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Detection of 2,4,6-Trinitrotoluene-Utilizing Anaerobic Bacteria by ^{15}N and ^{13}C Incorporation▽

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2,4,6-Trinitrotoluene (^{15}N or ^{13}C labeled) was added to Norfolk Harbor sediments to test whether anaerobic bacteria use TNT for growth. Stable-isotope probing (SIP)–terminal restriction fragment length polymorphism (TRFLP) detected peaks in the [^{15}N]TNT cultures (60, 163, and 168 bp). The 60-bp peak was also present in the [^{13}C]TNT cultures and was related to *Lysobacter taiwanensis*.

It has been estimated that there are over 1 million cubic yards of material contaminated with 2,4,6-trinitrotoluene (TNT) in the United States at concentrations as high as 600,000 to 700,00 mg/kg of material (9). Marine and estuarine sediments have also been impacted through the manufacturing, use, and/or disposal of TNT. Microbial biodegradation of these pollutants *in situ* is preferable due to the large volume of contaminated soils/sediments. However, it is unclear whether *in situ* bacteria can utilize TNT as a nitrogen or carbon source. Under aerobic conditions, TNT appears to be largely unavailable to bacteria but can be used by a variety of fungi as a carbon and nitrogen source (7). Under anaerobic conditions, only a few bacterial strains (*Clostridium* and *Desulfovibrio* strains and *Pseudomonas* sp. strain JLR11) have been reported to utilize TNT as a sole nitrogen source (6, 7). It is widely believed that nitroaromatic compounds cannot serve as growth substrates under anaerobic conditions *in situ* (11), and coamendment strategies are suggested for stimulating TNT transformation to 2,4,6-triaminotoluene (TAT) (1, 7, 18). Given these difficulties, there is no direct evidence that TNT can be biodegraded *in situ* and there is little proof that anaerobic bacteria can utilize TNT as a sole carbon or nitrogen source in organic-rich sediments. This study tested whether bacteria in Norfolk Harbor sediment are able to incorporate nitrogen (N) or carbon (C) from TNT into biomass under sulfidogenic conditions using stable-isotope probing (SIP). The findings indicate that bacteria assimilate ^{15}N and ^{13}C from TNT into their genomes during anaerobic incubations (2 to 35 days). Interestingly, one small-subunit (SSU) gene, related to *Lysobacter taiwanensis*, was observed in both the ^{15}N and the ^{13}C incubations.

Method optimization. An inoculum of Norfolk Harbor sediment was added to a minimal salts medium (10% sediment slurries) containing no additional nitrogen, with sulfate as the electron acceptor and TNT (100 μM) as the electron donor (method adapted from reference 13). There were 3 different treatments for this experiment: (i) inoculum in media with [^{14}N]TNT added, (ii) inoculum in media with [^{15}N]TNT only added, and (iii) inoculum in media with [^{13}C]TNT added. The live cultures were incubated in triplicate and destructively sampled at 0, 2, 7, 14, 21, and 28 days for the [^{15}N]TNT-amended cultures. The [^{13}C]TNT-amended cultures were sampled at day 35. DNA was extracted from the each biological sample in triplicate using a modified phenol-chloroform extraction procedure (12, 17).

For stable-isotope probing (15), enrichment DNA and *Halobacterium salinarium* carrier DNA (labeled using [^{15}N]- or [^{13}C]Isogro medium; Isotec, Miamisburg, OH) were added to cesium chloride gradients as described by Gallagher et al. (8). Since DNA has a lower number of nitrogen atoms than carbon, heavy N bands will migrate differently in cesium gradients with respect to heavy C (3, 5). We found that the distances between the light and heavy bands were routinely around 3.0 mm for N-SIP gradients and 5.5 mm for C-SIP gradients. These distances of separation remained constant for both pure culture gradients (data not shown) and environmental sample gradients (Fig. 1). For determining the identity of bacteria able to assimilate the ^{15}N or ^{13}C label, the 16S rRNA gene was amplified using 27F and 1100R primers (8). In all ^{14}N control incubations, no PCR amplification occurred in the heavy carrier band samples, as has been described previously (8) (data not shown).

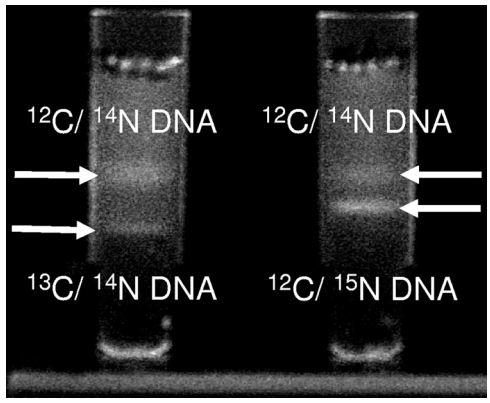

FIG. 1. Cesium gradients demonstrating separation of light and heavy isotopes of carbon and nitrogen.

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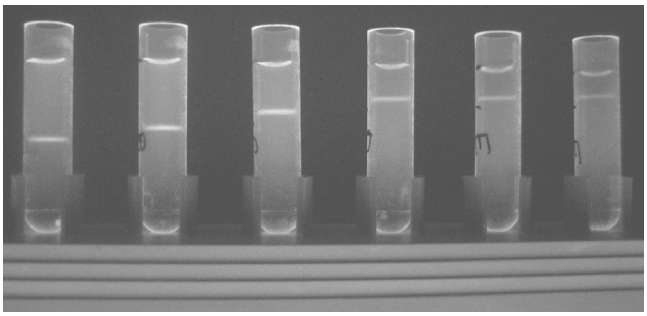

FIG. 2. Replicate CsCl gradients with identical DNA concentrations and increasing amounts of ethidium bromide (5 to 80 μg).

While there are prior reports of great difficulty in achieving distinct ^{15}N fractions with respect to genomic DNA GC content in cesium gradients (3, 5), our methods allow good recovery of uncontaminated DNA. One possible reason is the practice of DNA quantification before addition to the gradient and the use of ethidium bromide for visualization. It has long been known that intercalating reagents can influence the apparent buoyant density of DNA in cesium gradients. Figure 2 illustrates replicate gradients containing the same mass of [^{12}C]DNA that have been amended with increasing concentrations of ethidium bromide. As the visual evidence shows, the DNA can band in very different parts of the gradient depending on the DNA/ethidium bromide ratio. The addition of ethidium bromide appears to overwhelm the influence of GC content on buoyant density (mass and volume) by contributing to the DNA mass as well as changing the DNA volume (topology). This finding is in agreement with the report by Buckley et al. (3), where the intercalating reagent bisbenzimide is used for preparing ^{15}N.

Result from incubations. A time course of the various ^{15}N terminal restriction fragment length polymorphism (TRFLP) profiles from the incubations indicated there were 6 peaks (operational taxonomic units [OTUs]) present at day 2; this number increased through days 7, 14, and 21 and ultimately decreased by day 28. The largest of these peaks (60 bp) (Fig. 3) was observed throughout the incubation period (data not shown). To test whether a similar microorganism(s) also obtains carbon from TNT for growth, [^{13}C]TNT enrichment was performed. After 35 days, the ^{13}C label profiles contained 38 OTUs. When the TRFLP profiles from day 7 in the [^{15}N]TNT study and day 35 from the [^{13}C]TNT study are placed side by side, the terminal restriction fragment (TRF) present at 60 bp is discernible in both cultures (Fig. 3).

To identify the bacteria involved in N and C uptake from TNT, a TOPO TA clone library was established from the ^{15}N 7-day incubation sample and screened for various TRFLP peaks of interest. The SSU gene from the 60-bp peak was identified, though attempts to identify other major TRFLPs in the clone library by screening over 200 colonies were not successful. This cloning bias (i.e., missing many of the major TRFLP peaks in a sample) has been reported previously (4, 14, 21). Sequence analysis of the SSU gene for the 60-bp TRF clone (GenBank accession no. GU324324) indicated that the closest matches were to environmental clones obtained from a wetland treated for dichloroethene contamination in Germany (GenBank accession no. FM205963), a microbial mat above sulfidic waters in Movile Cave, Romania (GenBank accession no. EU662609), and a deep well in Siberia (GenBank accession no. AJ534675). Phylogenetic tree reconstruction using the Geneious Basic software package and maximum-likelihood methods (10) generated a bootstrapped consensus tree showing that the 60-bp SSU was related to *Lysobacter* and *Dokdonella* spp. (Fig. 4).

These findings differ from the wide belief that TNT is largely recalcitrant and not mineralized by bacteria in environmental samples due to its rapid transformation into amino derivatives and immobilization to soil particles (11). For example, a study

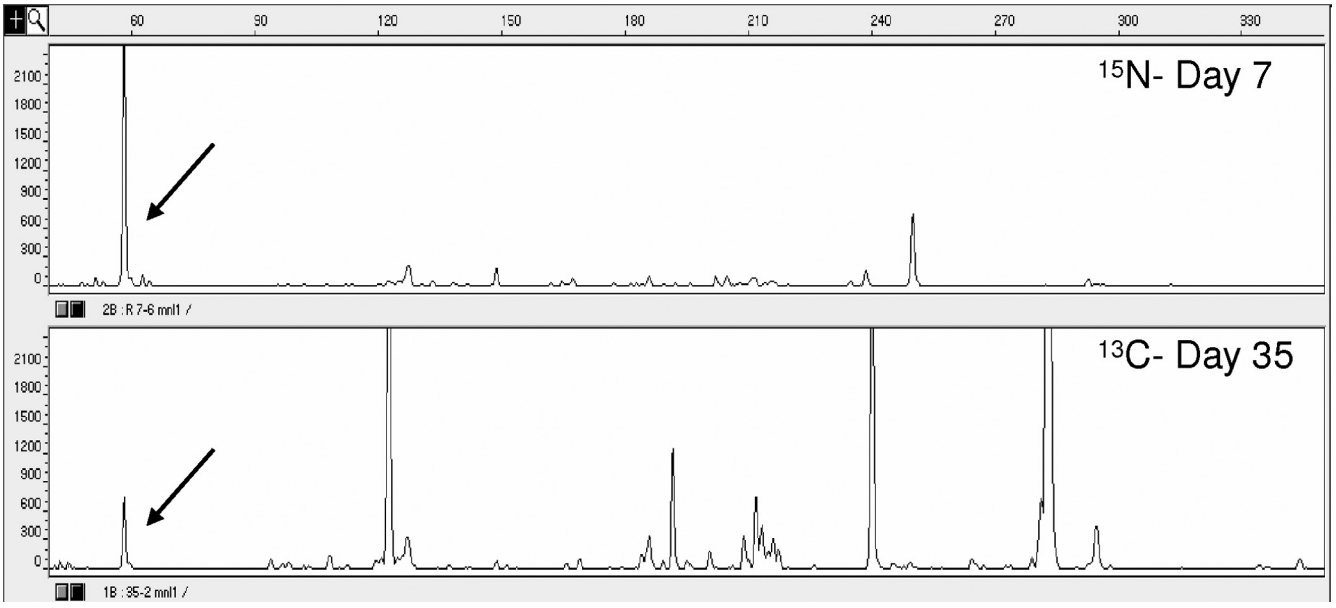

FIG. 3. Electropherogram of a ^{15}N- and ^{13}C-labeled community in microcosms. The 60-bp TRFLP peak is indicated.

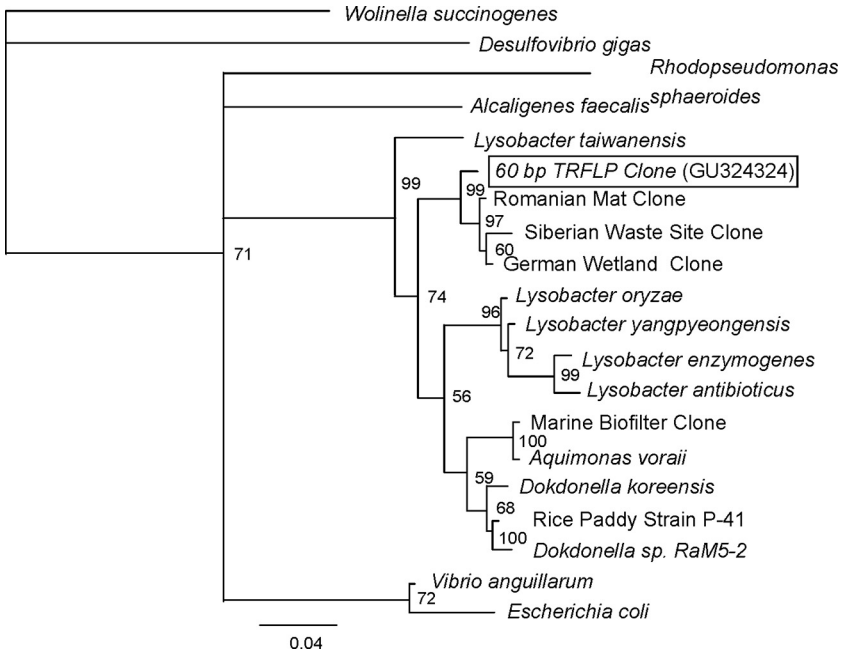

FIG. 4. Consensus maximum-likelihood tree reconstruction using SSU genes from known strains and closely related clones (based on 360 unambiguously aligned bp).

of ^{15}N- and ^{14}C-labeled TNT in soil incubations lasting from 108 to 176 days found the conversion of TNT into aminodinitrotoluene and diaminonitrotoluene metabolites (16 to 21%), with the bulk of the isotopic label associated with the nonextractable residue (50 to 30%) (22). In contrast, our report demonstrates that both the nitrogen and the carbon from TNT can become incorporated into bacterial DNA under short incubation times, even in the presence of organic-rich sediments. Our results are similar to a report of ^{15}N incorporation using the explosive RDX (cyclo-1,3,5-trimethylene-2,4,6-trinitramine) from samples collected at Picatinny Arsenal in New Jersey (16). The RDX study also utilized ^{14}N- and ^{15}N-labeled substrates to test for incorporation into bacterial biomass. However, each microcosm was coamended with cheese whey or yeast extract, initially provided with an aerobic headspace, and allowed to become anaerobic during a 25-day incubation. The results demonstrated difficulty in separating [^{14}N]DNA from [^{15}N]DNA with respect to 16S rRNA genes. The researchers, however, were able to differentiate the fractions using a functional gene approach (cytochrome P450; *xplA* gene). In contrast, our study using the archaeal carrier method indicated no detectable contamination in ^{15}N bands when using the ^{14}N-labeled substrate. Furthermore, the time frame necessary to detect the signal was significantly reduced, as has been described before (8).

Finally, our finding of a *Lysobacter*-like bacterium that is capable of both nitrogen and carbon uptake from TNT into biomass is consistent with reports of phytoremediation of explosives (19, 20). *Lysobacter* species are found in soil, aquatic environments, and the rhizosphere. In conclusion, these data unequivocally demonstrate that there are bacteria in estuarine sediments that are able to utilize TNT as a carbon and nitrogen source under anaerobic conditions. Ultimately, this SIP approach could be used in conjunction with compound-specific C and N isotopic analysis to observe biological fractionation *in situ* (2).

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