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Identification of a Toluene-Degrading Bacterium from a Soil Sample through $H_2{}^{18}O$ DNA Stable Isotope Probing▽†

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DNA stable isotope probing (DNA-SIP) with $H_2{}^{18}O$ was used to identify a toluene-degrading bacterium in soil amended with 48 ppm toluene. After quantification of toluene degradation rates in soil, DNA was extracted from soil incubated with $H_2{}^{18}O$, $H_2{}^{16}O$, $H_2{}^{16}O$ and 48 ppm toluene, or $H_2{}^{18}O$ and 48 ppm toluene. A single DNA band formed along a cesium chloride gradient after isopycnic centrifugation of extracts from soils incubated with $H_2{}^{16}O$. With extracts from soils to which only $H_2{}^{18}O$ was added, two distinct DNA bands formed, while three bands formed when DNA extracted from soil incubated with both $H_2{}^{18}O$ and toluene was analyzed. We suggest that this third band formed because toluene does not contain any oxygen atoms and toluene-degrading organisms had to transfer oxygen atoms from $H_2{}^{18}O$ into metabolic intermediates to form nucleic acids *de novo*. We extracted the third DNA band and amplified a large fraction of the bacterial 16S rRNA gene. Direct sequencing of the PCR product obtained from the labeled DNA, as well as cloned 16S rRNA amplicons, identified a known toluene degrader, *Rhodococcus jostii* RHA1. A toluene-degrading bacterial strain was subsequently isolated from soil and shown to be *Rhodococcus jostii* RHA1. Finally, quantitative real-time PCR analysis showed that the abundance of the 16S rRNA gene of *Rhodococcus jostii* RHA1 increased in soil after toluene exposure but not in soils from which toluene was withheld. This study indicates that $H_2{}^{18}O$ DNA-SIP can be a useful method for identifying pollutant-degrading bacteria in soil.

DNA stable isotope probing (DNA-SIP) with ^{13}C-labeled substrates is an effective means for identifying microorganisms that degrade and assimilate a substrate (3, 6, 8, 34). The soil microcosm is spiked with labeled substrate and incubated, a soil DNA extraction is performed after incubation, and the DNA of labeled organisms is separated from the DNA of unlabeled organisms on a CsCl density gradient generated by ultracentrifugation (17, 20). This has been a valuable method for identifying active pollutant degraders in terrestrial and aquatic systems (5, 12, 14, 18, 19, 27).

An important limitation in using DNA-SIP to characterize pollutant-degrading microorganisms *in situ* is that DNA-SIP studies often use a single labeled, freshly added substrate, while most waste sites are contaminated by multiple pollutants, some of which exhibit decreased bioavailability because of sorption or other processes rendering the compound inaccessible (2, 9, 4, 33). In DNA-SIP experiments, a cocktail of labeled substrates would simulate a real-world contamination scenario more accurately, but the costs of using many different labeled substrates may be prohibitive. Further, manufacturing a labeled complex cocktail of compounds, such as crude oil, is technically not feasible. Tracing newly added substrate into DNA also ignores the challenge of aged pollutants in soil. Often soil contamination has been present for extended periods of time and we are particularly interested in revealing the identities of microorganisms that degrade these less-bioavailable chemicals (24). We were therefore interested in developing a SIP approach that could investigate degradation of multiple or aged pollutants in soil.

We hypothesized that it would be feasible to characterize pollutant-degrading microorganisms in soil through DNA-SIP with $H_2{}^{18}O$ (25, 26). By comparing labeled DNA extracted from soils exposed to pollutants and $H_2{}^{18}O$ to labeled DNA isolated from soils incubated with only $H_2{}^{18}O$, it may be possible to identify the organisms that grow in the presence of the pollutant but do not replicate when the pollutant is absent. If it is feasible to identify pollutant-degrading microorganisms through $H_2{}^{18}O$ DNA-SIP, the technique would lend itself well to the study of degradation of contaminant cocktails, because any mixture of pollutants could be added to the treatment samples yet only one isotopically labeled substrate ($H_2{}^{18}O$) would need to be procured. Furthermore, the experimental approach may be suitable for study of the degradation of sorbed pollutants by comparing the growing microbial populations among samples to which (i) the pollutant was added well before the addition of $H_2{}^{18}O$, (ii) the pollutant was added with the $H_2{}^{18}O$, or (iii) only $H_2{}^{18}O$ was added.

Since $H_2{}^{18}O$ DNA-SIP has not yet been used in pollutant degradation studies, we tested the ability of the method to identify a toluene degrader in soil obtained from an automobile salvage yard. We hypothesized that small amounts of toluene added to mixtures used in $H_2{}^{18}O$ DNA-SIP experiments would stimulate the growth of toluene-degrading bacteria and that $H_2{}^{18}O$ DNA-SIP would allow us to identify them in soil by comparing results for samples spiked with toluene and those for samples without toluene.

MATERIALS AND METHODS

Biodegradation of toluene in soil microcosms. For all experiments, soil was collected from the top 6 cm at an automobile salvage yard located in the high desert of north-central Arizona. The site is at an elevation of ~960 m, has a mean annual temperature of 16.2°C, and receives ~368 mm of precipitation annually.

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At the time of collection, the gravimetric water content was 6.1%. Triplicate 20-g soil samples were prepared in 200-ml glass bottles for destructive sampling at 6 time points (18 treatments and 18 controls). Control soils were autoclaved twice for 30 min at 121°C, with 24 h between sterilizations. All bottles were spiked with 10 μl toluene (99.9% purity; J. T. Baker, Inc. Phillipsburg, NJ), sealed with Teflon septa and aluminum crimp tops, and incubated at 32°C. Gas-tight syringes were used to extract 10 μl of headspace gas, which was injected into a Shimadzu GC-14A instrument equipped with a flame ionization detector. The carrier gas used was He, and the column, injector, and detector temperatures were set at 110°C, 180°C, and 180°C, respectively. Toluene concentrations were analyzed using PeakSimple v3.29 software from SRI Instruments (Las Vegas, NV).

$H_2{}^{18}O$ DNA-SIP experiments. Soil microcosms were prepared in triplicate for $H_2{}^{18}O$ DNA-SIP experiments. $H_2{}^{18}O$ (97% ^{18}O) was obtained from Cambridge Isotope Laboratories (Andover, MA). Two treatment mixtures and two control mixtures were prepared in 10-ml glass vials as follows: 1.0 g of soil with 250 μl $H_2{}^{18}O$, 1.0 g of soil with 250 μl $H_2{}^{18}O$ and 0.5 μl toluene, 1.0 g of soil with 250 μl $H_2{}^{16}O$, and 1.0 g of soil with 250 μl $H_2{}^{16}O$ and 0.5 μl toluene. All vials were sealed with Teflon septa and aluminum crimp tops and incubated at 32°C with shaking (100 rpm). After 2 weeks, DNA was extracted using a FastDNA spin kit for soil (MP Biomedicals, Solon OH) following the manufacturer's instructions.

Five hundred nanograms of DNA was added to 3.6 ml of cesium chloride (1.9 g/ml), 0.3 ml of gradient buffer (200 mM Tris [pH 8.0], 200 mM KCl, 2 mM EDTA), and 0.5 μl of 10,000× SYBR green I (Invitrogen, Carlsbad CA), prepared in OptiSeal ultracentrifuge tubes (Beckman Coulter, Brea, CA). The tubes were spun at 60,000 rpm (149,990 × *g* at r_{av}) at 15°C for 144 h in a TLA-110 rotor in an Optima MAX benchtop ultracentrifuge (Beckman Coulter, Brea CA). Two hundred microliters of a DNA band was removed either by using a pipettor inserted through the tube top opening to retrieve the upper bands or by piercing the side of the tube just below the heavier bands using a 21-gauge, 1 1/2-in. needle attached to a 1-ml syringe. Densities of cesium chloride fractions were determined using an analytical balance.

Direct sequencing and cloning of 16S rRNA gene PCR products. Conserved bacterial primers 27F and 1492R (13) were used to target and amplify the nearly complete 16S rRNA gene using the following mixture: 0.1 to 50 ng band extract DNA template, 10 pmol primer 27F, 10 pmol primer 1492R, 0.2 mM deoxynucleoside triphosphate, 2.5 U *Taq* polymerase (Invitrogen, Carlsbad CA), 3 mM $MgCl_2$, and 1× *Taq* buffer. The protocol used was as follows: an initial 2-min period at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; and finally a 10-min incubation at 72°C. To achieve at least 2× coverage over the large gene fragment, the following internal primers were used in addition to 27F and 1492R for directly sequencing the PCR product: 357F (5′-CTACGGGAG GCAGCAG-3′), 520R (5′-ACCGFGGGGTGCTGGC-3′), 920F (5′-AAACTC AAAGGAATTGACGG-3′), and 1080R (5′-CCCAACATCTCACGAC-3′). All reactions were performed using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), analyzed on an ABI Prism 3730XL DNA sequencer (Applied Biosystems, Foster City, CA), and assembled using the Staden package pregap4 v1.5 and gap v4.10 assembly programs (28).

After direct sequencing, we also developed a bacterial clone library from the heavily labeled DNA extracts using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Using the provided M13 primer set, whole-cell PCR was performed on 30 colonies. Sequencing reactions, analyses, and assembly were performed as described above, and the consensus sequences carrying the 16S rRNA gene of *Rhodococcus jostii* were submitted to the Greengenes 16S rRNA gene database (http://greengenes.lbl.gov) for alignment and taxonomic classification.

Enrichment and isolation of toluene degraders. The vapor plate enrichment method was used to enrich for toluene degraders (29). Soil dilutions were plated on minimal salts agar (containing, in g/ml distilled water [dH_2O]: KH_2PO_4, 6.8E−4; K_2HPO_4, 1.73E−3; $MgSO_4 \cdot 7H_2O$, 1.0E−4; NH_4NO_3, 1.0E−3; trace elements, 1.0E−4 ml [trace element solution, in g/ml dH_2O: MgO, 0.01; $CaCO_3$, 0.002; $FeCl_3 \cdot 6H_2O$, 0.006; $ZnSO_4 \cdot 7H_2O$, 0.002; $CuSO_4 \cdot 5H_2O$, 2.63E−4; $CoSO_4 \cdot 7H_2O$, 2.95E−5; H_3BO_4, 6.53E−5; $Na_2MoO_4 \cdot 2H_2O$, 5.16E−4; 10 ml concentrated HCl; filter sterilized]; and purified agar, 17.0 [pH 7.0]) and incubated at 25°C in a sealed chamber with toluene vapor as the sole carbon source. Every 3 days, the lid was removed to aerate the chamber and replenish the toluene. Control plates were prepared and incubated in the same manner in a separate, sealed chamber, but toluene was withheld. When growth was detected, 6 colonies were selected and streaked successively 5 times. DNA was extracted from the isolates using an UltraClean microbial DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA), and 16S rRNA gene PCR, sequencing reactions, and analysis were performed as described above.

Toluene degradation in pure culture. Triplicate 20-ml minimal salts broth cultures of the isolate and sterile controls were prepared in 200-ml glass bottles for destructive headspace sampling at 4 time points (12 treatment and 12 control bottles). All bottles were spiked with 5 μl toluene, sealed with Teflon septa and aluminum crimp tops, and stored at 32°C with shaking (100 rpm) until destructive headspace sampling, which was performed as described above.

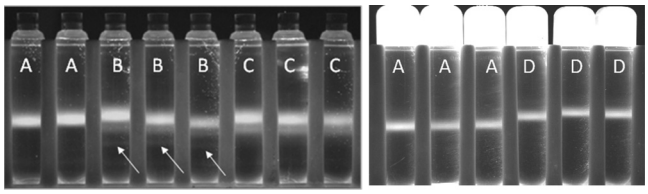

FIG. 1. Ultracentrifuge tubes after isopycnic centrifugation of DNA extracted from soil incubated with $H_2{}^{16}O$ (tubes A), $H_2{}^{18}O$ and 48 ppm toluene (tubes B), $H_2{}^{18}O$ (tubes C), or $H_2{}^{16}O$ and 48 ppm toluene (tubes D). The arrows indicate a third, more heavily labeled band, which is present only in soils exposed to $H_2{}^{18}O$ and toluene.

Phylogenetic characterization. Using the sequences shown in Table S1 in the supplemental material, we performed a multiple-sequence alignment using the ClustalW alignment algorithm (32). We then constructed a phylogenetic tree in MEGA4 (31), employing the neighbor-joining algorithm (23) and maximum composite likelihood method (30). A 1,000-replicate bootstrap test was performed for statistical support (7).

qPCR assays. For each of 7 time points, triplicate 1.0-g soil samples were moistened with 250 μl of sterile deionized water and incubated in 10-ml glass bottles (21 treatments and 21 controls). Treatments were spiked with 0.5 μl toluene, while toluene was withheld from control samples. The DNA was extracted with a Fast DNA Spin for Soil kit (MP Biomedicals, Carlsbad, CA) following the manufacturer's instructions. To develop a primer set specific to the 16S rRNA gene sequence of *Rhodococcus jostii* RHA1, clone sequences representing the 16S rRNA gene from RHA1 were checked against Greengenes-reported nearest neighbors for regions of variability and submitted to Primer3 v 0.4.0 for comparison. Primer pairs were then checked for exclusivity using the Primer-BLAST database (1). The primer pair RhodoF (5′-CGACCTTCGGCT GCATGGCT-3′) and RhodoR (5′-GCCCATCCTGCACCAGTAAACC-3′) was used in quantitative real-time PCR (qPCR) assays and formed a PCR product 53 bp in length. The primer pair was tested with both soil DNA and the RHA1 16S rRNA gene PCR product obtained from cloning. These PCR amplicons were used to generate a set of standards with known copy numbers of the target sequence. Standards were created by performing a 1:10 serial dilution to achieve a range from 10^2 to 10^9 copies of the 16S rRNA gene of *Rhodococcus jostii* RHA1. An r^2 value of greater than 0.975 and an amplification efficiency of 96.8% were achieved for the standard curves. Each 20-μl reaction mixture consisted of 0.2 μM RhodoF primer, 0.2 μM RhodoR primer, 20 ng template DNA, and iQ SYBR green Supermix. The optimized cycling protocol was 95°C at 5 min, followed by 40 cycles of 1 min at 94°C, 30 s at 64°C, and 1 min at 72°C, with a 10-min final extension at 72°C.

Nucleotide sequence accession numbers. The consensus sequences carrying the 16S rRNA gene of *Rhodococcus jostii* have been deposited in GenBank (accession numbers JF339138 to JF339141).

RESULTS

The toluene concentration in soil microcosms amended with 48 ppm toluene began to decline steadily after 4 days of incubation (see Fig. S1 in the supplemental material). By day 7, the toluene concentration had declined to 22.8 ppm, after which toluene degradation accelerated, so that by 8.5 days no toluene was detected. In sterile control microcosms, also amended with 48 ppm toluene, no decline in toluene concentration was observed.

After isopycnic centrifugation to separate labeled from nonlabeled DNA, one DNA band was observed in both controls exposed only to $H_2{}^{16}O$ and in controls exposed to $H_2{}^{16}O$ and 48 ppm toluene (Fig. 1). The average density of the DNA band extracted from $H_2{}^{16}O$-only controls was 1.65 g/ml, while the average density of the DNA band extracted from controls with

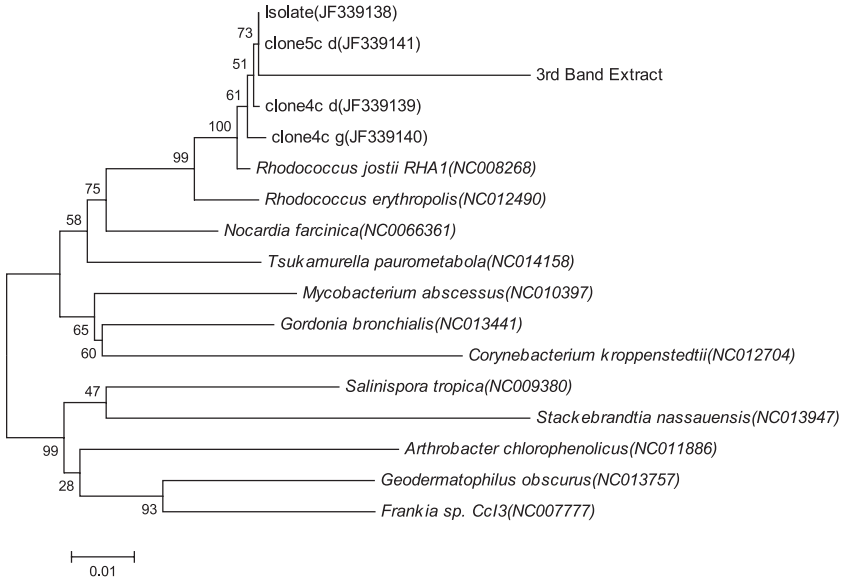

FIG. 2. Phylogenetic tree displaying relationships between the 16S rRNA gene sequences of the toluene-degrading isolate, the directly sequenced PCR product obtained from the third band extract, three clones generated from PCR products of the third band extract, and *Rhodococcus jostii* RHA1 (sequence obtained from GenBank). The evolutionary distances were computed in MEGA4 with a neighbor-joining algorithm using the maximum composite likelihood method and were bootstrapped with 1,000 replications for statistical support; bootstrap percentage values are shown at the nodes.

$H_2{}^{16}O$ plus 48 ppm toluene was 1.66 g/ml. In soils to which only $H_2{}^{18}O$ was added, two distinct DNA bands formed: the upper band, representing nonreplicating organisms, including dead cells, was comprised of nonlabeled DNA with an average density of 1.66 g/ml, while the lower band, representing actively replicating organisms, consisted of ^{18}O-labeled DNA with an average density of 1.68 g/ml. These two distinct bands also formed when DNA extracted from soil incubated with both $H_2{}^{18}O$ and toluene was centrifuged, but in addition, a third, lower band was present, consisting of DNA with a higher buoyant density. The average densities determined for the three bands extracted from these tubes were as follows: top band, 1.66 g/ml; middle band, 1.68 g/ml; and bottom band (henceforth referred to as the third band), 1.70 g/ml.

DNA from the third band, present only in $H_2{}^{18}O$ plus toluene treatments (Fig. 1, tubes B), was extracted and precipitated, the bacterial 16S rRNA gene was amplified, and the PCR product was sequenced directly. The resulting sequence reads were noisy but did indicate one 16S rRNA gene product as overwhelmingly dominant. Comparison of the sequence with the GenBank database revealed *Rhodococcus jostii* RHA1 as the closest match. To validate these results, we generated a clone library of the 16S rRNA gene products from the third band extract. Subsequent sequencing of our cloned gene products confirmed the presence of an organism with high similarity to *Rhodococcus jostii* RHA1, with 3 clones closely aligning with the strain in a phylogenetic tree (Fig. 2). BLAST results showed that these 3 cloned 16S rRNA gene products had a maximum identity of 99% with the 16S rRNA gene of *Rhodococcus jostii* RHA1 present in the GenBank database.

A toluene-degrading organism was isolated from plated soil dilutions incubated in a chamber with toluene vapors. The isolate grew on minimal medium plates exposed to toluene but did not grow on minimal medium when toluene was withheld from the chamber, indicating that it could use toluene as a sole carbon and energy source (see Fig. S2A in the supplemental material). Pure cultures of the isolate suspended in minimal salts broth amended with 24 ppm toluene were tested for their ability to degrade toluene. Twenty-four ppm toluene was depleted in 13 h in microcosms with the live isolate, while toluene did not decease in sterile controls (see Fig. S2B in the supplemental material).

The 16S rRNA gene of the isolate was sequenced and compared to the GenBank database. BLAST results determined a 99% maximum identity with *Rhodococcus jostii* RHA1, the same species detected in the third band in the SIP experiments. The 16S rRNA gene sequences obtained from the third band in the SIP experiments and the 16S rRNA gene sequence from the isolate all clustered together in a phylogenetic tree (Fig. 2).

The abundance of the *Rhodococcus jostii* RHA1 population in 1.0-g soil microcosms amended with 48 ppm toluene was measured with a qPCR assay. During 15 days of incubation, the number of 16S rRNA *Rhodococcus jostii* RHA1 gene copies per 20 ng of DNA increased from 13 to more than 9,000 in soil amended with toluene (Fig. 3). Most of the growth occurred within the first 5 days of the incubation. In contrast, when toluene was withheld from the incubation, the number of gene copies per 20 ng of DNA never exceeded 50.

DISCUSSION

The purpose of this study was to test whether it was feasible to identify a pollutant-degrading microorganism in soil through $H_2{}^{18}O$ DNA-SIP analysis. Commonly, after soil is exposed to $H_2{}^{18}O$ for 1 or 2 weeks, two bands of DNA appear in centrifugation tubes after isopycnic centrifugation

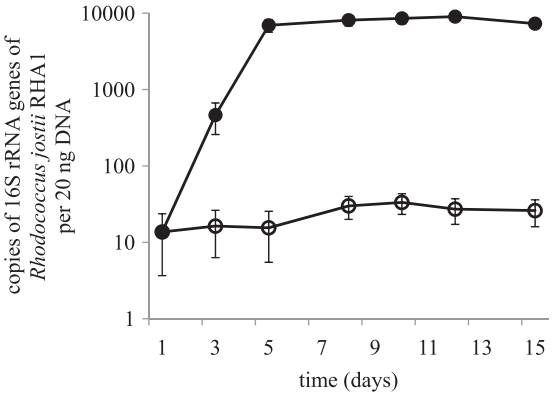

FIG. 3. Abundance of *Rhodococcus jostii* RHA1 16S rRNA genes over time in soil exposed to 48 ppm toluene (●) and in soil from which toluene was withheld (○). Error bars represent standard errors for three replicates. When error bars are not visible, the standard error is smaller than the symbol.

(25, 26) We hypothesized that sequences of pollutant-degrading microorganisms would be present in the labeled DNA fraction obtained from soils exposed to toluene but would be absent in labeled DNA extracted from soils without toluene. Unexpectedly, a third DNA band appeared after isopycnic centrifugation of DNA extracted from soil exposed to toluene and $H_2{}^{18}O$ (Fig. 1). Because formation of the third band was dependent on the presence of toluene, we altered our experimental approach and reasoned that genomic sequences of pollutant-degrading microorganisms were present in the third DNA band.

Several lines of evidence show that the genome of *Rhodococcus jostii* RHA1 was present in the third band and that this bacterium degraded toluene in our soil microcosms. First, direct sequencing of a bacterial 16S rRNA gene amplified with conserved bacterial primers showed that the genome of *Rhodococcus jostii* RHA1 was present in the third DNA band. *Rhodococcus jostii* RHA1 is a well-characterized Gram-positive actinomycete that is commonly found in arid and semiarid soils and known to degrade a broad range of aromatics, including toluene (15). Further, RHA1's unusually large genome (9.7 Mb) has been extensively characterized, and 203 oxygenase-encoding genes have been identified (16). This is consistent with the organism's ability to hydroxylate and cleave a wide range of aromatics. Considering both its known niche and its metabolic capabilities, the detection of RHA1 as a toluene degrader in a high-desert contaminated soil was not surprising. Second, a strain with a 16S rRNA gene highly similar to the sequence detected in the third DNA band was isolated from the soil and shown to degrade toluene in pure cultures (see Fig. S2 in the supplemental material). Third, the abundance of *Rhodococcus jostii* RHA1 increased in soils amended with toluene but did not in soils without toluene, indicating that the presence of toluene was required for growth (Fig. 3).

The observation that the bottom third band forms only in the presence of toluene may have important implications for the study of pollutant degradation in soil. In studies with pure cultures of *Escherichia coli*, Richards and Boyer (22) showed that the branch oxygen atoms within the phosphodiester linkage rapidly became labeled with ^{18}O from $H_2{}^{18}O$, while the C-3 and C-5 bridge oxygen atoms were derived from glucose, the carbon source employed in their studies. Exchange of oxygen atoms between inorganic phosphate, ATP, and water occurs rapidly, and these oxygen atoms may be incorporated into specific positions in the phosphodiester backbone of DNA (4, 10, 11, 21). The toluene-degrading isolate characterized in this study used toluene as its sole carbon and energy source and converted the pollutant into all biomolecules, including DNA. Toluene does not contain any oxygen atoms, and therefore, to generate nucleotides when toluene is an organism's sole carbon source, ^{18}O atoms from water not only are incorporated into the phosphodiester linkage but may be incorporated into other parts of the nucleotide as well, resulting in heavier DNA. If this interpretation is correct, the formation of the third band would occur whenever a microorganism is unable to salvage nucleotides from catabolic pathways and is required to produce nucleotides from a carbon source that lacks oxygen atoms. Because most organic pollutants that pose environmental concerns lack oxygen atoms, it is possible that most pollutant-degrading microorganisms will form a third band in $H_2{}^{18}O$ DNA-SIP experiments.

Future research on the application of $H_2{}^{18}O$ DNA-SIP to the study of pollutant degradation in soil should combine multiple contaminants in a cocktail in order to identify microbial populations that assimilate these pollutants. In $H_2{}^{18}O$ DNA-SIP, heavy isotopes from the pollutant substrate do not become incorporated into nucleic acids of contaminant-degrading organisms. Therefore, there is no direct evidence that soil organisms with DNA that is labeled after incubation with $H_2{}^{18}O$ have actually assimilated the pollutants. It will be necessary to show that the DNA sequences associated with putative pollutant degraders identified from the labeled DNA increase in abundance in soils exposed to the pollutant but are not more abundant in soils from which the pollutant was withheld. Even then it will not be possible to identify which substrate in the pollutant cocktail the organism grew on without further research, including characterizing the population dynamics of the organism in soil exposed to a single pollutant derived from the cocktail or using ^{13}C-based DNA-SIP.

This study lays the groundwork for application of $H_2{}^{18}O$ SIP to future biodegradation studies. We suggest that $H_2{}^{18}O$ SIP may offer novel insights into pollutant degradation experiments by characterizing microorganisms that grow after pollutant exposure and may especially be valuable as a cost-effective means for studying the degradation of complex cocktails of pollutants in soil.

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