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Phylogenetic Characterization of a Mixed Microbial Community Capable of Degrading Carbon Tetrachloride

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Abstract

Two bacterial communities (NO92 and GBS) capable of degrading carbon tetrachloride (CT) were enriched from in-house CT-contaminated water. These communities are able to degrade CT in the presence of toluene. To characterize the community structure and diversity, one enrichment (NO92) was subjected to 16S ribosomal RNA (rRNA) gene-based molecular analysis. The 16S rRNA genes were amplified from the bulk genomic community DNA and cloned into plasmid vectors. Unique 16S rRNA gene clones, i.e., phylotypes, were detected by four tetrameric restriction enzymes. Together, 123 16S rRNA gene clones were obtained; thirty-one showed different restriction fragment length polymorphism (RFLP) patterns. About 73% of the clones belong to two dominant RFLP patterns. Phylogenetic analysis based on the partial 16S rRNA gene sequences of 10 major phylotypes showed that all the phylotypes that were sequenced were affiliated with the high G+C Gram-positive bacteria. Whereas seven of the phylotypes (~80% of the clones) were closely related to *Rhodococcus*, the other three (~5% of the clones) were related to *Curtobacterium*. These results suggest that this CT-degrading community is diverse but is predominated by closely related bacterial groups.

Index Entries: Carbon tetrachloride; bioremediation; phylogeny; microbial community.

Introduction

Carbon tetrachloride (CT) is one of the major environmental pollutants in groundwater. It has been widely used as a solvent, industrial

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degreasing agent, fire extinguisher, and grain fumigant. It is listed as a priority pollutant by the US Environmental Protection Agency (EPA) because of its carcinogenic effects.

CT has been shown to be reductively dechlorinated to lesser chlorinated compounds by different types of anaerobic bacteria such as denitrifiers *(1)*, methanogens *(2,3)*, sulfate-reducing bacteria *(4)*, and iron-reducing bacteria *(5)*, but little is known about the organisms responsible for these activities. Under anaerobic conditions, the transformation of CT typically results in the accumulation of chloroform, a compound that is persistent and potentially harmful to human health *(6)*. An exception is *Pseudomonas stutzeri* KC, which is capable of hydrolyzing CT to carbon dioxide, formate, and nonvolatile products without the production of chloroform *(7)*. However, the mechanism for this transformation is poorly understood.

Recently, we established two enrichment communities (NO92 and GBS) capable of degrading CT without producing chloroform *(8,9)*. In culture with toluene (5 mg/L) and a mineral salts media, these cultures completely degraded the toluene and significantly reduced CT concentration (from 1 mg/L). To understand the CT-degrading community structure and diversity, one enrichment community (NO92) was subjected to 16S rRNA gene-based molecular analysis, which showed this CT-degrading community to be diverse but predominantly *Rhodococcus* species.

Materials and Methods

Enrichments and DNA Extraction

The CT-degrading community used for this study was enriched initially under bulk aerobic conditions *(8,9)* in 15-mL EPA vials containing 14 mL of a modified minimal salt medium *(10)* with 5 ppm toluene, 1 ppm CT, and 0.01% yeast extract. In this work the modified mineral salt medium contained nitrate but no iron and copper. Duplicate vials were inoculated with the cell suspensions incubated inverted at room temperature for 30 d prior to headspace analyses by gas chromatography. Then 20 mL of the enrichment cultures from the vials positive for CT degradation *(8)* were used for DNA extraction. Under these conditions, toluene was completely degraded by the NO92 culture and six other cultures tested *(9)*. However, only a few of the cultures showed CT degradation, and NO92 and GBS achieved the greatest reduction in CT.

A combination of physical and chemical lysis methods *(11)* was used to isolate the total community DNA from the CT-degrading community. The samples were ground with a mortar and pestle in the presence of sterile sand and liquid nitrogen before adding the extraction buffer *(11)*. The mortar and pestle were washed with the extraction buffer, and the preparations were then frozen at –70°C and thawed by microwave heating until the preparation briefly boiled a total of three times. The genomic DNA was then extracted by a sodium dodecyl sulfate–based lysis method *(12)*.

16S rRNA Gene Amplification and Cloning

The primer set of fD1 and rP1 oligonucleotides, with modifications to the linker sequences *(12)*, was used for amplifying eubacterial 16S rRNA genes as described by Weisburg et al. *(13)*. All stocks for polymerase chain reaction (PCR) amplification were made, and procedures were performed with the precautions suggested by Kwok and Higuchi *(14)*. One microliter of the purified DNA was used as a template in each 20-μL reaction. The conditions for amplifying 16S rRNA genes were described previously *(12)*.

The PCR-amplified 16S rRNA gene products were quantified by comparing the band intensity on agarose gels to the known concentrations of standard lambda DNA. The amplified PCR products were directly ligated to the pCR™ II vector from Invitrogen (San Diego, CA). Ligation and transformation were carried out according to the manufacturer's instructions. The ratio of inserts to vectors in ligation was 0.5–1:1. Two microliters of the ligation reaction mixture were transformed by heat pulse into *Escherichia coli* INVaF' competent cells (Invitrogen).

A set of primers specific to the polylinker of the vector pCR II was designed to screen the 16S rDNA inserts *(15)*. The sequences of the primers used were 5'GCCGCCAGTGTGCTGGAATT3', and 5'TAGATGCAT GCTCGAGCGGC3'. The cloned inserts were directly amplified from transformant cells using these primers. A toothpick was used to transfer cells to standard PCR mix from a colony on a Luria broth (LB) agar plate containing ampicillin. The reaction mix included a 1X *Taq* polymerase buffer, 0.5 U of *Taq* polymerase (Promega, Madison, WI), 200 μ*M* dNTPs and 20 pmole of each primer in a 20-μL reaction. The reaction conditions consisted of denaturation at 92°C for 2 min, 30 cycles at 94°C for 30 s, 68°C for 1 min, and 72°C for 2 min, and one additional cycle with a final 6 min of chain elongation. The amplified PCR products were analyzed for 16S rDNA inserts by gel electrophoresis on 1.5% agarose gels.

The 16S rDNA clones were numbered (CT-1 to CT-123) based on the order of initial detection.

16S rDNA Restriction Fragment Length Polymorphism Analysis

To detect unique 16S rDNA clones and thus reduce the amount of sequencing required, restriction fragment length polymorphism (RFLP) analysis was carried out. One-fifth of the 20-μL PCR amplified products was digested by using 0.1 U of each tetrameric endonuclease pairs *Msp*I plus *Rsa*I, and *Hha*I plus *Hae*III (Gibco BRL, Gaithersburg, MD) overnight at 37°C. The resulting RFLP products were separated by gel electrophoresis in 3.5% Metaphor agarose (FMC Bioproducts, Rockland, ME) in 1X TBE at 4°C with 7 V/cm for 4 h. Metaphor agarose gel was prepared according to the manufacturer's instructions. The gel was stained with 0.5 mg of ethidium bromide per milliliter and visualized by ultraviolet excitation. The RFLP patterns were compared by visual inspection. As in other studies *(16–18)*, we designated each unique RFLP pattern as an operational taxonomic unit (OTU).

To estimate the diversity of the enrichment and to determine whether *in situ* bacterial diversity was well represented by the clones examined, we performed a rarefaction analysis *(19)*. The abundance data on the 16S rDNA clones identified in the RLFP analysis were used in this analysis. OTUs were numbered in order of abundance, and the cumulative number of OTUs was plotted as a function of clone number. The saturation level was estimated using the nonlinear regression analysis routine in SigmaPlot 4.01 (SPSS) using the model $y = a(1 - \exp^{-bx})$, where y is the saturation level for the number of OTUs.

rDNA Sequencing

To analyze the phylogenetic diversity of the CT-degrading communities, 10 of the most dominant clones were selected for sequencing using the primer 529R. The 529R sequencing primer for 16S rRNA gene used in this study spanned *E. coli* 16S rRNA gene positions of 529-512. The DNA sequences of the 16S rRNA genes were determined directly using the PCR-amplified DNA as the sequencing template. The amplified PCR products were purified using the Wizard™ PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. DNA sequences were determined with automated fluorescent *Taq* cycle sequencing using the ABI Catalyst 800 and ABI 373A Sequencer (Applied Biosystems, Forester City, CA). Approximately 100 ng of the purified DNA was used for one automated fluorescent sequencing reaction.

Phylogenetic Analysis

Sequences were assembled using assembling programs in the Genetic Computer Group software package *(20)*, and preliminary analysis was done by searching the current databases (GenBank release 92.0 and EMBL release 45.0) using the program FASTA. Sequences were then aligned manually to the 16S rDNA sequences of the species, which showed high similarity scores in the outputs of FASTA, in the previously aligned 16S rDNA sequence database, Ribosomal Database Project (RDP) *(21)* using the GDE multiple sequence editor program from RDP. Initial phylogenetic screening was constructed using the DNA distance program, Neighbor-Joining, in the PHYLIP package *(22)* based on all 16S rDNA sequences of high G+C Gram-positive bacteria in RDP plus the closely related 16S rDNA sequences that were absent in RDP. DNA distances were estimated by the method of Kimura *(23)*. Based on the initial phylogenetic results, appropriate subsets of 16S rDNA sequences were selected and subjected to final phylogenetic analysis through the maximum likelihood method with the program fastDNAml in RDP. Final phylogenetic trees were constructed with the transition/transversion ratio of 2.0 by using jumbled orders of 10 for the addition of taxa. Comparison of the phylogenetic analyses of the 5'- and 3'-half portions of the sequences indicated that the obtained sequences showed no obvious evidence of chimeric artifacts.

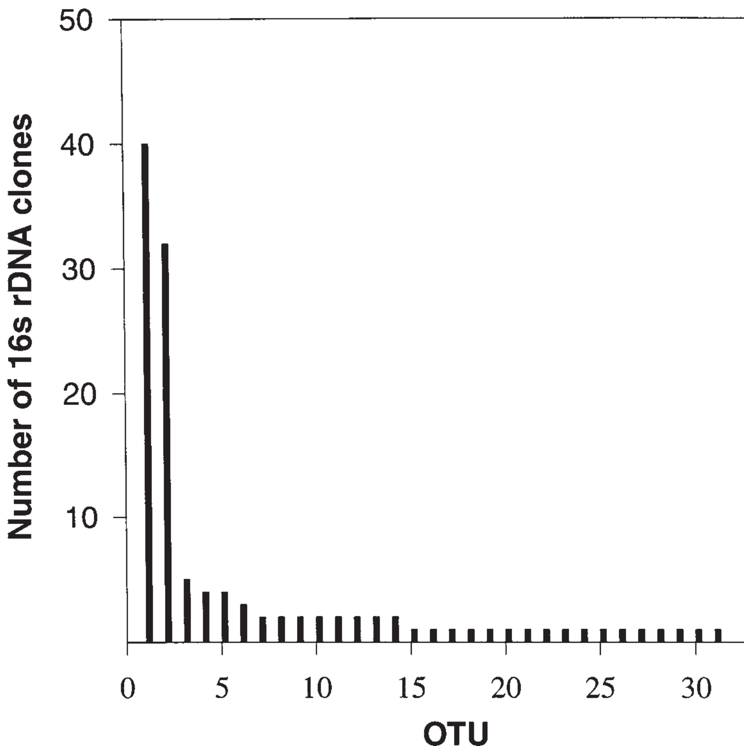

Fig. 1. Distribution of 16S rDNA clones from the CT-degrading community among different OTUs each as defined as a unique RFLP pattern. The OTUs are shown in order of the clone abundance.

Results

A total of 202 colonies that tested positive for α-complementation of β-galactosidase were obtained by the cloning procedure. Whereas 33 (16.3%) of these clones contained no detectable inserts, 163 clones (83.7%) had an insertion detectable by PCR amplification with the vector-specific primers. A total of 123 of these clones contained the entire 1.5-kb 16S rDNA insert.

The RFLP patterns of the 123 intact 16S rDNA clones indicated that 20 RFLP patterns were obtained after restriction with the *Msp*I and *Rsa*I restriction enzymes. Whereas five of these had two clones, nine had only one representative. The RFLP patterns containing more than two clones were subjected to secondary digestion with the restriction enzymes *Hha*I plus *Hae*III. An additional 11 RFLP patterns were obtained with the second set of enzymes. Thus, a total of 31 OTUs were detected with the four restriction enzymes.

The majority of the clones were represented by the two dominant RFLP patterns. OTUs 1 and 2 accounted for 32.5 and 26.0% of the 16S rDNA clones, respectively (Fig. 1). The remaining 51 16S rDNA clones were distributed among 29 OTUs. Whereas 13 of the remaining RFLP patterns con-

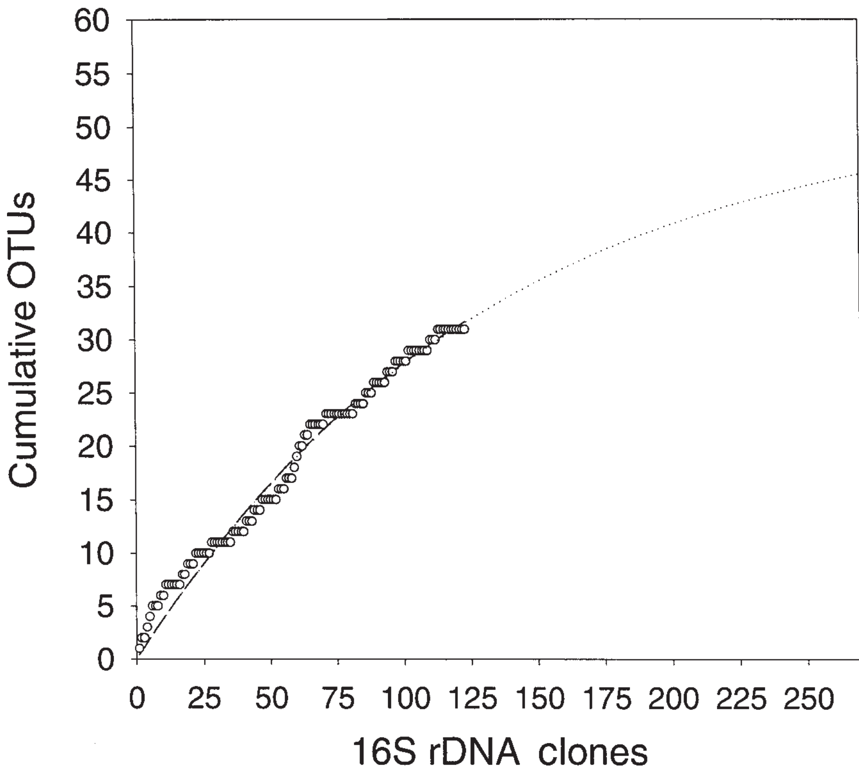

Fig. 2. Evaluation of the representation of the clones obtained from the CT-degrading community by rarefraction analysis *(11,18)*. The 16S rDNA clones were numbered based on their order of initial detection, which is assumed to be stochastic relative to the distribution of clones in the clone library. Both the data (○) and the results of the nonlinear regression analysis (– – –) are plotted. The most uncertain portion of the regression is the extrapolation (· · ·) past the final data point.

tained more than one clone, 16 of the RFLP patterns were represented by only a single 16S rDNA clone.

Based on a rarefaction analysis, our methods appeared to capture about 54% of the diversity present in this enrichment (Fig. 2). After the first 71 16S rDNA clones (52%) had been screened, 23 (74%) of the 31 OTUs had been detected (Fig. 2). Only eight additional OTUs were detected among the remaining 52 clones for the 31 total OTUs detected. The results of the nonlinear regression analysis [adjusted R^2 of 0.98, for the model $y = 1 \times (1 - \exp^{(-b \times x)})$] suggest saturation at approx 52 OTUs. This estimate is highly uncertain owing to the necessary extrapolation to the missing portion of the curve and could also vary with the regression model used. One of the dominant RFLP patterns, OTU 1, was detected prior to any other OTUs, whereas the other dominant one, OTU 2, was detected after the first 19 16S rDNA clones were examined.

Phylogenetic analysis, established by maximum likelihood methods for all the clones sequenced, revealed the presence of two groups that belong

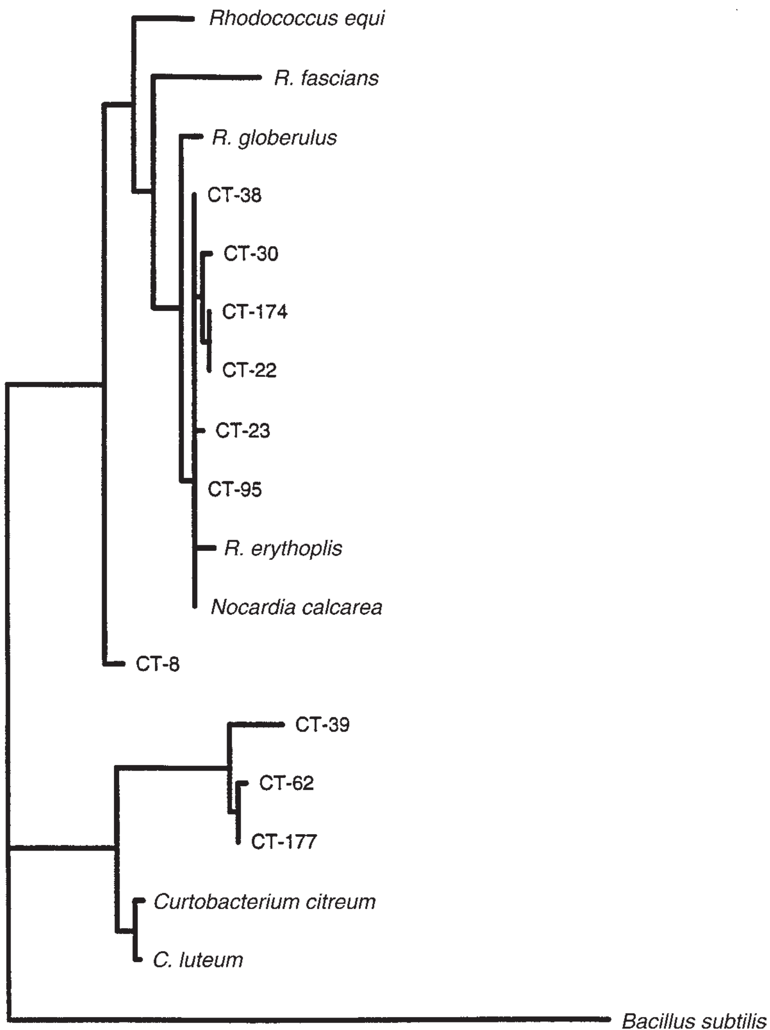

Fig. 3. Phylogenetic relationships of the 10 phylotypes sequenced from the CT-degrading community. The tree was established by the maximum likelihood method based on the partial 16S rRNA gene sequences with *Bacillus subtilis* as the outgroup.

to the high G+C subdivision of Gram-positive bacteria (Fig. 3). The most frequently encountered group of clones was the CT-38 cluster. This cluster was composed of clones CT-38 (OTU 1), CT-23 (OTU 2), CT-95 (OTU 4), CT-22 (OTU 7), CT-174 (OTU 5), and CT-30 (OTU 8). The similarities of the sequenced regions for this ranged from 99.4 to 100% among these clones (Table 1). This cluster is affiliated with the *Nocardia* subgroup within the *Mycobacterium* group of the high G+C Gram-positive bacteria, and is closely related to *Rhodococcus erythropolis*, *R. globerulus*, and *N. calcarea*, with similarities of >99% (Fig. 3, Table 1). We referred to this cluster as the *Rhodococcus* cluster, and it accounted for 72.9% of the clones. Clone CT-8 clustered more distantly to this group.

Table 1
Pairwise Levels of Sequence Identity and Evolutionary Distances Among the Clones in the CT-Degrading Community and Some Closely Related Species in High G+C Gram-Positive Bacteria

Clone or species	Sequence identity or evolutionary distance (10^2)[a] (%)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. CT-177		99.7	98.1	89.1	89.4	88.0	88.8	89.0	88.9	91.1	95.1	95.1	88.1	88.3	88.1	88.3
2. CT-62	0.3		97.9	88.8	89.1	88.1	88.4	88.7	88.6	90.8	94.7	94.7	87.8	88.4	87.8	88.1
3. CT-39	1.9	2.1		90.8	91.1	89.2	90.4	90.7	90.5	89.3	93.8	93.5	89.2	88.8	89.3	89.5
4. CT-22	12.0	12.3	10.0		100.0	100.0	100.0	99.7	99.7	98.2	90.6	90.6	99.4	95.0	99.7	99.7
5. CT-95	11.7	12.0	9.7	0.0		100.0	100.0	99.7	99.7	98.1	91.2	91.2	99.7	95.8	100.0	100.0
6. CT-174	13.3	13.3	11.9	0.0	0.0		100.0	99.7	99.7	98.2	89.7	89.7	99.5	95.2	99.7	99.7
7. CT-38	12.4	12.8	10.5	0.0	0.0	0.0		99.7	99.7	98.0	90.8	90.8	99.7	95.5	100.0	100.0
8. CT-30	12.1	12.4	10.1	0.3	0.3	0.3	0.3		99.4	97.8	90.7	90.7	99.4	95.2	99.7	99.7
9. CT-23	12.2	12.6	10.4	0.3	0.3	0.3	0.3	0.6		97.8	90.8	90.8	99.4	95.1	99.7	99.7
10. CT-8	9.6	10.0	11.9	1.9	1.9	1.9	2.0	2.2	2.2		91.7	92.0	97.9	94.8	98.1	98.2
11. *Curtobacterium citreum*	5.0	5.6	6.5	10.1	9.5	11.3	9.9	10.0	9.9	8.9		99.6	90.4	90.7	90.8	91.0
12. *C. luteum*	5.0	5.6	6.8	10.2	9.5	11.3	10.0	10.0	10.0	8.5	0.4		90.4	90.7	90.8	91.0
13. *Rhodococcus globerulus*	13.2	13.5	11.8	0.6	0.3	0.5	0.3	0.6	0.6	2.2	10.4	10.4		95.1	98.3	98.9
14. *R. fascians*	13.0	13.0	12.4	5.2	4.6	5.1	4.8	5.0	5.1	5.5	10.1	10.1	5.1		95.5	95.4
15. *R. erythropolis*	13.2	13.5	11.8	0.3	0.0	0.3	0.0	0.3	0.3	1.9	9.9	9.9	1.7	4.6		99.2
16. *Nocardia calcarea*	12.9	13.2	11.5	0.3	0.0	0.3	0.0	0.3	0.3	1.9	9.8	9.7	1.1	4.8	0.7	

[a]The values on the upper right are levels of sequence identity, and the values on the lower left are evolutionary distance.

Clone CT-8 was more distantly related to the clones in cluster CT-38 with similarities of 97.8–98.2% (Table 1). It branched before *R. erythropolis*, *R. globerulus*, and *N. calcarea* separated, but after the other *Rhodococcus* species such as *R. equi* and *R. fascians* diverged (Fig. 3). It had 97.9–98.2% similarities to these species (Table 1). 16S sequence similarities and phylogenetic analysis suggest that clone CT-8 should be a member of the genus *Rhodococcus* and thus should also belong to the CT-38 cluster. Clones CT-12, CT-54, CT-79, CT-87, CT-190, and CT-197 were not sequenced but could also be related to clone CT-8 because they had the same RFLP patterns generated with the enzymes *Msp*I plus *Rsa*I in the primary screening. Together, the *Rhodococcus* cluster accounted for 78.8% of the clones.

Another distantly related phylogroup was the CT-39 cluster, which was composed of CT-39, CT-177, and CT-62. The CT-39 cluster was affiliated with the *Arthrobacter* group of the high G+C Gram-positive bacteria, and is closely related to *Curtobacterium citreum* and *C. luteum*, with similarities of 93.5–95.1% (Table 1, Fig. 3). We defined this cluster as the *Curtobacterium* cluster, and it accounted for 4.9% of the clones. There were 97.9–99.7% similarities among these three clones, and 88.0–91.1% similarities among these three clones and the other CT clones in the *Rhodococcus* cluster (Table 1).

Discussion

RFLP analysis showed that the NO92 CT-degrading community is diverse but dominated by only a few clones. Thirty-one distinct OTUs were detected and our estimates are that an additional 26 OTUs may have been present. Only two OTUs were present in great abundance, accounting for 58.5% of the total number of clones. The other 29 OTUs were present at low levels, <4.2% each. This distribution may be typical for enrichment communities because they are highly selected for specific conditions. The number of types (31) seen in the enrichment was comparable to that (21–36 types) found in four communities by Liu et al. *(24)* using a terminal RFLP method on 16S rRNA.

A significant decrease in the rate of OTU detection was observed in the rarefaction curve (Fig. 2). Whereas 23 of the 31 OTUs were detected among the first 71 16S rDNA clones, only 8 additional OTUs were detected among the remaining 52 clones. Thus, the level of analysis was sufficient to detect the community's predominant OTUs and infer their distribution within the CT-degrading community. However, our estimates suggest that only a bare majority (54%) of the diversity in the enrichment culture was detected by our methods. The remaining OTUs must represent very minor components of the enrichment since we detected 17 isolates at levels of 0.8% each.

Phylogenetic analysis showed that 7 of the 10 phylotypes sequenced (about 73% of the clones) were affiliated with high G+C Gram-positive bacteria and closely related to *Rhodococcus* species: *R. erythropolis*, *R. globerulus*, and *N. calarea*. These *Rhodococcus* species are widely distributed and abun-

dant in soils and are able to use a wide range of organic compounds as sole sources for energy and growth, and are able to transform a variety of xenobiotics *(25)*. Thus, the *Rhodococcus*-related strains appear to be the key players for CT degradation in this community.

Curtobacterium was also found in this CT-degrading community. However, their possible functions in this community are less certain because the physiological and biochemical characteristics of this group are not well understood. Most of the members of this genus have been isolated from plants. *C. citrum* and *C. luteum* were from rice, but their phytopathogenicity is unknown *(25)*. In addition, some strains of *C. pusillum* were also isolated from oil brine in an oil field *(25)*. They could be directly or indirectly involved in CT degradation, or they may have no role.

To estimate rapidly the phylogenetic diversity of the CT-degrading community sequences averaging about 350 bp sequences were obtained with one sequencing reaction from the hypervariable region of the 16S rRNA gene for all of the clones. Such short sequences are sufficient to allow us to distinguish most organisms from their relatives and to estimate their approximate phylogenetic relationships. However, such short sequences may be inadequate to resolve deep phylogenetic relationships. Thus, the branching orders on some of the phylogenetic trees are only approximate.

These cultures were grown in the presence of a relatively complex substrate (toluene) in addition to CT. Under these conditions, there was complete toluene removal but some CT remained *(9)*. Other enrichment cultures grown in parallel under similar conditions also removed the toluene but did not reduce the CT *(9)*. The relative complexity of the substrate may help maintain a high diversity owing to the potential for use of degradation products as the toluene is utilized. However, any toxic effects of the CT would have worked to reduce the diversity.

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