Microbial Ecology

Chlorobenzoate-Degrading Bacteria in Similar Pristine Soils Exhibit Different Community Structures and Population Dynamics in Response to Anthropogenic 2-, 3-, and 4-Chlorobenzoate Levels

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Abstract

A study was conducted to determine the diversity of 2-, 3-, and 4-chlorobenzoate (CB) degraders in two pristine soils with similar physical and chemical characteristics. Surface soils were collected from forested sites and amended with 500 μg of 2-, 3-, or 4-CB g^{-1} soil. The CB levels and degrader numbers were monitored throughout the study. Degraders were isolated, grouped by DNA fingerprints, identified via 16S rDNA sequences, and screened for plasmids. The CB genes in selected degraders were isolated and/or sequenced. In the Madera soil, 2-CB and 4-CB degraded within 11 and 42 d, respectively, but 3-CB did not degrade. In contrast, 3-CB and 4-CB degraded in the Oversite soil within 14 and 28 d, respectively, while 2-CB did not degrade. Approximately 10^7 CFU g^{-1} of degraders were detected in the Madera soil with 2-CB, and the Oversite soil with 3- and 4-CB. No degraders were detected in the Madera soil with 4-CB even though the 4-CB degraded. Nearly all of the 2-CB degraders isolated from the Madera soil were identified as a *Burkholderia* sp. containing chromosomally encoded degradative genes. In contrast, several different 3- and 4-CB degraders were isolated from the Oversite soil, and their populations changed as CB degradation progressed. Most of these 3-CB degraders were identified as *Burkholderia* spp. while the majority of 4-CB degraders were identified as *Bradyrhizobium* spp. Several of the 3-CB degraders contained the degradative genes on large plasmids, and there was variation between the plasmids in different isolates. When a fresh sample of Madera soil was amended with 50, 100, or 200 μg 3-CB g^{-1}, 3-CB degradation occurred, suggesting that 500 μg 3-CB g^{-1} was toxic to the degraders. Also, different 3-CB degraders were isolated from the Madera soil at each of the three lower levels of 3-CB. No 2-CB degradation was detected in the Oversite soil even at lower 2-CB levels. These results indicate that the development of 2-, 3-, and 4-CB degrader populations is site-specific and that 2-, 3-, and 4-CB are degraded by different bacterial populations in pristine soils. These results also imply that the microbial ecology of two soils that develop under similar biotic and abiotic environments can be quite different.

Introduction

Chlorobenzoates are common soil pollutants that occur at polychlorinated biphenyl (PCB)-contaminated sites as products of microbial PCB-metabolism [5]. Historically, much of the efforts to isolate chlorobenzoate (CB) degraders from the environment have focused on these contaminated sites [17, 24, 46]; however, several relatively recent studies have found widespread occurrence of chlorobenzoate degrading bacteria even at noncontaminated sites [18, 19, 47]. For example, one study successfully isolated 3-CB degraders from pristine ecosystems in five different continents [18]. The study also found that geography and vegetation affected which degraders developed at each site.

It is likely that chlorobenzoate-degrading bacteria develop and are maintained in pristine sites because of naturally occurring halogenated compounds. Plants, insects, bacteria, and fungi are known to release halogenated chemicals into the environment [22]. These halogenated compounds may be intentionally produced, such as chemical defenses, or may be by-products of metabolism [16]. In fact, at least 68 different genera of Basidiomycetes have been shown to produce halogenated, primarily chlorinated, metabolites [10]. It also ap-

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pears that chlorobenzoates specifically are naturally produced in the environment [35].

With the widespread occurrence, albeit at low levels, of chlorinated hydrocarbons naturally in the environment, it is not surprising that many different bacteria have been identified that degrade the three monochlorobenzoates, 2-, 3-, and 4-CB. Isolated bacteria that degrade 2-CB include *Alcaligenes* spp., *Burkholderia* spp., and *Pseudomonas* spp. [23, 24, 31, 41, 42]. The 3-CB degraders include *Acinetobacter* spp., *Alcaligenes* spp., *Bacillus* spp., *Burkholderia* spp., *Comamonas* spp., *Pseudomonas* spp., and *Ralstonia* spp. [6, 13, 20, 28, 33, 36, 38, 39, 46]. The 4-CB degraders include *Alcaligenes* spp., *Arthrobacter* spp., *Burkholderia* spp., *Nocardia* spp., *Pseudomonas* spp., and *Sphingomonas* spp. [1, 4, 8, 14, 27, 30, 43].

Even though many of the same genera of bacteria degrade 2-, 3-, and 4-CB, the three compounds are typically degraded by different enzymes and pathways. In addition, there is diversity in the known degradation pathways for 2-, 3-, and 4-CB. Some researchers have found overlap in the degradation pathways of 2-, 3-, or 4-CB and have even isolated bacteria capable of degrading two or more mono CBs and even di- and trichlorobenzoates [1, 24]. Chlorobenzoate degradative genes are also commonly found on transmissible plasmids or transposons, which may explain the widespread distribution of chlorobenzoate degradative genes in diverse bacterial genera—possibly resulting from horizontal gene transfer events [13, 21, 40, 45, 47, 48].

While there has been considerable characterization of chlorobenzoate degraders isolated from individual sites, and the diversity of 3-CB degraders has even been compared on several different continents, to our knowledge a study has not been conducted to examine the diversity of the three possible monochlorobenzoate (2-, 3-, and 4-CB) degraders at similar sites. Knowledge of this diversity along with an understanding of the microbial ecology of the degrader community that develops when a pristine soil is first exposed to high levels of CBs may help determine the mechanisms of microbial adaptation and degrader development, such as horizontal gene transfer, that occur as a result of the contamination event. The sky islands of the southwestern United States and northwestern Mexico provide a unique opportunity to examine the issue of CB degrader diversity in similar environments. The sky islands are composed of about 27 mountain ranges that rise from the desert floor with vegetation progressively changing from desert scrub to oak woodland to pine forest as the elevation increases. Even though the sky islands have similar climates at a given elevation, there is considerable biogeographical diversity between the islands due to their spatial isolation [9].

For this study, we collected soil from two different sky islands and exposed the samples to 2-, 3-, or 4-CB. We monitored changes in CB concentrations and determined the effects on total bacterial and CB-degrader populations. Degraders were also isolated throughout the study to assess changes in the degrader community structure. The isolated degraders were screened for the presence of plasmids and were identified based on 16S rDNA sequences. Additionally, CB genes in selected degraders were characterized.

Methods

Soils. Soils were collected from the surface horizon of pristine, forested sites of the Coronado National Forest in Madera Canyon near Green Valley, AZ, and Oversite Canyon near Sierra Vista, AZ. Both sites contained scrub oak vegetation and were approximately 1670 m in elevation. The Madera and Oversite soils had pH values of 6.5 and 6.8, respectively. Percent organic C, N, sand, silt, and clay values were 2.6, 0.17, 76, 20, and 4, respectively, for the Madera soil and 2.8, 0.23, 74, 21, and 5 for the Oversite soil. Field-moist soil was passed through a 2-mm sieve and 100 g dry weight equivalent added to polypropylene jars. Sterile deionized water was added to bring the soil water content to 140% of −0.3 MPa soil water potential. Microcosms were incubated for 7 d at 27°C to allow for acclimation of indigenous microorganisms prior to imposing treatments.

Addition of Chlorobenzoate to Soils. Stock solutions of 2-CB (98%, Aldrich Chemical Co. Inc., Milwaukee, WI), 3-CB (99%, Sigma Chemical Co. Inc., St. Louis, MO), and 4-CB (99%, Aldrich) were prepared by a method similar to that previously described for 2,4-dichlorophenoxyacetic acid (2,4-D) [11]. Microcosms, containing Madera or Oversite Canyon soil, were amended with 0 or 500 μg of the respective CB g^{-1} dry soil. All treatments were conducted in triplicate. Following addition of all amendments, soil was brought to approximately field capacity (175% of −0.3 MPa soil water potential) and incubated at 27°C.

Quantification of Chlorobenzoate Biodegradation. The CB was extracted from soil by placing 1.2 g moist soil into 9.5 ml of extracting solution (6 μM Zwittergent detergent and 0.2% sodium hexametaphosphate) [7], and mixing on a horizontal shaker at high setting (280 osc min^{-1}) for 5 min. A 1.0-mL aliquot of the soil-extraction solution was placed in a 1.5-mL microcentrifuge tube and centrifuged at 16,000 g for 10 min. Supernatants were passed through 0.2 μm pore-size polypropylene filters (Pall Gelman, Ann Arbor, MI) prior to HPLC analysis. The concentration of CB was

determined with a Waters Associates LC Module HPLC system and a Waters C_{18} column (3.9 mm × 150 mm) at a wavelength of 235 nm. Elution was isocratic, and the mobile phase was acetonitrile-acidified water (50:50, v/v) with a flow rate of 1 mL min^{-1}. Waters Millennium32 (version 3.05) software was utilized for peak integrations. Acidified water, pH 2.6, was prepared with phosphoric acid. Several noncontaminated soil samples were analyzed to confirm that no natural soil components eluted at the same time as CB.

Enumeration of Culturable Bacteria. The soil extraction solution from the CB quantification was serially diluted in 0.85% NaCl buffer. Total numbers of culturable CB degraders were determined by spread-plating 0.1 mL of the appropriate dilution onto Bushnell-Haas Agar (BH) containing 500 mg of the appropriate CB L^{-1} as a C source [3]. Noble agar (Difco, Detroit, MI) was used as the solidifying agent in BH in order to reduce undesired carbon compounds in the media. Additionally, BH was amended with 50 mg bromthymol blue L^{-1} as an indicator. Culturable, heterotrophic bacteria were enumerated on R2A (Difco). Plate counts were conducted after incubation of plates at 27°C for 6 d (R2A) or 14 d (BH).

Isolation and Characterization of Dominant Indigenous Degraders. Dominant chlorobenzoate degraders were isolated during the study. Bacteria were selected from plates at the highest (most dilute) dilution that produced a countable number of bacterial colonies. Selected bacteria were streaked to purity on the appropriate CB medium and then ultimately streaked onto R2A. Approximately one colony of bacterial cells was harvested, placed into 20 μL of sterile, molecular-grade water, and stored at −20°C. Isolated degraders were grouped based on enterobacterial repetitive intergenic consensus (ERIC) PCR of the harvested cells with a method modified from that described by Versalovic et al. [44]. The FailSafe PCR System (Epicentre, Madison, WI) was used to generate ERIC fingerprints by adding primers (0.1 μM of each in final reaction) from Versalovic et al. [44] to FailSafe PCR 2× PreMix F and Enzyme Mix according to the manufacturer's instructions. The PCR reaction was conducted in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) with an initial denaturation step for 7 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 4 min at 68°C. The PCR products were visualized on a 1.2% agarose gel following electrophoresis and ethidium bromide staining. Selected bacteria from each ERIC profile were also screened for the presence of plasmids [25]. Plasmid extracts were separated with gel electrophoresis and visualized with ethidium bromide staining along with the 85-kb plasmid from *Comamo testosteroni* BR60, which was utilized as a size standard [33]. The ability of isolated degraders to metabolize CB was confirmed by inoculating each isolate into modified M9 broth (without glucose or thiamine) [3] containing 50 mg of the respective CB L^{-1}, shaking cultures at 150 rpm and room temperature, and monitoring CB levels at 230 nm with UV spectroscopy.

Phylogenetic Analysis. Selected bacteria from each ERIC profile were identified by 16S rDNA PCR using the forward primer 338F (5′-CTCCTACGG GAGGCAGCAG-3′) and reverse primer 784R (5′-GGACTACCAGGGTATCTAATCC-3′) (J. McQuaid, pers. comm.). The 100-μL reaction contained 1× PCR Buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 2.5 U AmpliTaq Gold polymerase (PerkinElmer, Foster City, CA), and 5 μL of harvested bacterial cells. The reaction was conducted in a GeneAmp PCR System 2700 and consisted of an initial hot-start denaturation step for 10 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C prior to a final 5-min extension step at 72°C. Positive PCR products were confirmed using agarose gel electrophoresis and ethidium bromide staining. The PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). The forward primer was used for dye termination PCR sequencing performed at the University of Arizona's Laboratory of Molecular Systematics and Evolution sequencing facility. Following sequencing, the reverse primer sequence was removed from the 3′ end of the PCR product sequence. The sequence was then compared to sequences in GenBank using BLASTN 2.2.5 [2]. Additionally, the 16S rDNA sequences of isolated degraders were aligned with CLUSTAL W (v. 1.8), and a phylogenetic tree was generated using PAUP and GCG Wisconsin Package (v. 10.2, Accelrys, San Diego, CA) with Neighbor-Joining construction, Jukes–Cantor distance correction, and a bootstrapping value of 100.

Characterization of Chlorobenzoate Degradative Genes in Selected Degraders. We initially attempted to amplify regions of CB degradative genes from selected 2-, 3-, and 4-CB degraders. We utilized the primers designed by Suzuki et al. [41] for *cbdA* (2-halobenzoate dioxygenase) to amplify the gene for 2-CB degradation (Table 1). The 3-CB primers were designed based on the *tfdC* (chlorocatechol 1,2-dioxygenase) and *tfdD* (chloromuconate cycloisomerase) genes in the 3-CB degrader *Ralstonia eutropha* JMP134 (GenBank accession no. M35097) [37]. Primers were also designed based on the putative 4-chlorobenzoate Co-A ligase gene in *Bradyrhizobium japonicum* USDA 110 (GenBank accession no. AP005948). The PCR amplifications were performed

Table 1. Primers and probes used for characterization of 2-, 3-, and 4-chlorobenzoate (CB) genes in isolated degraders

CB	*Probe*	*Primer*	*Primer sequence (5′–3′)*	*Source*
2-CB	*cbdA*	cbdA-F	TTTCTCGCACACGAAAGTCAG	41
		cbdA-R	ATCCTCGTATTGACGGATTCG	41
3-CB	*tfdC*	tfdC-F	AGTGAACAAAAGAGTCAAGGATG	This study
		tfdC-R	CTCGATAACGAAGTTGATGTTC	This study
	tfdD	tfdD-F	AGATGTCGATCACTACCGTGCA	This study
		tfdD-R	GACCTACGCCAGTGGGAACCT	This study
4-CB	—	4CB-F	GCGATCCAATGCGATTGCCT	This study
		4CB-R	ATGATCTCGCCCTCCTCGCCA	This study

using *Taq* DNA polymerase (Promega, Madison, WI) with one cycle at 94°C for 5 min followed by 30 cycles of 95°C for 0.5 min, 56°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 10 min. The amplified products were analyzed by electrophoresis in a 1% agarose gel and purified using the Qiaquick Gel Extraction kit (Qiagen). Approximately 300 ng of purified DNA from positive PCR reactions was used for dye termination PCR sequencing with each the corresponding forward and reverse primers at the University of Arizona's Laboratory of Molecular Systematics and Evolution sequencing facility. The sequence was then compared to sequences in GenBank using BLASTN and BLASTX programs [2].

Additionally, genomic and plasmid DNA in the 2- and 3-CB degraders above were assayed for the presence of CB degradative genes using Southern blot analysis. Genomic DNA was isolated using the Blood & Cell Culture DNA mini kit (Qiagen). Plasmid DNA was isolated according to the method of Kado and Liu [25], except that two phenol–chloroform extractions were performed followed by a precipitation step with the same volume of isopropanol. One microgram of the appropriate DNA was digested with *Bam*HI or *Eco*RI, separated in a 0.8% agarose gel, transferred to nylon membranes, and hybridized according to the manufacturer's instructions (MSI Inc., Westborough, MA). Gene specific primers for *cbdA* were used to PCR amplify the probe (~900 bp) from *Burkholderia* sp. TH2 (Table 1) [41] according to the methods described above. Probes for *tfdC* (~800 bp) and *tfdD* (~1100 bp) were generated from *R. eutropha* JMP134 using gene specific primers (Table 1). The PCR amplification products were purified from an agarose gel with the Qiaquick Gel Extraction kit (Qiagen). Each probe was randomly labeled with $\alpha-^{32}P$ (dCTP) using the RadPrime DNA labeling system (Invitrogen, Carlsbad, CA). Probes were hybridized with the DNA gel blot overnight at 62°C in buffer composed of 5× SSC, 1× Denhardt's, and 0.5% SDS. The membranes were washed once with a solution (2× SSC, 0.1% SDS) at room temperature and twice with a second solution (0.2× SSC, 0.1% SDS) for 20 min at 65°C prior to overnight exposure to Kodak BioMax MS film at −80°C.

Chlorobenzoate Dose Response Assays. Lower CB amendment levels were tested for soils in which specific CBs did not degrade in order to determine if the initial level of CB (500 μg g^{-1} soil) was responsible for inhibition of indigenous degraders or if the soil in fact had no detectable degraders even at lower CB concentrations. Uncontaminated soils were amended with 50, 100, 200, or 500 μg of the respective CB g^{-1}. Soils were incubated and sampled using the procedures outlined for the original microcosms except that degraders were enumerated, after 47 d of incubation, on BH containing 100 mg of the appropriate CB L^{-1} from which selected degraders were isolated and characterized. The ability of isolated degraders to metabolize CB was confirmed by

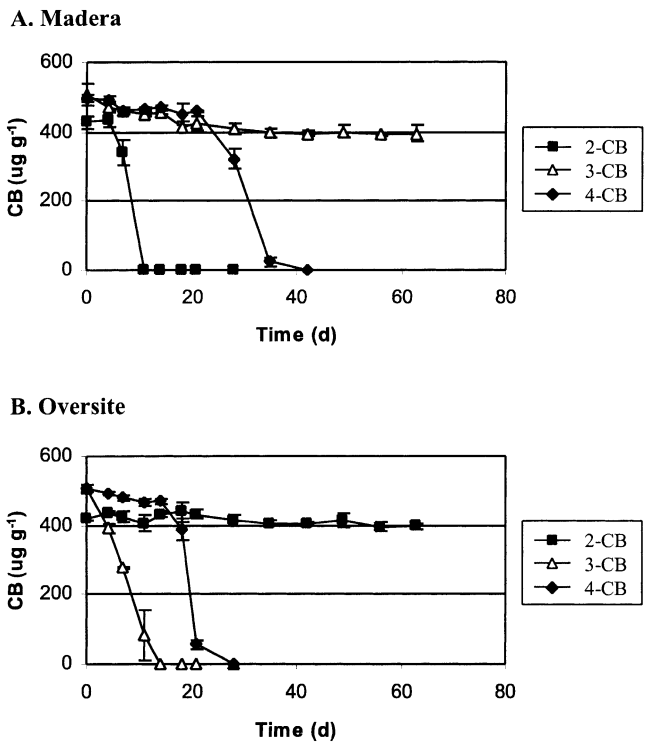

Figure 1. Levels of 2-, 3-, and 4-chlorobenzoate (CB) in (A) Madera and (B) Oversite soils after amendment with 500 μg CB g^{-1}. Error bars represent the standard deviation of three replicate microcosms.

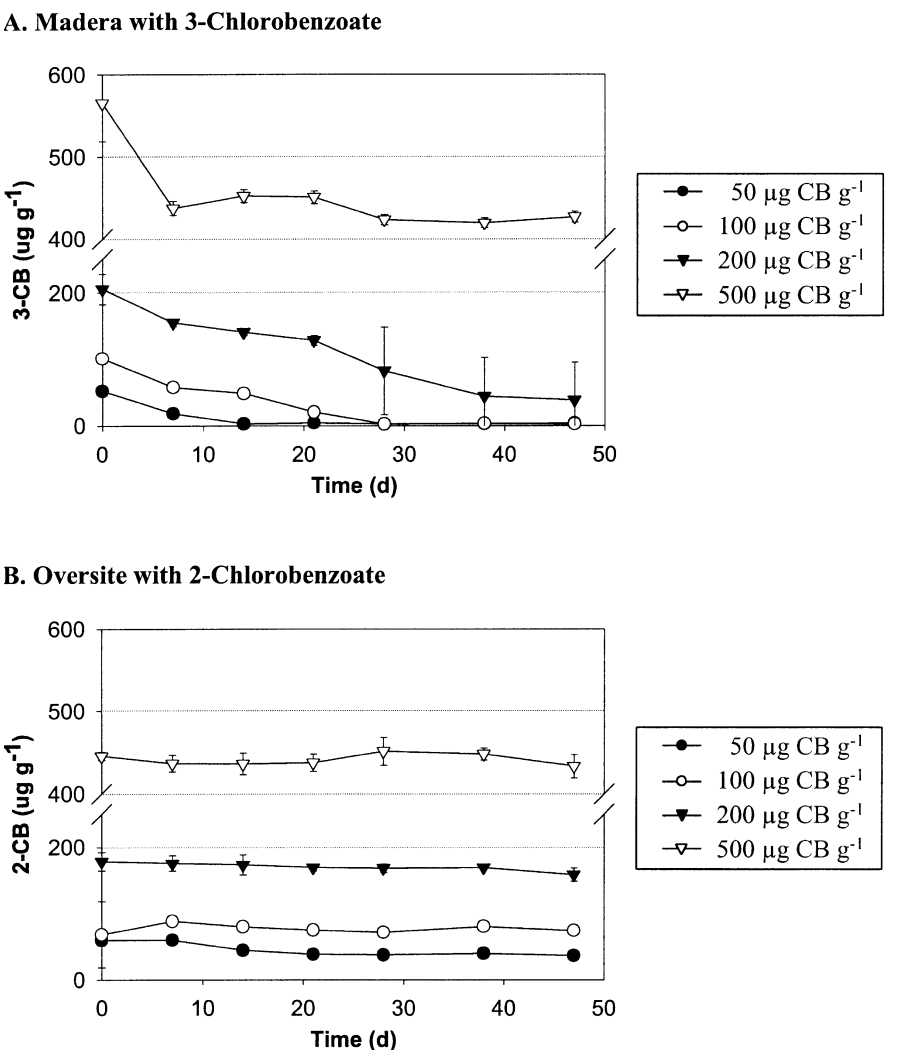

Figure 2. Levels of (A) 3-chlorobenzoate (CB) in Madera soil and (B) 2-CB in Oversite soil following amendment with 50, 100, 200, or 500 µg CB g^{-1} in the dose-response assays. Error bars represent the standard deviation of three replicate microcosms.

inoculation into M9 broth containing 10 mg of the appropriate CB L^{-1}.

Results

Chlorobenzoate Degradation Following Bioaugmentation. In the Madera soil, 2-CB and 4-CB levels dropped from the original, 500 µg CB g^{-1} soil, level to below the limit of detection (~5 µg g^{-1}) within 11 and 42 d of incubation, respectively (Fig. 1). Little or no 3-CB degradation was observed in the Madera soil. In contrast, 3-CB and 4-CB degraded within 14 and 28 d, respectively, in the Oversite soil while only negligible degradation of 2-CB occurred within 63 d.

In the dose response assay, the 50 and 100 µg 3-CB g^{-1} amendments were degraded in the Madera soil within 14 and 28 d, respectively (Fig. 2). Likewise, the 3-CB in the 200 µg 3-CB g^{-1} soil microcosms was mostly degraded by 49 d while the 3-CB concentrations in the 500 µg 3-CB g^{-1} soil microcosms remained level as in the original Madera microcosms. In contrast, no 2-CB degradation was detected in the Oversite soil at any amendment level.

Enumeration of Culturable Bacterial Populations and Chlorobenzoate Degraders. Both the Madera and Oversite soils had large culturable bacterial populations of approximately 10^8 CFU g^{-1} soil (Fig. 3). Following addition of 3- and 4-CB to Madera soil, culturable bacterial numbers decreased slightly and then eventually increased to above the initial levels after about 21 to 28 d of incubation. The 2-CB contamination did not appear to affect the culturable bacterial numbers in the Madera soil. Likewise, culturable bacterial numbers first decreased slightly in the Oversite soil after addition of 3- and 4-CB, but then increased above initial levels after about 14 d. Again, 2-CB did not appear to affect the culturable bacterial numbers in the Oversite soil.

In the Madera soil, culturable 2-CB degrader numbers increased from below the limit of detection to over

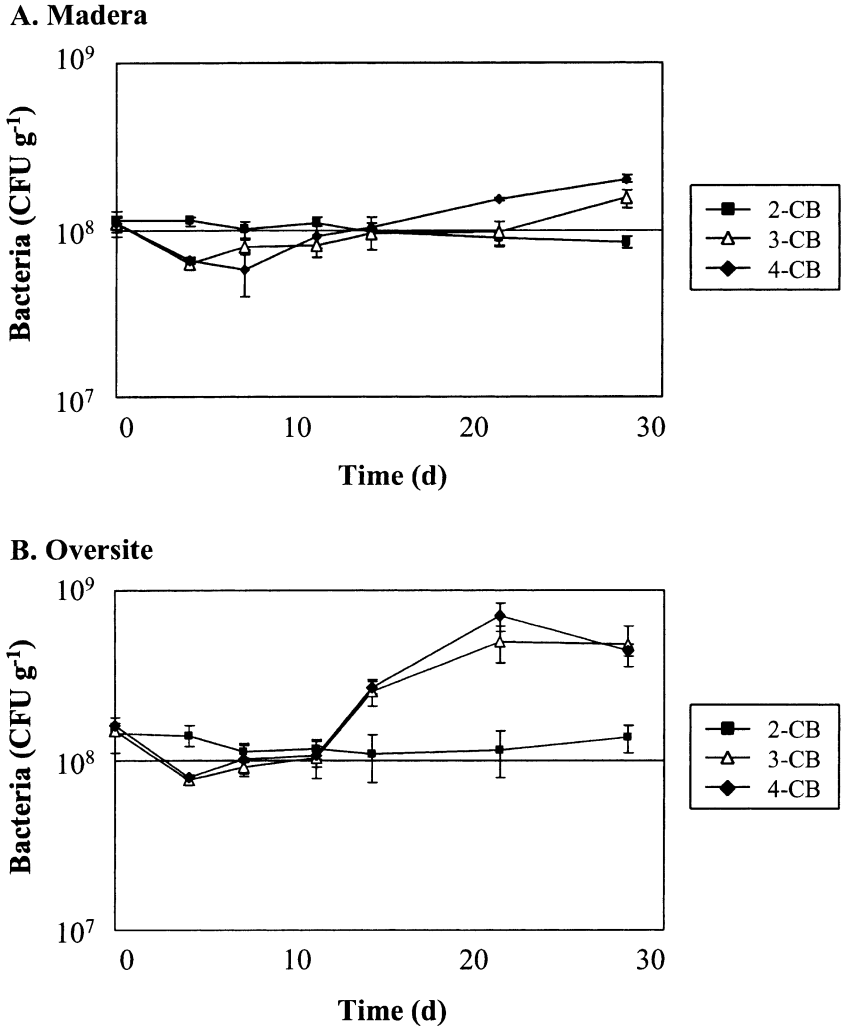

Figure 3. Number of culturable bacteria in (A) Madera and (B) Oversite soils after amendment with 500 μg 2-, 3-, or 4-chlorobenzoate (CB) g^{-1}. Error bars represent the standard deviation of three replicate microcosms.

10^7 CFU g^{-1} soil within 7 d and mirrored the degradation of 2-CB (Fig. 4). No 3- or 4-CB degraders were detected. Conversely, 3- and 4-CB degraders in the Oversite soil increased from $\sim 10^3$ CFU g^{-1} initially (<30 degrader colonies per plate were detected at 0 and 4 d for 3-CB and 0, 4, and 7 d for 4-CB) to 10^7 and 10^9 CFU g^{-1} within 14 and 21 d, respectively, and followed 3- and 4-CB degradation. No 2-CB degraders were detected in the Oversite soil throughout the study.

In the dose-response assay, small populations of 3-CB degraders, about 10^4 CFU g^{-1} soil, were detected in the Madera soil amended with 50, 100, and 200 μg 3-CB g^{-1} soil (Table 2). No 3-CB degraders were detected in the 500 μg 3-CB g^{-1} microcosms. The 2-CB degraders in the Oversite soil were not assessed since no degradation was detected at any of the tested 2-CB levels.

Chlorobenzoate Degrader Community Dynamics. Approximately 500 isolated CB degraders were characterized based on ERIC fingerprints. All of the 2-CB degraders isolated from Madera soil at days 4 and 7 belonged to ERIC fingerprint *a* while 94% and 6% of 2-CB degraders at 11 d belonged to fingerprints *a* and *ab*, respectively. Conversely, 3- and 4-CB degraders in Oversite soil belonged to numerous different ERIC fingerprints and the populations shifted over time. As shown in Fig. 5A, fingerprint *c* comprised the largest portion of 3-CB degraders in the Oversite soil at day 0 but gradually decreased at day 7 and 14 while degraders belonging to other fingerprints, including *f*, *i*, and *m*, became dominant. Likewise, Fig. 5B demonstrates that the majority of 4-CB degraders in the Oversite soil at day 0 belonged to fingerprint *ah*, but the degraders with this fingerprint decreased over time. At day 4, 4-CB degraders were evenly divided among several different fingerprints with *ag3* becoming dominant after 11 d along with *ah3* at 21 d.

In the Madera 3-CB dose response assay, degraders isolated from the 50, 100, and 200 μg 3-CB g^{-1} microcosms differed in their ERIC fingerprints (Fig. 6). In the 50 μg 3-CB g^{-1} microcosms, the dominant fingerprint was *3n2* while the dominant fingerprints were *3a* in the

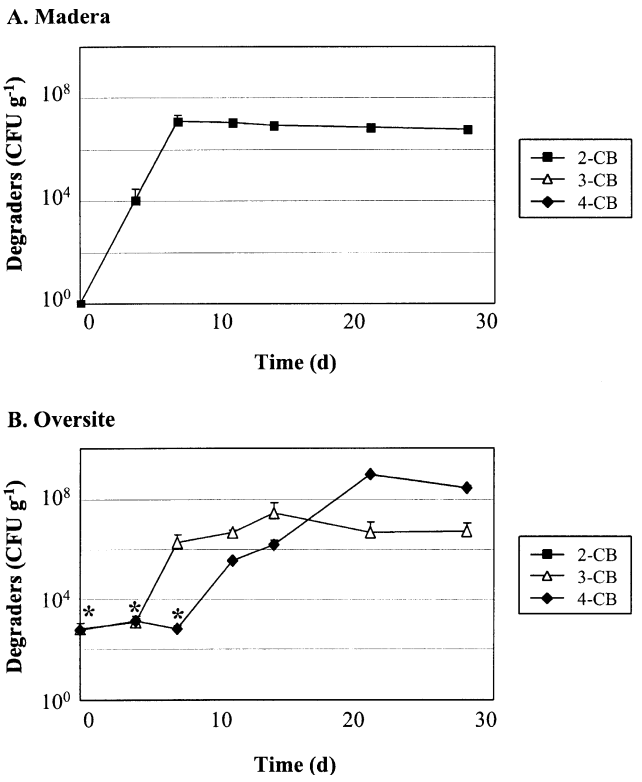

Figure 4. Number of 2-, 3-, and 4-chlorobenzoate (CB) degraders in (A) Madera and (B) Oversite soils after amendment with 500 μg CB g^{-1}. Error bars represent the standard deviation of three replicate microcosms. *Fewer than 30 degrader colonies per plate detected at indicated data points.

100 μg 3-CB g^{-1} microcosms and *3b* and *3 h* in the 200 μg 3-CB g^{-1} microcosms.

Plasmid Isolation and Description. Large plasmids were detected in several of the 3-CB degraders isolated from the Oversite soil with at least three differently sized plasmids occurring (Fig. 7). Plasmids in lanes 6, 8, 10, and 11 are from degraders with ERIC fingerprint *s2*. Plasmids in lanes 3, 5, and 7 are from degraders with ERIC fingerprint *n3*. The plasmids in lanes 4 and 9 are from degraders with ERIC fingerprints *j* and *r*, respectively. Plasmids were not as apparent in the 2-CB degraders isolated from the Madera soil (lane 2) or 4-CB degraders isolated from the Oversite soil (lanes 14 and 15); however, there were some 4-CB degraders that produced extremely faint bands which would correspond to very large plasmids, but the bands are not visible on the gel photograph. Plasmids were also not apparent in the majority of 3-CB degraders isolated from the 50, 100, or 200 μg 3-CB g^{-1} Madera microcosms (lanes 12 and 13).

Identification of Isolated Chlorobenzoate Degraders. The majority of 2-CB degraders (97%) isolated from Madera soil were identified as a *Burkholderia* sp.

Table 2. Number of 3-chlorobenzoate (3-CB) degraders and culturable bacteria in the 3-CB dose response assay Madera soil 47 d after soil was amended with 50, 100, 200, or 500 μg 3-CB g^{-1}

3-Chlorobenzoate level	*3-Chlorobenzoate degraders (CFU g^{-1})*	*Culturable bacteria (CFU g^{-1})*
50 μg 3-CB g^{-1}	0.9 (±1.2) × 10^{4a}	2.9 (±1.0) × 10^6
100 μg 3-CB g^{-1}	1.2 (±0.7) × 10^4	3.7 (±1.0) × 10^6
200 μg 3-CB g^{-1}	6.1 (±4.4) × 10^4	4.2 (±3.5) × 10^6
500 μg 3-CB g^{-1}	ND[b]	8.8 (±1.0) × 10^6

[a]<30 degrader colonies per plate detected at indicated data point.
[b]No chlorobenzoate degraders detected.

(Table 3). Likewise, the 3-CB degraders isolated from Oversite soil were primarily composed of *Burkholderia* spp., with 62% having 16S rDNA sequences most similar to *Burkholderia* sp. AZ163. In contrast, most (51%) of the 4-CB degraders from Oversite soil were classified as a *Bradyrhizobium* sp. The 3-CB degraders isolated in the Madera 3-CB dose response assay were predominately *Burkholderia* spp. Furthermore, there appeared to be limited phylogenetic overlap between 2-, 3-, and 4-CB degraders isolated from the two soils (Fig. 8).

GenBank Accession Numbers for 16S Sequences of Isolated Degraders. The partial, 16S rDNA sequences for isolated 2-, 3-, and 4-CB degraders representing each unique ERIC fingerprint were submitted to GenBank as a PopSet and assigned accession numbers AY267468–AY267531. The CB degraders were named for the GenBank entry by using the closest genus match from GenBank, the chlorobenzoate the isolate degraded, and its ERIC fingerprint. For example, the 2-CB degrader with ERIC fingerprint *a* that was classified as a *Burkholderia* sp. is identified in GenBank as *Burkholderia* sp. 2CBA.

Characterization of Chlorobenzoate Degradative Genes in Selected Degraders. Since the *cbdA* (2-halobenzoate dioxygenase) gene appears to be very conserved among different *Burkholderia* strains [41], we performed PCR on the 2-CB degrading *Burkholderia* sp. having ERIC fingerprint a (*Burkholderia* sp. 2CBA) that we isolated in this study using the primers designed from *Burkholderia* sp. TH2. An ~900 bp DNA fragment was amplified and sequenced. Database analysis of this sequence showed a 97% amino acid identity and 97% nucleotide identity with the *cbdA* sequence from *Burkholderia* sp. TH2 (GenBank accession no. AB035324). The putative *cbdA* sequence from *Burkholderia* sp. 2CBA was submitted to GenBank and assigned accession number AY297949. Additionally, Southern blot analysis indicated that the *cbdA* probe hybridized to the genomic DNA of *Burkholderia* sp. 2CBA (Fig. 9A). Two hybridized restriction fragments were shown for each the *Eco*RI and *Bam*HI digested genomic DNA.

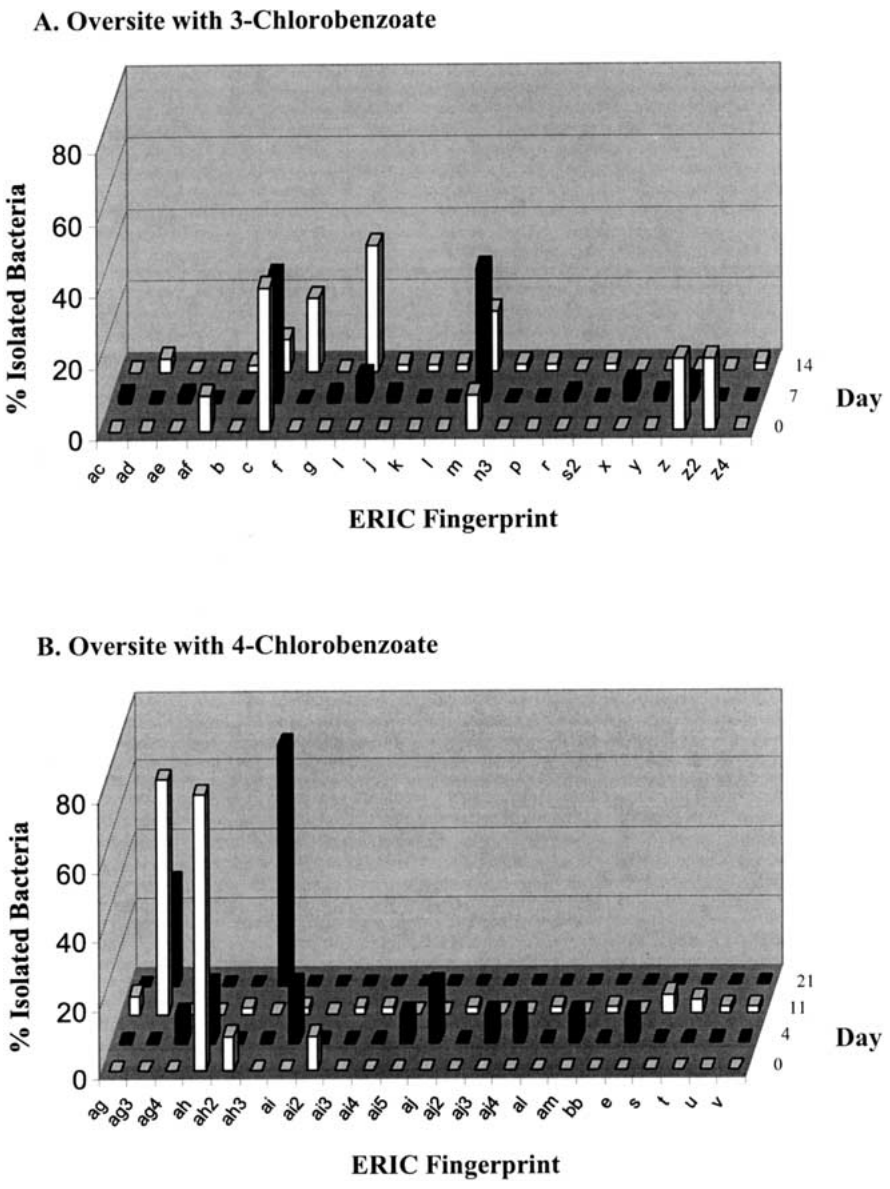

Figure 5. Semiquantitative analysis of (A) 3-chlorobenzoate (CB) degraders and (B) 4-CB degraders isolated at various times from Oversite soil following amendment with 500 μg CB g^{-1}. ERIC fingerprints of isolated degraders are plotted as percentage of total isolates against sampling day.

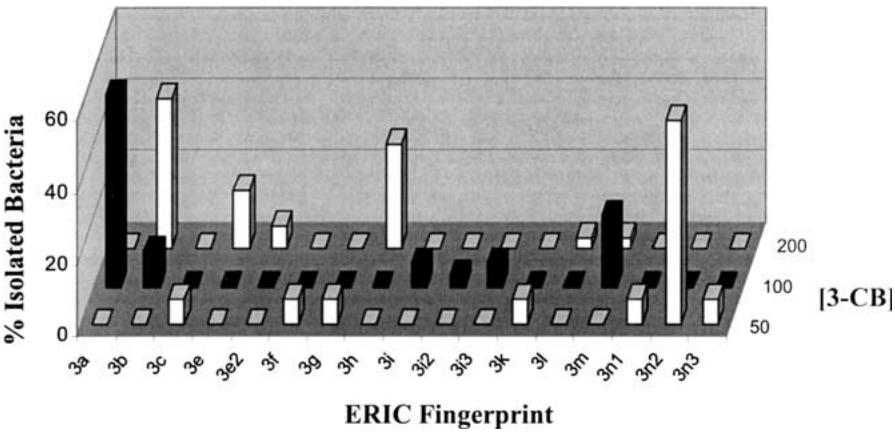

Figure 6. Semiquantitative analysis of 3-chlorobenzoate (3-CB) degraders isolated from Madera soil in dose-response assay following amendment with 50, 100, or 200 μg 3-CB g^{-1}. ERIC fingerprints are plotted as a percentage of total isolates against 3-CB concentration of originating microcosm.

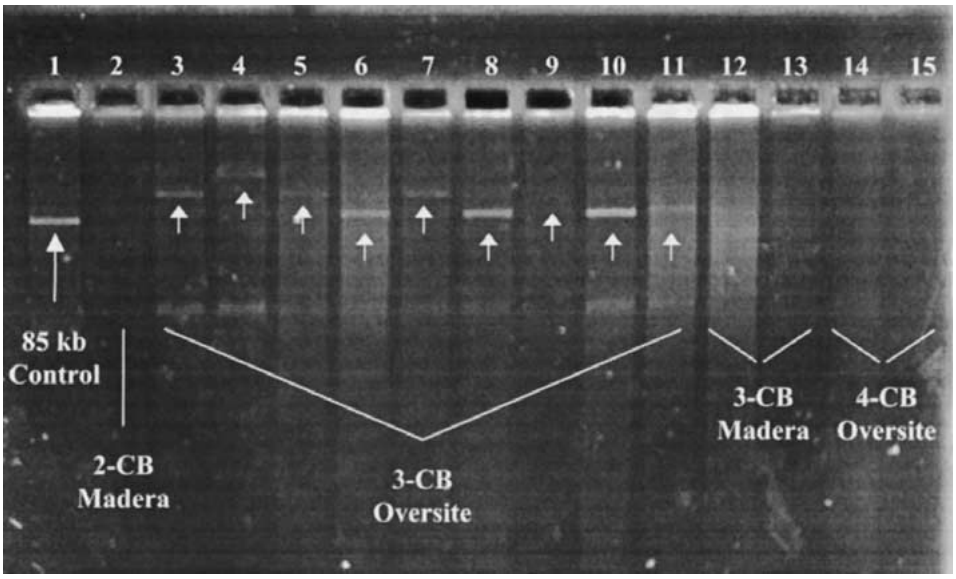

Figure 7. Plasmid screens of selected 2-, 3-, or 4-chlorobenzoate (CB) degraders isolated from Madera and Oversite soils. The 85 kb plasmid from the 3-CB degrader *Comamo testosteroni* BR60 was ran in lane 1 as a positive control. A negative control was run on another gel.

We were unable to obtain gene-specific PCR amplification for the tested 3-CB degraders having ERIC fingerprints *j*, *p*, and *n3* (these isolates were selected because they contained the three different sizes of plasmids most common in our 3-CB isolates). However, in the Southern blot analysis, the *tfdC* and *tfdD*

Table 3. Identification of 2-, 3-, and 4-chlorobenzoate (CB) degraders isolated from Madera and Oversite soils[a]

Isolate identity	*ERIC Fingerprint(s)*	*% of isolates*
Madera 2-CB		
Burkholderia sp. N3P2	a	97
Lysobacter sp. XL1	ab	3
Madera 3-CB		
Bradyrhizobium sp. ORS206	3g, 3k	3
Burkholderia glatheiz	3f, 3n1, 3n2, 3n3	15
Burkholderia sp. JS150	3a	21
Burkholderia sp. LMG 21262	3b, 3h, 3i, 3i2, 3i3, 3m	49
Ralstonia campinensis LMG 20576	3c	1
Ralstonia campinensis LMG 20579	3e, 3e2, 3l	11
Oversite 3-CB		
Burkholderia brasiliensis M130	ad, i, n3	22
Burkholderia sp. AZ163	ac, ae, c, g, j, m, p, s2, x, z, z2	62
Burkholderia sp. JRB1	r	1
Microbacterium arabinogalactanolyticum	f	9
Microbacterium sp. YK18	k, l, z4	3
Pseudomonas putida	af	1
Pseudomonas sp. K23	y	1
Rhodanobacter sp. D46	b	1
Oversite 4-CB		
Alcaligenes fecalis	ai3, u, v	3
Bradyrhizobium japonicum	ag, ag3, ah, aj2	51
Microbacterium sp. YK18	ah2, ah3, al, s	33
Pseudomonas aeruginosa PAO1	ai4, t	2
Pseudomonas mosselii	ai5	1
Pseudomonas putida	ag4, aj, aj3, e	5
Pseudomonas sp. HhSoUsc	aj4, am	2
Ralstonia sp. S23	bb	1
Stenotrophomonas maltophilia	ai, ai2	2

[a]Isolates representing each ERIC fingerprint are listed along with the closest species match in GenBank based on 16S rDNA sequences. Homology levels ranged from 97 to 100% similarity between the query and database sequences except for *Burkholderia* sp. AZ163 and *ae* (96%), *Microbacterium* sp. YK18 and *k* (95%), and *Rhodanobacter* sp. D46 and *b* (94%).

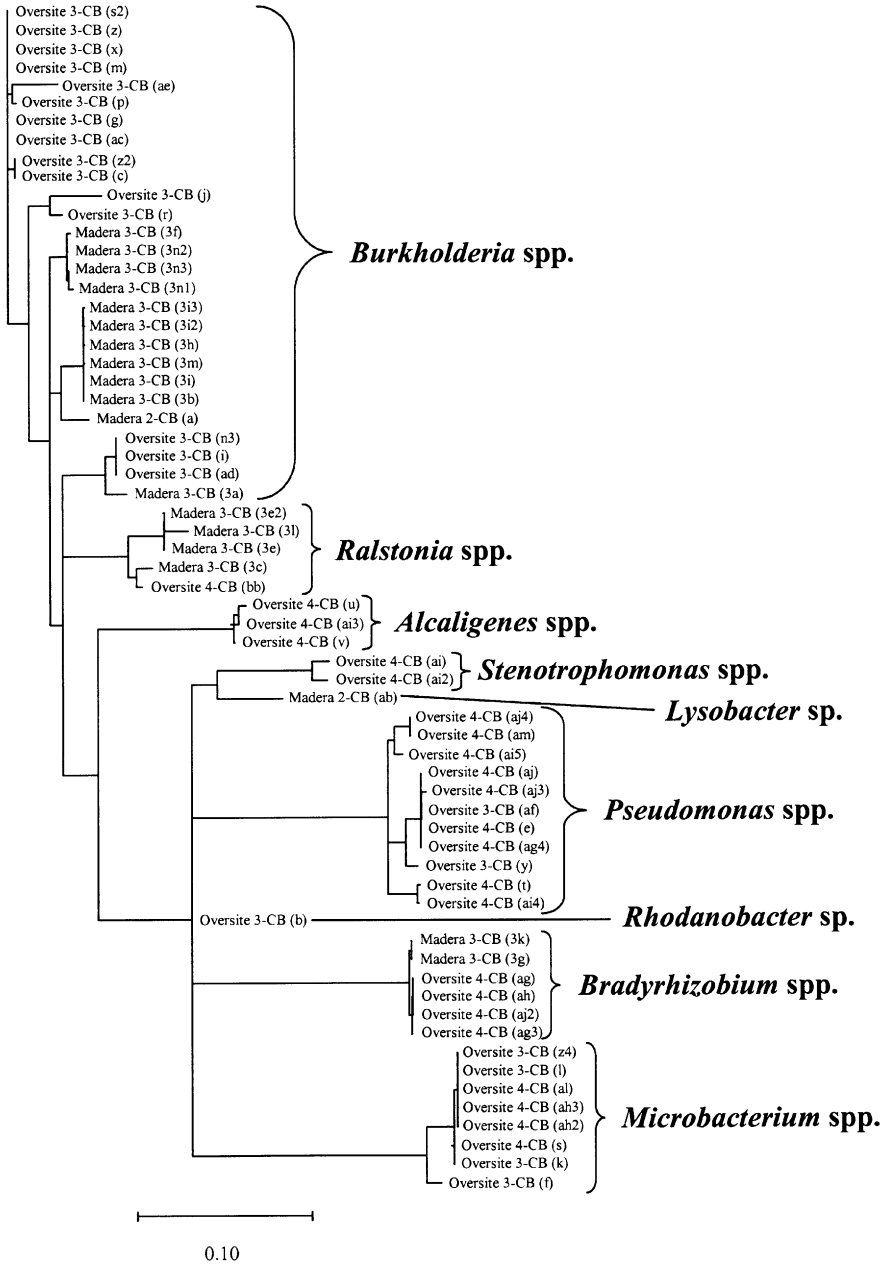

Figure 8. Phylogenetic tree of isolated 2-, 3-, and 4-chlorobenzoate (CB) degraders based on partial 16S rDNA sequences. Degraders are identified by the soil isolated from and CB degraded along with their ERIC fingerprint in parentheses.

probes generated from the 3-CB degradative genes in *R. eutropha* JMP134 both hybridized with single restriction fragments of the plasmid DNA from the isolated 3-CB degraders with fingerprints *j* and *n3* (Fig. 9B, C). These two degraders were previously identified as *Burkholderia* spp. (*Burkholderia* sp. 3CBJ and *Burkholderia* sp. 3CBN3, respectively). The molecular sizes of the restriction fragments were polymorphic between the two isolates. No hybridization signal was observed for the 3-CB degrader with fingerprint *p* (*Burkholderia* sp. 3CBP). Additionally, we were unable to obtain gene-specific PCR amplification for the dominant 4-CB degrader isolate (ERIC fingerprint *ag3*).

Discussion

Microbial ecology can be defined as the study of the relationships or interactions of microorganisms between themselves and also the biotic and abiotic characteristics of the environment around them. Intuitively, one might expect that soils which develop under similar soil-forming factors would contain similar microbial communities. However, the two soils in this study contained strikingly different populations of 2-, 3-, and 4-CB degraders that adapted differently to the addition of anthropogenic levels of 2-, 3-, and 4-CB. The isolated 3- and 4-CB degraders were more diverse than the 2-CB degraders. Each

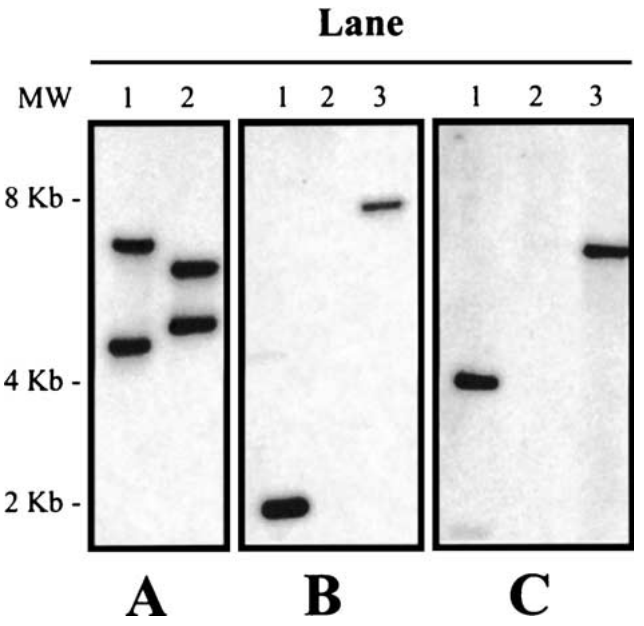

Figure 9. Southern blot analysis of selected 2- and 3-CB degraders. (A) Genomic DNA of *Burkholderia* sp. 2CBA hybridized with *cbdA*. Lanes, 1: *Bam*HI digestion; 2: *Eco*RI digestion. (B) Plasmid DNA from 3-CB degraders digested with *Bam*HI and hybridized with *tfdC*. DNA in lanes 1, 2, and 3 were from *Burkholderia* spp. 3CBJ, 3CBP, and 3CBN3, respectively. (C) Plasmid DNA from 3-CB degraders digested with *Bam*HI and hybridized with *tfdD*. DNA in lanes 1, 2, and 3 were from *Burkholderia* spp. 3CBJ, 3CBP, and 3CBN3, respectively.

individual soil also appeared to contain distinct microorganisms that specifically degraded only a single CB, either 2-, 3-, or 4-CB. To our knowledge, this was the first study that investigated the diversity of 2-, 3-, and 4-CB degraders in similar pristine soils. Interestingly, our results, indicating that the 3- and 4-CB degraders in two similar soils were more diverse than the 2-CB degraders, are consistent with the data in the literature even though most of those reported degraders were isolated in separate studies and from different environments [1, 4, 6, 8, 13, 14, 20, 23, 24, 27, 28, 30, 31, 33, 36, 39, 41–43, 46].

The differences in the degrader species diversity contained in the soils have potential implications for successful microbial adaptation following environmental contamination with chlorobenzoates or PCBs. A more diverse community, such as the 3- and 4-CB degrader communities in the Oversite soil, would be expected to be active throughout a wider range of conditions and be more functionally stable following perturbation than would a less diverse community, such as the 2-CB degrader population in the Madera soil [15, 32]. The ability of a more diverse community to adapt to the presence of a contaminant is illustrated by the shifts in the 3- and 4-CB degrader species composition throughout the study in the Oversite soil, in contrast to 2-CB degraders in the Madera soil, which were dominated by a single species. The inability of the Madera degrader community in this study to degrade 500 μg 3-CB g^{-1}, in contrast to results from a previous study in 2000 [20], further indicates the need for degrader communities to be adaptable to different environmental conditions. In the previous study, there were primarily two different 3-CB degraders in the Madera soil—*Burkholderia* spp. AZ102 and AZ163. These two degraders were not even detected in the current study. Their populations appear to have diminished over time, possibly because of a long drought in the region, resulting in the inability of the higher 3-CB level to be degraded in the Madera soil. In fact, the 3-CB degrader populations detected in the Madera soil from the current experiment were less similar to *Burkholderia* spp. AZ102 and AZ163 than were many of the 3-CB degraders that were isolated from the Oversite soil.

One potential explanation for the large diversity of 3-CB degraders in the Oversite soil is the horizontal transfer of degradative genes. We did not detect direct evidence of plasmid transfer as an adaptation mechanism during the duration of this study. However, it is probable that horizontal gene transfer was responsible for the origin of degradative genes in some of the 3-CB degraders isolated from Oversite soil. The genes from the modified *ortho*-3-CB cleavage pathway (*tfdC* and *tfdD*) that hybridized to plasmids from the Oversite 3-CB degraders are commonly found on self-transmissible plasmids [37]. Other 3-CB degradative genes are also found on transmissible plasmids or transposons [33]. Additionally, it is possible that the shifts in 3-CB degrader species in the Oversite soil that occurred following addition of 3-CB may have been due to gene transfer events, but it could also have been due to selection of more competitive degrader populations initially existing at the site [12, 29, 34]. Newby et al. [34] found that the populations of 2,4-D degraders in Madera soil had similar shifts in diversity following the addition of 2,4-D to the soil. The 2,4-D degraders had received a self-transmissible plasmid, containing the 2,4-D genes, from an introduced *E. coli* culture. While the degrader populations changed over time, it is unknown if the diversity occurred due to *E. coli* transferring the plasmid to different indigenous soil bacteria or from successive plasmid transmission from one soil bacterium to a different soil bacterium.

In contrast, plasmid transfer probably does not explain the diversity of 4-CB degraders in the Oversite soil or 3-CB degraders in the Madera soil since plasmids were not as apparent in these isolates. The genome of *Bradyrhizobium japonicum* USDA110 has recently been sequenced and contains a putative 4-CB-CoA ligase [26]. The existence of this gene in our *Bradyrhizobium* spp. isolates would explain their ability to degrade 4-chlorobenzoate and also their apparent lack of plasmids, since the 4-CB-CoA ligase gene would probably be chromo-

somal. However, we cannot confirm the presence of this gene in our degraders because we could not PCR-amplify it using the primers designed from *B. japonicum* USDA110. It is possible that our *Bradyrhizobium* sp. isolate contained a 4-CB-CoA ligase with a slightly different sequence than that *of B. japonicum* USDA110 resulting in no PCR amplification. Nevertheless, it is also possible that other 4-CB degradative genes were present in the microbial community at the site and were exchanged between some of the 4-CB degraders via other mobile forms, such as transposons, prior to this experiment [47]. However, the 4-CB genes in the isolated degraders would have to be sequenced to confirm or refute this.

In conclusion, even though the Madera and Oversite Canyon soils were from similar locations and were chemically and physically comparable, the microbial ecology of the communities that developed resulted in varying abilities to degrade 2-, 3-, and 4-CB. Studies by other researchers have found soils with similar vegetation to contain similar populations of CB degraders, but in this case there appear to be other factors, perhaps even temporal fluctuations, which had an impact on the presence or absence of 2-, 3-, and 4-CB degraders. In addition, 2-, 3-, and 4-CB were degraded by different bacteria within a given soil. Populations of specific 3- and 4-CB degraders shifted over time in the Oversite soil. The 3-CB degraders isolated from Madera soil that received 50, 100, or 200 μg of 3-CB g^{-1} differed depending on the 3-CB level, possibly indicating that different degrader populations developed in response to amendment with higher levels of 3-CB. Additionally, the origin of degradative genes in many of the isolated 3-CB degraders may have been due to gene transfer events, as several of the isolated 3-CB degraders contained large plasmids, and at least two of the different plasmids appeared to contain homologous 3-CB degradative genes.

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References

1. Ajithkumar, PV, Kunhi, AAM (2000) Pathways for 3-chloro- and 4-chlorobenzoate degradation in *Pseudomonas aeruginosa* 3mT. Biodegradation 11: 247–261
2. Altschul, SF, Madden, TL, Schaffer, AA, Zhang, J, Zhang, Z, Miller, W, Lipman, DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402
3. Atlas, RM (1993) Handbook of Microbiological Media. CRC Press, Boca Raton, FL
4. Babbitt, PC, Kenyon, GL, Martin, BM, Charest, H, Sylvestre, M, Scholten, JD, Chang, K-H, Liang, P-H, Dunaway-Mariano, D (1992) Ancestry of the 4-chlorobenzoate dehalogenase: analysis of amino acid sequence identities among families of acyl:adenyl ligases, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases. Biochemistry 31: 5594–5604
5. Barriault, D, Sylvestre, M (1993) Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. Can J Microbiol 39: 594–602
6. Bott, TL, Kaplan, LA (2002) Autoecological properties of 3-chlorobenzoate-degrading bacteria and their population dynamics when introduced into sediments. Microb Ecol 43: 199–216
7. Brendecke, JW, Axelson, RD, Pepper, IL (1993) Soil microbial activity as an indicator of soil fertility; the long term effects of municipal sewage sludge on an arid soil. Soil Biol Biochem 25: 751–758
8. Davison, AD, Jardine, DR, Karuso, P (1999) 5-chloropicolinic acid is produced by specific degradation of chlorobenzoic acid by *Sphingomonas paucimobilis* BPSI-3. J Ind Microbiol Biotechnol 23: 347–352
9. DeBano, LF, Ffolliott, PF, Ortega-Rubio, A, Gottfried, GJ, Hamre, RH, Edminster, CB, tech. coords (1995) Biodiversity and management of the madrean archipelago: the sky islands of southwestern United States and northwestern Mexico. 1994 Sept. 19–23; Tucson, AZ. Gen. Tech. Rep. RM-GTR 264. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. p 669
10. de Jong, E , Field, JA (1997) Sulfur tuft and turkey tail: biosynthesis and biodegradation of organohalogens by Basidiomycetes. Ann Rev Microbiol 51: 375–414
11. Di Giovanni, GD, Neilson, JW, Pepper, IL, Sinclair, NA (1996) Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl Environ Microbiol 62: 2521–2526
12. Di Mattia, E, Grego, S, Cacciari, I (2002) Eco-physical characterization of soil bacterial populations in different states of growth. Microb Ecol 43: 34–43
13. Don, RH, Weightman, AJ, Knackmuss, H-J, Timmis, KN (1985) Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J Bacteriol 161: 85–90
14. Dunaway-Mariano, D, Babbitt, PC (1994) On the origins and functions of the enzymes of the 4-chlorobenzoate to 4-hydroxybenzoate converting pathway. Biodegradation 5: 259–276
15. Fernandez, AS, Hashsham, SA, Dollhope, SL, Raskin, L, Glagoleva, O, Dazzo, FB, Hickey, RF, Criddle, CS, Tiedje, JM (2000) Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microbiol 66: 4058–4067
16. Fetzner, S (1998) Bacterial dehalogenation. Appl Microbiol Biotechnol 50: 633–657
17. Focht, DD, Shelton, D (1987) Growth kinetics of *Pseudomonas alcaligenes* C-O relative to inoculation and 3-Chlorobenzoate metabolism in soil. Appl Environ Microbiol 53: 1846–1849
18. Fulthorpe, RR, Rhodes, AN, Tiedje, JM (1998) High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. Appl Environ Microbiol 64: 1620–1627
19. Fulthorpe, RR, Rhodes, AN, Tiedje, JM (1996) Pristine soils mineralize 3-chlorobenzoate and 2,4-dichlorophenoxyacetate via different microbial populations. Appl Environ Microbiol 62: 1159–1166
20. Gentry, TJ, Newby, DT, Josephson, KL, Pepper, IL (2001) Soil microbial population dynamics following bioaugmentation with a 3-chlorobenzoate-degrading bacterial culture. Biodegradation 12: 349–357

21. Ghosal, D, You, IS, Chatterjee, DK, Chakrabarty, AM (1985) Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. Proc Natl Acad Sci USA 82: 1638–1642
22. Gribble, GW (1998) Naturally occurring organohalogen compounds. Ace Chem Res 31: 141–152
23. Haak, B, Fetzner, S, Lingens, F (1995) Cloning, nucleotide sequence, and expression of the plasmid encoded genes for the two-component 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS. J Bacteriol 177: 667–675
24. Hickey, WJ, Focht, DD (1990) Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Appl Environ Microbiol 56: 3842–3850
25. Kado, CI, Liu, ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365–1373
26. Kaneko, T, Nakamura, Y, Sato, S, Minamisawa, K, Uchiumi, T, Sasamoto, S, Watanabe, A, Idesawa, K, Iriguchi, M, Kawashima, K, Kohara, M, Matsumoto, M, Shimpo, S, Tsuruoka, H, Wada, T, Yamada, M, Tabata, S (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110. DNA Res 9: 189–197
27. Klages, U, Lingens, F (1979) Degradation of 4-chlorobenzoic acid by a *Nocardia* species. FEMS Microbiol Lett 6: 201–203
28. Lang, E (1996) Diversity of bacterial capabilities in utilizing alkylated benzenes and other aromatic compounds. Lett Appl Microbiol 23: 257–260
29. Lipson, DA, Schadt, CW, Schmidt, SK (2002) Changes in soil microbial community structure and function in an alpine dry meadow following spring snow melt. Microb Ecol 43: 307–314
30. Marks, TS, Smith, ARW, Quirk, AV (1984) Degradation of 4-chlorobenzoic acid by *Arthrobacter* sp. Appl Environ Microbiol 48: 1020–1025
31. Miguez, CB, Greer, CW, Ingram, JM (1990) Degradation of mono- and dichlorobenzoic acid isomers by two natural isolates of *Acaligenes denitrificans*. Arch Microbiol 154: 139–143
32. Müller, AK, Westergaard, K, Christensen, S, Sørensen, SJ (2002) The diversity and function of soil microbial communities exposed to different disturbances. Microb Ecol 44: 49–58
33. Nakatsu, CH, Providenti, M, Wyndham, RC (1997) The *cis*-diol dehydrogenase *cbaC* gene of Tn*5271* is required for growth on 3-chlorobenzoate but not 3,4-dichlorobenozate. Gene 196: 209–218
34. Newby, DT, Gentry, TJ, Pepper, IL (2000) Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. Appl Environ Microbiol 66: 3399–3407
35. Niedan, V, Schöler, HF (1997) Natural formation of chlorobenzoic acids (CBA) and distinction between PCB-degraded CBA. Chemosphere 35: 1233–1241
36. Ogawa, N, Miyashita, K (1995) Recombination of a 3-chlorobenzoate catabolic plasmid from *Alcaligenes eutrophus* NH9 mediated by direct repeat elements. Appl Environ Microbiol 61: 3788–3795
37. Perkins, EJ, Gordon, MP, Caceres, O, Lurquin, PF (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J Bacteriol 172: 2351–2359
38. Ramírez-Saad, HC, Sessitsch, A, de Vos, WM, Akkermans, ADL (2000) Bacterial community changes and enrichment of *Burkholderia*-like bacteria induced by chlorinated benzoates in a peat-forest soil-microcosm. System Appl Microbiol 23: 591–598
39. Reber, HH, Thierbach, G (1980) Physiological studies on the oxidation of 3-chlorobenzoate by *Acinetobacter calcoaceticus* strain Bs-5. Eur J Appl Microbiol Biotechnol 10: 223–233
40. Rensing, C, Newby, DT, Pepper, IL (2002) The role of selective pressure and selfish DNA in horizontal gene transfer and soil microbial community adaptation. Soil Biol Biochem 34: 285–296
41. Suzuki, K, Ogawa, N, Miyashita, K (2001) Expression of 2-halobenzoate dioxygenase genes (*cbdSABC*) involved in the degradation of benzoate and 2-halobenzoate in *Burkholderia* sp. TH2. Gene 262: 137–145
42. Tsoi, TV, Plotnikova, EG, Cole, JR, Guerin, WF, Bagdasarian, M, Tiedje, JM (1999) Cloning, expression, and nucleotide sequence of the *Pseudomonas aeruginosa* 142 *ohb* genes coding for oxygenolytic *ortho* dehalogenation of halobenzoates. Appl Environ Microbiol 65: 2151–2162
43. van den Tweel, WJJ, Kok, JB, de Bont, JAM (1987) Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by *Alcaligenes denitrificans* NTB-1. Appl Environ Microbiol 53: 810–815
44. Versalovic, J, Koeuth, T, Lupski, JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823–6831
45. Wackett, LP, Hershberger, CD (2001) Biocatalysis and Biodegradation. ASM Press, Washington DC
46. Wyndham, RC, Singh, RK, Straus, NA (1988) Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60. Arch Microbial 150: 237–243
47. Yi, H-R, Min, K-H, Kim, C-K, Ka, J-O (2000) Phylogenetic and phenotypic diversity of 4-chlorobenzoate-degrading bacteria isolated from soils. FEMS Microbiol Ecol 31: 53–60
48. Zhou, J-Z, Tiedje, JM (1995) Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium. Mol Ecol 4: 613–618