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Identification of a Novel Toluene-Degrading Bacterium from the Candidate Phylum TM7, as Determined by DNA Stable Isotope Probing▽

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The dominant bacterium responsible for carbon uptake from toluene in an agricultural soil was identified by stable isotope probing. Samples were amended with unlabeled toluene or labeled [ring-$^{13}C_6$]toluene, and DNA was extracted over time. Sequencing indicated that the organism involved belongs to the candidate phylum TM7. Microorganisms in this candidate phylum are of particular interest because although they have been found in a variety of habitats, no stable culture of any species exists, so their general metabolic capabilities are largely unknown.

The application of PCR technology has uncovered the impressive diversity of the microbial world. It has been estimated that less than half of the recognized bacterial phyla include cultured representatives (14). Here, stable isotope probing (SIP) (a method that links function to identity in mixed microbial samples) was used to identify dominant toluene degraders in an environment previously unexposed to the contaminant but likely containing a diverse microbial community of previously undiscovered toluene degraders.

Soil samples were collected from a field in Michigan previously under corn production. This field received biosolids from a wastewater treatment plant 2 to 3 years before sample collection. Following collection, soils were homogenized, sieved (4-mm screen), and stored at 4°C until use (<1 year). The microcosms consisted of phosphate-buffered mineral medium (20 ml) (10) and soil (6 g [wet weight]) in serum bottles (150 ml). The bottles were sealed with rubber stoppers and an aluminum seal. The treatment groups included no-toluene controls, autoclaved controls, and samples amended with unlabeled (1 μl, 99%; Chem Service) or labeled (1 μl, ring-$^{13}C_6$, 99%; Cambridge Isotope Laboratories) toluene. Eight samples for each treatment (two for each time point) were incubated at room temperature (~20°C) with reciprocal shaking. The concentrations of toluene in headspace samples (200 μl) after toluene addition (~2 h) and at each time point were determined with a gas chromatograph (Perkin Elmer) equipped with a flame ionization detector and a capillary column (DB-624 [diameter, 0.53 mm]; J&W Scientific). The injector and detector temperatures were set at 200°C, and the column temperature was 80°C.

At four time points (3, 5, 6, and 8 days after toluene addition), two samples from the labeled and unlabeled treatment groups were sacrificed for soil DNA extraction using a Powersoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA). At each time point, DNAs from two microcosms were pooled, and SIP involved terminal restriction fragment length polymorphism (TRFLP) analysis of all fractions (labeled and unlabeled). Approximately 10 μg DNA (quantified with an ND-1000 spectrometer; Nanodrop) was added to Quick-Seal polyallomer tubes (13 by 51 mm, 5.1 ml; Beckman Coulter), along with a Tris-EDTA (pH 8.0)–CsCl solution. Before the tubes were sealed (Quick-Seal tube topper; Beckman Coulter), buoyant density (BD) was determined (~1.77 g ml^{-1}) with a model AR200 digital refractometer (Leica Microsystems, Inc.) and adjusted by adding CsCl solution or Tris-EDTA buffer. The tubes were centrifuged at 178,000 × *g* (20°C) for 48 h in a Stepsaver 70 V6 vertical titanium rotor (eight tubes, 5.1-ml capacity each) within a Sorvall WX 80 Ultra Series centrifuge (Thermo Scientific). Following centrifugation, a fraction recovery system (Beckman Coulter) was used for fraction (150 μl) collection. The BD of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation.

The fractions were PCR amplified using 27F-FAM (5′-AGAGTTTGATCMTGGCTCAG [5′ end labeled with carboxyfluorescein]) and 1492R (5′-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) as previously described (4). Briefly, this involved the following conditions: 94°C (5 min); 30 cycles at 94°C (30 s), 55°C (30 s), and 72°C (1.5 min); and 72°C (5 min). The presence of PCR products was confirmed by gel electrophoresis. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Inc.) (~150 ng) and digested with HaeIII (New England Biolabs). In addition, three other enzymes (MspI, MseI, and HincII; New England Biolabs) were used for digestion of a number of heavy fractions. DNA fragments were separated by capillary electrophoresis (ABI Prism 3100 genetic analyzer; Applied Biosystems) at the Research Technology Support Facility at Michigan State University. Data were analyzed with Gene-

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TABLE 1. Ranges for percentages of toluene remaining in soil-liquid slurries over time

Time (days)	Range of % toluene in:		
	Sterile controls	Samples treated with toluene	
		^{12}C	^{13}C
3	66–78	61–81	65–81
5	63–67	47–57	50–65
6	43–75	23–37	25–38
8	50–76	0.3–0.5	0.3–0.9

Scan software (Applied Biosystems), and the percent abundance of each fragment was determined (18). Heavy-fraction ^{13}C-labeled DNAs (day 8 fractions with BD values of 1.744 g ml^{-1}) were amplified, as described above, with unlabeled primers and cloned into *Escherichia coli* TOP10 by using a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria-Bertani medium solidified with 15 g agar liter^{-1} in the presence of 50 μg ampicillin liter^{-1} for 16 h at 37°C. Colonies with inserts were verified by PCR with primers M13 forward (5′-TGTAAAACGACG GCCAGT-3′) and M13 reverse (5′-AACAGCTATGACCA TG-3′), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.), and the insertions were sequenced (using primers M13 forward and M13 reverse) at the Research Technology Support Facility at Michigan State University. The partial 16S rRNA gene sequences obtained were aligned and edited with Chromas Pro (Technelysium, Pty. Ltd.). The Ribosomal Database Project (Center for Microbial Ecology, Michigan State University) analysis tool "classifier" (16) was utilized to assign taxonomic identity. In addition, the Ribosomal Database Project classifier checked the deposited sequence for chimeras.

Toluene removal occurred rapidly, starting after 3 days and reaching completion after 8 days (Table 1). The low percentage of recoveries was likely caused by toluene sorption to the soil; however, the difference between the controls and samples clearly illustrates a biological removal mechanism. DNAs extracted over time (on days 3, 5, 6, and 8) from both labeled and unlabeled samples were subjected to ultracentrifugation, fractionation, and TRFLP on every fraction. TRFLP analyses indicated that one fragment (394 bp) was highly enriched in the heavy fractions (>1.732 g ml^{-1}) obtained from [^{13}C]toluene microcosms but not in fractions with similar BD values obtained from the unlabeled controls, and further, the level of enrichment increased with time (Fig. 1). At day 3, the fragment was present in only one fraction TRFLP profile, indicating a low natural relative abundance of this organism. The peak TRFLP relative abundance values for the 394-bp fragment were 11.3%, 41.5%, and 62.1% on days 5, 6, and 8, respectively (Fig. 2). Consistent with this trend, the BD of this fragment also increased with time, with the peak relative abundances being found at BD values of 1.732 g ml^{-1}, 1.740 g ml^{-1}, and 1.744 g ml^{-1} on days 5, 6, and 8, respectively (Fig. 2). These trends indicate that the organism represented by this fragment is directly involved in toluene transformation. A number of other TRFLP peaks were found in the heavy ^{13}C fractions; however, because they were also present in the heavy ^{12}C fractions, they were excluded from further analysis.

The microorganism represented by the 394-bp TRFLP fragment was determined both by partial 16S rRNA gene sequencing of cloned DNA and by TRFLP analysis with additional restriction enzymes. TRFLP analysis with the restriction enzymes MspI, MseI, and HincII on ^{13}C-enriched heavy fractions resulted in a unique dominant peak for each enzyme (Fig. 3). The fragment lengths of these dominant peaks were compared

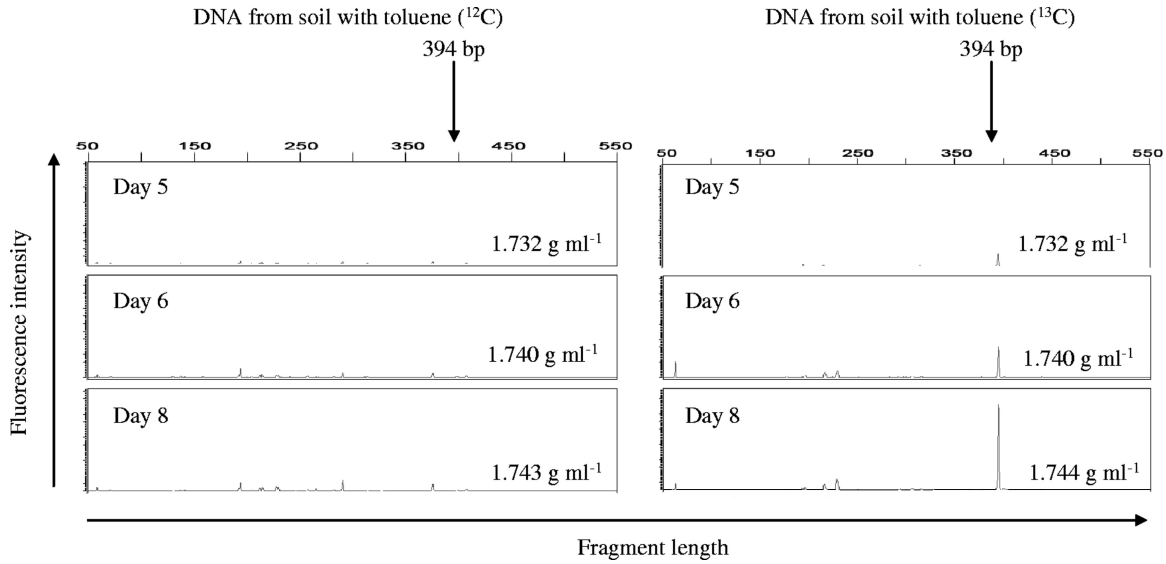

FIG. 1. Dominance of the 394-bp fragment over time in TRFLP profiles from heavy fractions of soil samples amended with unlabeled (^{12}C) or labeled (^{13}C) toluene. The pattern is representative of other heavy-fraction samples at these time points. The BD values are given in the lower-right corners of the profiles.

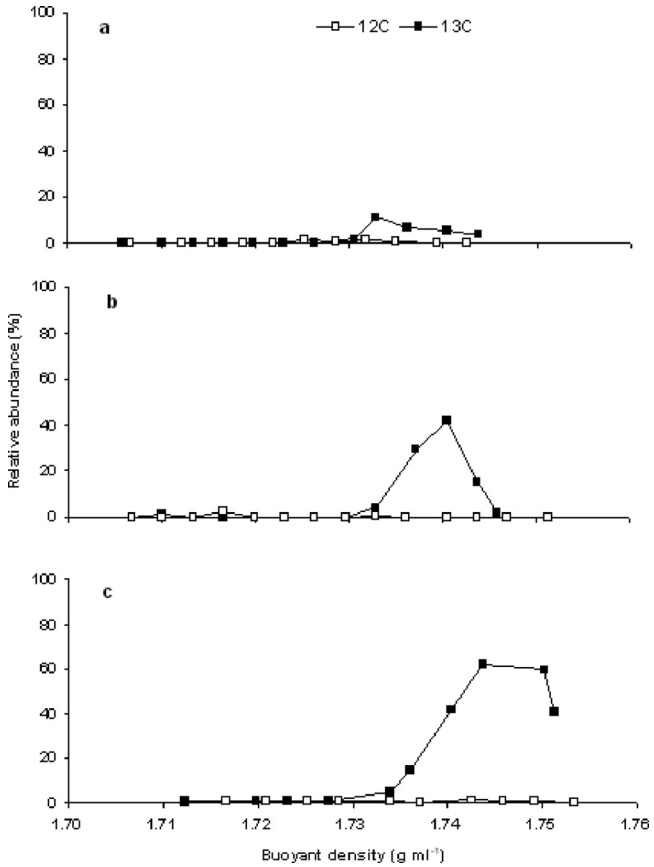

FIG. 2. Relative abundances of the dominant 394-bp fragment over a range of BD values from DNA extracted after 5 days (a), 6 days (b), or 8 days (c) from soil amended with either labeled (^{13}C) or unlabeled (^{12}C) toluene.

to those obtained from sequence data for in silico digests of cloned 16S rRNA genes. Of 20 clones sequenced, 17 contained restriction enzyme-cut sites that matched the TRFLP results (Table 2). The slight difference (2 or 3 bases) between the TRFLP fragment lengths and those predicted using sequence

TABLE 2. Comparison of dominant fragments in heavy-fraction TRFLP profiles to those in clone restriction enzyme-cut sites, as predicted by sequence analyses

Restriction enzyme	Fragment length (bp)	
	TRFLP	Clones
HaeIII	394	396
MspI	455	457
MseI	182	184
HincII	254	256

data has also been noted by others (2, 4, 11). The partial 16S rRNA gene sequences obtained from 17 clones (~1,300 bp for each) were all highly similar (>99%) and classified as belonging to the candidate phylum TM7. The three most similar (95%) sequences in GenBank (EU431823.1, EU431720.1, and EU431818.1) represented uncultured clones originating from calcium carbonate muds in Italy. The TM7 toluene degrader identified here could have originated from a wastewater treatment plant or could be a member of the soil community. Others have also found TM7 organisms in soils (13) and wastewater-associated samples (15, 17).

Microorganisms belonging to the candidate phylum TM7 have been referred to as biology's "dark matter" problem (9), being a focus of study because although they have been identified (via clone sequences) in a wide variety of habitats, from hydrothermal sediments and chlorinated solvent sites to the human mouth (1, 3, 5–8, 12), researchers have yet to obtain a stable culture of any isolate. A number of novel approaches have been used to investigate TM7 organisms, including the use of microfluidic devices (9) and cell separation by fluorescent in situ hybridization and flow cytometry (13) to obtain single cells for genetic analysis. The data presented here contribute to the limited pool of information on the functional abilities of TM7 bacteria and provide the first report directly linking toluene removal to this phylum.

Nucleotide sequence accession number. The 16S rRNA gene sequence of the bacterium classified as belonging to candidate

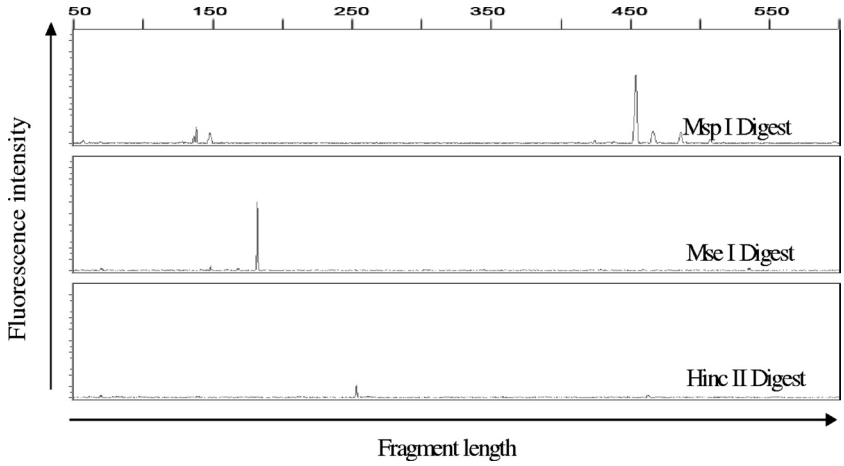

FIG. 3. TRFLP profiles from heavy fractions (1.732 to 1.751 g ml^{-1}) of labeled toluene-amended soil samples with three different restriction enzymes (digestion with HincII contained a lower mass of DNA).

phylum TM7 was deposited in GenBank under accession number FJ629383.

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