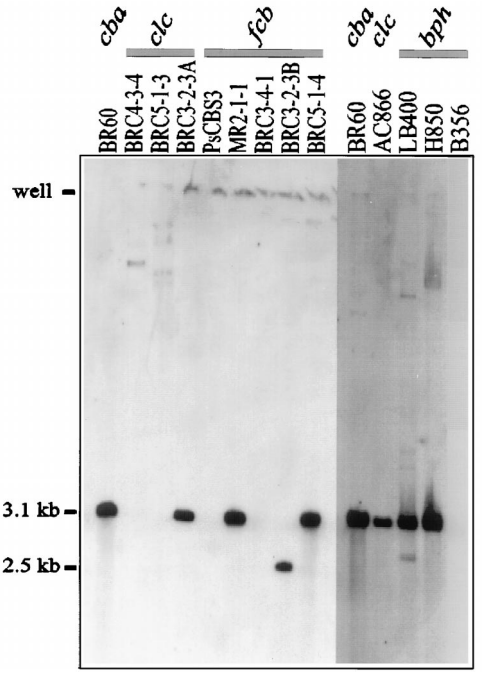

FIG. 3. Hybridization of the IS*1071* internal probe H4 to genomic DNA treated with *Nhe*I for selected isolates that carried *clcABD* and *fcbB* genes for chlorobenzoate degradation. Control genomic DNA digests for *C. testosteroni* BR60 (*cbaABC*); *P. putida* AC866 (*clcABD*); and the biphenyl-CBP-degrading strains *Burkholderia* sp. strain LB400 (*bph clc*), *Alcaligenes* sp. strain H850 (*bph*), and *Pseudomonas* sp. strain B-356 (*bph*) are also shown. The size of hybridizing DNA fragments was determined relative to the mobility of *Hin*dIII-digested λ DNA. Note that the copy number of IS*1071* is not apparent in these digests because the *Nhe*I recognition sites are located in the inverted repeats of the element. For example, the genomic DNA of strain BR60 contains seven copies of IS*1071*.

The results of genomic DNA digestion with *Nhe*I and hybridization to the insertion sequence IS*1071* showed that this element was not limited to the *cbaABC* collection. All nine *cbaABC* isolates contained IS*1071*, and 4 of 11 (nonsibling) isolates carrying the *clcABD* genes and 3 of 4 isolates carrying the *fcbB* gene also hybridized to the H4 probe for this element (Table 3 and Fig. 3). All isolates yielded identical 3.1-kb fragment sizes expected for IS*1071* with the single exception of one of the *fcbB* isolates, BRC3-2-3B, which yielded a 2.5-kb fragment (Fig. 3). Additional restriction digestions of genomic DNAs of selected IS*1071*-containing isolates showed no variation in the expected fragment sizes other than that noted above for isolate BRC3-2-3B (data not shown). Several of the control strains including the *clcABD* control *P. putida* AC866 and the biphenyl-degrading controls *Burkholderia* sp. strain LB400 (*clcABD*) and *Alcaligenes* sp. strain H850 contained the IS*1071* element. There was no correlation between high-molecular-weight plasmid content and the incidence of IS*1071* in any of the isolates or controls, consistent with previous observations of the plasmid and chromosomal location of this element (8, 25).

DISCUSSION

All three of the well-characterized *clc*, *cba*, and *fcb* genotypes for CBA degradation can be found in roughly equivalent but low proportions among the isolates from the contaminated water sources at Hyde Park. None of these genotypes were enriched from the noncontaminated sites along Fish Creek. This observation in combination with the study of pristine soils by Fulthorpe et al. (12, 13) may indicate that prolonged selection by chloroaromatic pollutants in the environment is required to increase the incidence of these genotypes to detectable levels. Additional evidence for an influence of prior CBA exposure on community composition comes from the 3-CBA selection studies. Almost 30% of isolates from the contaminated sites were obtained by direct enrichment on 3-CBA, whereas no isolates were obtained in this way from Fish Creek. Preenrichment steps on 3-CBP were required in order to isolate 3-CBA degraders from this noncontaminated site, and the known genes were not detected among these isolates.

Evidence presented here shows that multiple genotypes for CBA degradation may occur simultaneously in the bacterial community degrading these pollutants. This conclusion is supported by our observation of multiple genotypes isolated from the same samples, for example, isolates WellD1-3A and -B (*clcABD*) with WellD1-3C (*cbaABC*) and also BRC3-2-3A (*clcABD*) with BRC3-2-3B (*fcbB*) (Fig. 2). Without additional biochemical and genetic studies on each isolate to show the involvement of each operon in CBA degradation, we cannot be certain of the link between genotype and phenotype. Nevertheless, in all cases the utilization by each isolate of the different CBA congeners matched the expected substrate range for the genotype. Also, for isolates carrying the *cbaABC* operon the evidence linking the presence of the genes to the degradative phenotype is strong. All of these *cbaABC*-containing isolates were unstable, showing spontaneous deletion at high frequencies of the operon and loss of the CBA-degradative phenotype (data not shown). We have shown previously that this is due to recombination between the IS*1071* copies flanking the *cbaABC* genes (8, 25).

This study has not addressed particular advantages of one genotype over another at this site, nor the frequency of these genotypes in situ, which may differ substantially from the frequencies reported in Tables 2 and 3. Nevertheless, in combination with data showing enhanced CBA biodegradation potentials as a result of chloroaromatic pollutant exposure (35), the complete study demonstrates that there exist ecological niches for each of the well-characterized CBA degradation operons at the sediment-water interface of the contaminated streams and that they are potentially involved in contaminant removal at these sites. The findings point to the necessity for comprehensive screening of microbial communities involved in contaminant removal in situ in order to assess the overall potential for bioremediation and for bioaugmentation. Screening for single genotypes will in most cases be insufficient. Furthermore, this study has shown that the largest part (>90%) of the isolate collection failed to hybridize with the known *clc*, *cba*, and *fcb* probes, even under relatively relaxed stringency. Clearly, the genetic diversity for CBA degradation is much greater than previously suspected. The results for the 3-CBA (3-CBP) enrichments in which *cba* and *clc* genes were found in a total of 8% of the isolates (Table 2) indicate that communities of aerobic bacteria involved in polychlorinated biphenyl degradation also show genetic diversity for CBA degradation, with much of that diversity yet uncharacterized. These findings emphasize the need to continue to characterize new pollutant biodegradation pathways and their genetic determinants.

Some part of the isolate collection that failed to hybridize to the known CBA catabolic genes may carry genes for other CBA degradation pathways. Krooneman et al. have shown that *Alcaligenes* sp. strain L6 uses a novel 3-CBA degradation pathway via gentisate to grow at low oxygen tensions and dilution rates (21). These environmental conditions may well occur at the sediment-water interface of the sites we sampled; however, genetic probes for this pathway are not yet available. The high proportion of nonhybridizing isolates within the collection may also carry homologues of the *clcABD*, *cbaABC*, or *fcbBAC* operons that have diverged in sequence sufficiently to escape detection by hybridization. Fulthorpe et al. obtained chlorocatechol 1,2-dioxygenase (*clcA*-like) gene sequences by PCR with redundant primers with 3-CBA-degrading bacteria from pristine soils (12, 13, 23). They showed that the PCR-amplified sequences were less than 60% similar to the *clcA* gene of *P. putida* AC866(pAC27) and were more closely related to the *tfdC* gene from 2,4-D-degrading isolates. As reported by Schlomann (37), this level of sequence divergence suggests that evolution of chlorocatechol dioxygenases has been ongoing for the past 70 to 90 million years.

The *fcbBAC* operon also shows evidence of extensive sequence divergence. For example, the *fcbB* (dehalogenase) and *fcbA* (CoA ligase) genes in the gram-positive isolate *Arthrobacter* sp. strain SU are 81 and 75% identical, respectively, to the genes in *Pseudomonas* sp. strain CBS3, while the putative *fcbC* (thioesterase) genes are nonhomologous (38). The gene orders also differ in these isolates (*fcbABC* in strain SU).

There is no evidence that similar variation exists in the *cbaABC* operon. In all isolates examined to date from the Hyde Park site (references 26 and 47 and this study), as well as an isolate from polychlorinated biphenyl-contaminated soils from Italy (8), the *cbaABC* operon was completely conserved. In the latter study comparing isolates from different continents, the sequence similarity in the *cbaA* gene was 99.3% and the operons were carried on similar, although not identical, composite transposons. The RFLP data described in Table 3 and additional data for hybridization with other Tn*5271*-derived probes (not shown) indicate that the nine *cbaABC* isolates found in this study carry composite transposons similar or identical to Tn*5271* (34). The evidence therefore suggests that horizontal transfer has been more important for the recent dissemination of the *cbaABC* genotype than for the other genotypes we examined. There is now substantial evidence for the involvement of gene mobilization and horizontal transfer in the biodegradation of pollutants in situ, for instance, in the natural attenuation of chlorobenzene contamination of an aquifer at Kelly Air Force Base in Texas (43) and the degradation of coal-tar-derived naphthalene in groundwater seeps (39). The observed noncongruency of the phylogenetic trees of the *tfdA* and 16S ribosomal DNA genes of 2,4-D-degrading bacteria shows that interspecies gene transfer has been an important factor in their evolution (24).

The *clcABD* operon was found to be broadly distributed in all five phenotypic groups of CBA-degrading bacteria (Fig. 2). This may reflect the broad distribution of relaxed-specificity benzoate and toluate dioxygenases needed to initiate this pathway. It may also reflect the involvement of this operon in the degradation of other chlorinated aromatic compounds that are converted to chlorocatechols. For instance, the study of chlorobenzene-degrading isolates from a contaminated aquifer at Kelly Air Force Base noted above has shown that genes almost identical to *clcABD* have combined in situ with chlorobenzene dioxygenase genes in *Ralstonia* species to naturally attenuate the pollutants at the site (43). Chlorobenzenes and chlorotoluenes are among the contaminants at Hyde Park (49), so that

the observed selection of *clc* genes in a variety of different hosts may have been in response to other chlorinated aromatic pollutants in addition to CBA and CBPs. We have not tested our *clc* isolate collection for degradation of other chlorinated aromatic compounds.

In contrast to the *clc* genes, the *cba* genes appear to be involved only in CBA and CBP degradation (27, 29, 47). The *cbaABC* host range, which is primarily in the β-*Proteobacteria*, has previously been correlated with the distribution of the protocatechuate *meta*-ring-fission pathway (26). This is a consequence of the preferential metabolism of the 3-CBA metabolites protocatechuate and 5-chloroprotocatechuate through the *meta*-ring-fission pathway in *cbaABC* hosts (26, 29). There are very few reports of 3,4-DCBA mineralization by pure cultures (8, 29); nevertheless, *Acinetobacter* sp. strain 4-CB1 carrying an *fcbBAC*-like dehalogenase operon is able to cometabolize 3,4-DCBA in the presence of 4-CBA (1). The *cbaABC* operon was the only one associated with 3,4-DCBA-degrading isolates in our collection. Metabolism of this congener through the *cbaABC* pathway also requires the (5-chloro)-protocatechuate *meta*-ring-fission pathway (29).

The uniform association of the *cbaABC* genes with IS*1071* in all isolates that carry this operon (Table 3) (8, 25, 34, 46, 47) suggests that horizontal transfer of the composite transposon Tn*5271* is the primary mode of dissemination of these genes. An unexpected finding was the occurrence of IS*1071* in the genomes of isolates that carried nonhomologous operons (Table 3 and Fig. 2 and 3). Here we show that this element occurs in 6 of 15 (40%) of the *clcABD* isolates and 3 of 4 (75%) of the *fcbB* isolates (Table 3) and that it occurs in all five phenotypic groups defined in Fig. 2. In addition, IS*1071* was detected in control strains AC866 (*clcABD*), LB400 (*bph clcABD*), and H850 (*bph*) (Fig. 3). A previous review of the distribution of IS*1071* linked this element with a diverse collection of genes for the degradation of aliphatic and aromatic contaminants (8). Many of these contain composite transposon structures flanked by direct repeats of IS*1071*. Recently, an example of this kind of transposon coding for the degradation of 2,4-D has been described (48). In the latter case, the IS*1071* elements are both interrupted by a class I insertion sequence, IS*1471*, inserted at identical positions in the *tnpA* genes of the flanking elements. A nested insertion of another transposon within IS*1071* may explain the 2.5-kb hybridizing fragment that we observed for isolate BRC3-2-3B (Fig. 3) for which the restriction pattern differs from those of all other isolates. Despite the natural instability of IS*1071* composite transposons, this element is likely a major contributor to biodegradative gene rearrangements and mobilization in bacteria in contaminated environments.

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