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^{13}C-Carrier DNA Shortens the Incubation Time Needed To Detect Benzoate-Utilizing Denitrifying Bacteria by Stable-Isotope Probing

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The active bacterial community able to utilize benzoate under denitrifying conditions was elucidated in two coastal sediments using stable-isotope probing (SIP) and *nosZ* gene amplification. The SIP method employed samples from Norfolk Harbor, Virginia, and a Long-Term Ecosystem Observatory (no. 15) off the coast of Tuckerton, New Jersey. The SIP method was modified by use of archaeal carrier DNA in the density gradient separation. The carrier DNA significantly reduced the incubation time necessary to detect the ^{13}C-labeled bacterial DNA from weeks to hours in the coastal enrichments. No denitrifier DNA was found to contaminate the archaeal ^{13}C-carrier when [^{12}C]benzoate was used as a substrate in the sediment enrichments. Shifts in the activity of the benzoate-utilizing denitrifying population could be detected throughout a 21-day incubation. These results suggest that temporal analysis using SIP can be used to illustrate the initial biodegrader(s) in a bacterial population and to document the cross-feeding microbial community.

Many bacteria are able to degrade anthropogenic pollutants through metabolism or respiration in the environment (4, 28, 31). Although the ability of bacteria to degrade environmental contaminants is widely known, it is difficult to determine the bacteria that perform this metabolism. Since it is widely believed that <1% of the bacteria present in environmental samples can be cultured in the lab (6, 8), it is unlikely that the active degraders of any particular environmental contaminant will fall within the easily culturable portion of bacteria. Recently, stable-isotope probing (SIP) techniques have been used to elucidate the active population of bacteria among the total population of bacteria contributing to a particular metabolic pathway (16, 18, 20, 22, 25, 30). The SIP method utilizes a ^{13}C-labeled substrate and PCR techniques to discern the members in a microbial community that incorporate the ^{13}C into their DNA. SIP capitalizes on the ability to separate DNA containing different carbon or nitrogen isotopic labels using cesium chloride (CsCl) gradient centrifugation (14, 20). In theory, the approach can ascertain the microorganism that initiates pollutant degradation if an appropriate time course can be utilized during a SIP experiment. However, the majority of SIP studies incubate for extended periods to generate DNA with sufficient label incorporation to visibly separate the DNA on a gradient (typically more than 20 days [16, 21, 22, 30]). Within shorter time frames, there were difficulties utilizing SIP to its full capabilities (3, 15, 27). RNA-based SIP studies (5, 11, 12, 13, 17), on the other hand, have generally used shorter incubation periods. RNA is labeled faster than the genome since RNA is growth rate regulated in many bacteria (9, 10). However, there have been complications reported in RNA purification or separation using stable isotopes (5, 12, 22).

In this study, genetic signatures from denitrifying bacteria capable of benzoate utilization in coastal sediments were discerned using DNA-SIP and ^{13}C-carrier DNA for the physical separation of ^{13}C-DNA. The addition of an archaeal carrier DNA to the CsCl gradient allows for a significant reduction in incubation time. For example, the benzoate-utilizing denitrifying community present at these coastal sites that had incorporated the ^{13}C label could be detected within a 1-hour time frame. Furthermore, shifts in this benzoate-utilizing community could be observed over the course of a 21-day incubation. The study demonstrates that SIP methodology can be used on short timescales, reducing the possibility of stable-isotope transfer in alternate chemical forms (cross-feeding). This advance will also allow for the determination of active in situ microbial populations and may enable the mapping of microbial food webs where the metabolic intermediates are not known.

MATERIALS AND METHODS

Multiple tests were performed to determine if the addition of carrier DNA could be combined with SIP studies using both pure cultures and environmental enrichments. To test for cesium chloride gradient contamination of the ^{13}C-DNA band with ^{12}C-DNA, a pure culture of *Thauera aromatica* strain T1 (26) was grown on [^{12}C]benzoate in a denitrifying medium, sparged with a 70%–30% mixture of N_2-CO_2, and amended with 100 μM NO_3^-. After the culture was grown for approximately 30 days, the cells were transferred 1:1 into fresh medium and amended with either 100 μM of uniformly ^{13}C-labeled benzoate or [^{12}C]benzoate as the sole carbon source. The culture was incubated for approximately 30 days, and the DNA was extracted using a phenol-chloroform method (19, 23).

For determining natural populations of benzoate-utilizing denitrifying bacteria, sediments were enriched with benzoate and nitrate from a Long-Term Ecosystem Observatory (LEO-15) located off the coast of Tuckerton, New Jersey, representing a nonimpacted condition, while sediment from Norfolk Harbor in Virginia was considered a contaminated site. A 10% (vol/vol) sediment slurry was sparged with a 70%–30% mixture of N_2-CO_2 and amended with 100 μM NO_3^- and 100 μM of uniformly ^{13}C-labeled benzoate, [^{12}C]benzoate, or no additional carbon source in 24 tubes (20-ml capacity). Triplicate tubes were sacrificed for each amendment at 1 h and 5-, 7-, 14-, and 21-day time points (three active tubes from each site with labeled substrate or unlabeled substrate). For each of the sampling points, approximately 400 μl of the sacrificed slurry was extracted using a modified phenol-chloroform extraction procedure, and the

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DNAs from the triplicate samples were pooled and resuspended in 100 μl of sterile deionized water (19, 23). The carrier DNA used for visualization of ^{13}C-DNA in this study was from *Halobacterium salinarium* grown in a ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg, OH). The stable-isotope-enriched medium was prepared for halophilic bacteria (7) and grown aerobically at 25°C for approximately 20 days before the cells were harvested and DNA was extracted, as described above.

Approximately 300 ng of environmental-sample DNA and 300 ng of ^{13}C-carrier DNA were added to a 500-μl CsCl density gradient (1 g/ml) containing 20 μg ethidium bromide and separated in a TLA 120 rotor on a Beckman Optima ultracentrifuge (Palo Alto, CA) at 225,000 × *g* (29). After 16 to 24 h, the bands were visualized using UV light and pulled from the gradient by first removing the ^{12}C-DNA band, changing the pipette tip, releasing a small air bubble above the height of the ^{12}C-DNA band, and proceeding to remove the ^{13}C-DNA band from the gradient. After band extraction, the genomic DNA was dialyzed using a 0.025-μm Millipore mixed cellulose ester dialysis filter (Bedford, MA) floating in a petri dish containing 10 mM Tris-HCl (pH 8.2).

For determination of the ^{13}C-assimilating denitrifying bacteria, terminal restriction fragment length polymorphism analysis (T-RFLP) of the nitrous oxide reductase (*nosZ*) gene was utilized (23). The primers used in the *nosZ* amplification were 752F (ACC GAY GGS ACC TAY GAY GG) and 1773R (ATR TCG ATC ARC TGB TCG TT), using a thermocycling program of 95°C for 5 min and then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 10 min. The forward primer was labeled with 6-carboxyfluorescien (6-FAM; Applied Biosystems) on the 5′ end. The amplicon provided by *nosZ* PCR amplification was then run on a 1% agarose gel and quantified. Fifteen nanograms of the PCR product was digested with MnlI endonuclease (New England Biolab, Beverly, MA). All digests were in 20-μl volumes for 6 h at 37°C. Precipitation of digested DNA was performed by adding 2 μl of 0.75 M sodium acetate solution and 0.3 μl glycogen (20 mg/ml) to the enzyme digest and precipitating with 37 μl of 95% ethanol. The precipitated DNA was washed with 70% ethanol and dried briefly. The dried DNA pellets were resuspended in 19.7 μl deionized formamide and 0.3 μl ROX 500 size standard (Applied Biosystems) for 15 min before analysis. T-RFLP fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software.

RESULTS

To test whether contamination between ^{12}C- and ^{13}C-DNA bands in a CsCl gradient could be observed, an experiment was designed using a benzoate-degrading bacterial strain, *Thauera aromatica* strain T1, that was either ^{12}C or partially ^{13}C labeled by growth on [^{12}C]benzoate or [^{13}C]benzoate after growth on [^{12}C]benzoate, as described above. The different T1 DNAs were mixed with ^{13}C-labeled "carrier" archaeal DNA and separated by ultracentrifugation, as described above. The ^{12}C- and ^{13}C-DNA bands were isolated, and the presence of *Thauera* DNA was tested by amplifying the 16S rRNA gene from each band using bacterium-specific primers (Fig. 1). Successful bacterium-specific amplification was observed in the ^{12}C band when either ^{12}C- or partially ^{13}C-labeled *Thauera* DNA was added to the gradients (lanes E and B). Likewise, a bacterial 16S rRNA gene product was detected in the ^{13}C band when partially ^{13}C-labeled *Thauera* DNA was added to the gradient (lane C). There was no bacterium-specific amplification product in the ^{13}C-DNA band when the *Thauera* DNA was labeled only with ^{12}C (lane F), suggesting that any ^{12}C contamination in the carrier DNA was below the PCR detection limit.

In addition to testing ^{13}C-DNA band contamination under pure culture conditions, control incubations using [^{12}C]benzoate amendments were performed on LEO-15 and Norfolk Harbor sediments on days 5 and 21 of the incubation. These ^{12}C-labeled samples were incubated and processed as the [^{13}C]benzoate-amended samples and *nosZ* amplifications were performed on the ^{13}C bands isolated from the cesium chloride gradients to identify the denitrifying bacteria (Fig. 2). No *nosZ* amplification could be seen in the ^{13}C-DNA bands that were supplemented with [^{12}C]benzoate in the day 5 or 21 incubations from the LEO-15 or Norfolk Harbor sites (lanes C to F). However, when the environmental slurry was amended with [^{13}C]benzoate, a *nosZ* PCR product was detected in the ^{13}C-labeled "carrier" band (lane G). This result illustrates that any bacterial ^{12}C-DNA from these environmental samples that may contaminate the ^{13}C band is below the *nosZ* PCR detection limit.

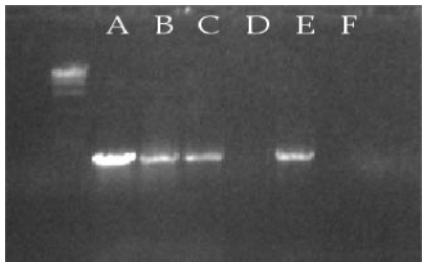

FIG. 1. Agarose gel showing bacterial 16S rRNA gene amplification using pure-culture *Thauera aromatica* strain T1 that was either ^{12}C or ^{13}C labeled in combination with ^{13}C-labeled "carrier" archaeal DNA. Lanes: A) positive control, B) ^{13}C-fed ^{12}C-*T. aromatica* DNA band, C) ^{13}C-fed partially ^{13}C-*T. aromatica* DNA band, D) blank, E) ^{12}C-fed ^{12}C-*T. aromatica* DNA band, F) ^{12}C-fed partially ^{13}C-*T. aromatica* DNA band.

To assess whether short incubations and site-specific differences in benzoate denitrifying populations could be discerned using SIP, a time course of ^{13}C-labeled enrichment samples was characterized by T-RFLP analysis. The results are shown in Fig. 3. Both the nonimpacted and the contaminated sites show distinct peaks at day 0 (60 min after substrate addition). The contaminated site, however, demonstrated a greater peak area at the 1-hour time point than the nonimpacted site, implying that the bacterial community at Norfolk Harbor was primed for the anaerobic degradation of aromatic compounds from the chronic exposure of petroleum hydrocarbons. These results establish that ^{13}C-labeled DNA can be generated within short incubation times (hours) and that small amounts of ^{13}C-

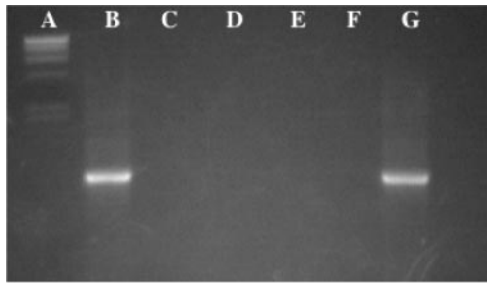

FIG. 2. Agarose gel showing *nosZ* gene amplification using carrier DNA in an environmental sample. Lanes: A) λ standard (125 ng), B) positive control, C) ^{13}C-DNA band fed [^{12}C]benzoate from LEO-15 incubation day 5, D) ^{13}C-DNA band fed [^{12}C]benzoate from LEO-15 incubation day 21, E) ^{13}C-DNA band fed [^{12}C]benzoate from Norfolk Harbor incubation day 5, F) ^{13}C-DNA band fed [^{12}C]benzoate from Norfolk Harbor incubation day 21, G) ^{13}C-DNA band fed [^{13}C]benzoate from Norfolk Harbor incubation day 5.

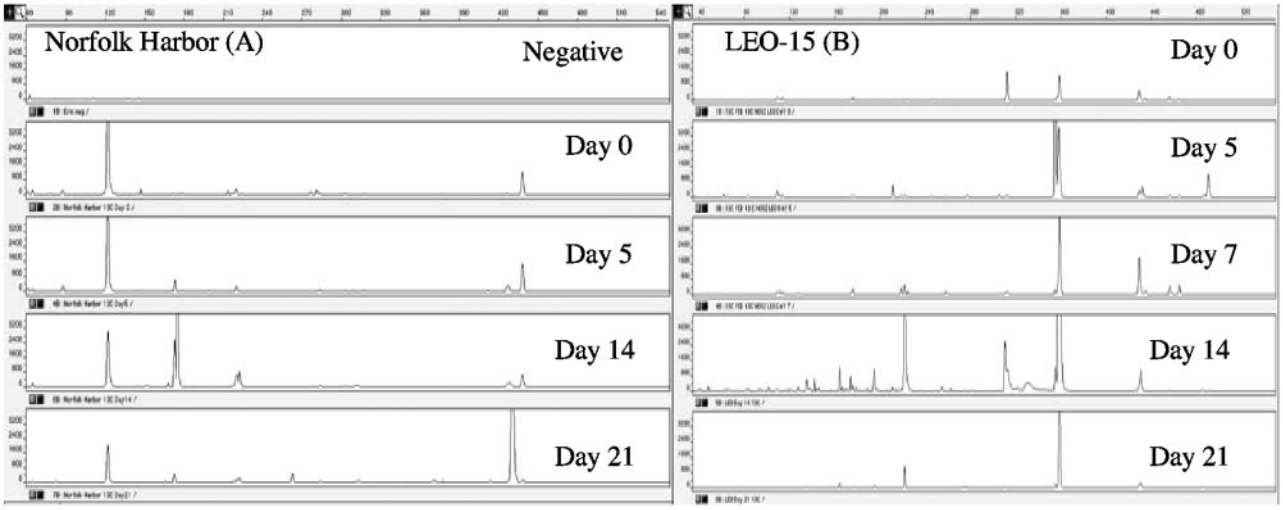

FIG. 3. Electropherogram illustrating different terminal restriction fragments (TRFs) over the time course from both environmental sites, Norfolk Harbor (A) and LEO-15 (B).

labeled DNA can be readily isolated with the use of carrier DNA.

For each sediment site, an increase in both peak number and peak area was observed during the first 2 weeks of the incubation (Fig. 4A). Both the LEO-15 and the Norfolk Harbor samples exhibited a five- to sixfold increase in the number of peaks by day 14. After the 2-week period, the community diversity of ^{13}C-labeled denitrifiers began to decrease. This increase in T-RFLP peaks during the SIP incubation implies the passing of ^{13}C-labeled metabolic intermediates or cellular constituents from the initial denitrifying degrader to another segment of the denitrifying community. (The use of *nosZ* genes precludes the detection of nondenitrifying members of the bacterial community involved in benzoate degradation.) In addition to changes in peak number, the relative area of the different peaks changed over the course of the incubation (Fig. 4B). For the Norfolk Harbor sample, the terminal restriction fragment (TRF) at 122 base pairs showed an initially high peak area within an hour after [^{13}C]benzoate amendment that decreased during the course of the incubation. In the LEO-15 amendment, the TRF at 349 base pairs showed a similar pattern, with a slight lag in the 1-hour time point. This rapid incorporation of ^{13}C from benzoate suggests that these particular denitrifying microorganisms are the primary utilizers of benzoate in the enrichments. Conversely, the TRF at 427 base pairs from the Norfolk Harbor and the TRF at 211 base pairs from LEO-15 were not initially detected at the 1-hour or day 5 time points. However by the day 21 incubation, the peak area

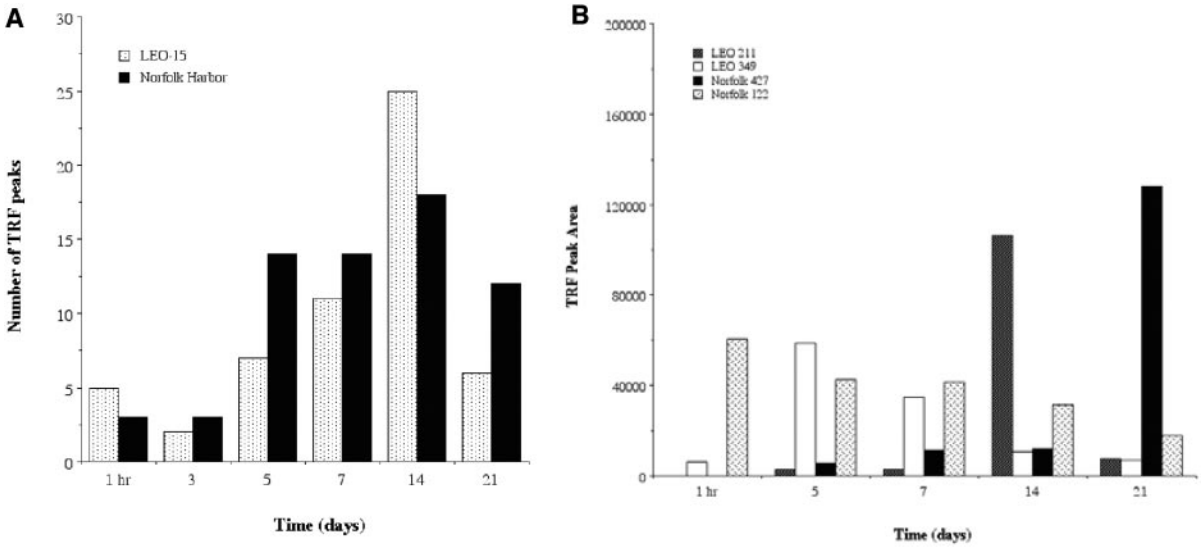

FIG. 4. (A) Change in peak numbers seen in the T-RFLP data during the time course of the SIP experiment. Data derived from the T-RFLP fingerprints are shown in Fig. 3. (B) The peak area of select TRFs was traced over time; for Norfolk Harbor, the TRFs were at 122 base pairs and at 427 base pairs, and for LEO-15, the TRFs at 349 base pairs and at 211 base pairs were presented. Data derived from the TRFLP fingerprints are shown in Fig. 3.

of these TRFs dominated the fingerprints, implying that these denitrifying bacteria are secondary utilizers of the carbon from benzoate. At both sites, the total area of TRFs from the primary utilizers decreased slowly over time, and the total area of the secondary utilizers increased over time.

DISCUSSION

In this report, we describe the metabolism of [^{13}C]benzoate under denitrifying conditions, using inocula from both a nonimpacted and contaminated coastal sediment site. While the benzoate concentration present in the microcosms was not directly measured, the incorporation of the ^{13}C label into DNA demonstrated the metabolism of [^{13}C]benzoate during the incubation. Previous studies have shown that bacteria grown with nitrate as a terminal electron acceptor under anoxic conditions in the laboratory are able to metabolize approximately 200 mg chemical oxygen demand per liter of benzoate in 48 to 50 h in the laboratory (1, 2). This benzoate degradation in hours in laboratory cultures is consistent with the incorporation of ^{13}C label into bacterial DNA in environmental enrichments within 60 min using carrier DNA. The addition of ^{13}C-labeled archaeal carrier DNA ensures that small amounts of ^{13}C-labeled bacterial DNA in the enrichment (below the visible-level detection limit) can still be removed from the gradient and amplified. Clearly, the use of carrier DNA greatly shortens the time necessary for performing a SIP study. While archaeal DNA was used in this study, any DNA in principle could be used as a carrier for SIP. The only requirements of the carrier DNA are that it does not contain the functional gene of interest (e.g., the *nosZ* gene in this study), or the target gene can be differentiated from the carrier gene (e.g., by the use of group PCR primers), and that the DNA is uniformly labeled with the stable isotope. For example, *Halobacterium* genomic DNA can be an ideal choice of carrier DNA in SIP studies looking at either bacterial or eukaryotic communities using rRNA target genes, since kingdom-specific PCR primers can prevent the amplification of the carrier ribosomal genes and select for the target community.

This ability to perform short-term SIP experiments is important for eliminating bottle effects and experimental artifacts. Although there are SIP experiments with incubations shorter than 20 days, there were no studies of DNA-based SIP utilizing PCR amplification and incubation times of 1 h prior to this report. One possible explanation for the detection of bacterial DNA in the archaeal carrier is contamination. For example, it has been reported that the buoyant density of DNA is affected by G+C content (22), and it is conceivable that ^{12}C-DNA with high G+C content may comigrate with ^{13}C-labeled DNA. However, in this study, there was no evidence of contamination in the ^{13}C-DNA band in both pure culture and environmental samples amended with the ^{12}C-labeled substrate, suggesting that the variable G+C content in natural samples will not impede environmental SIP studies using carrier DNA.

Furthermore, the use of a time course incubation study and the ability to use molecular techniques on the ^{13}C-labeled DNA have allowed for the detection of ^{13}C label incorporation into the DNA of what is possibly the initial benzoate degrader in the community. Previously, the use of long incubation times made the identity of the first microorganism to incorporate ^{13}C into its DNA difficult to distinguish because of the possibility of cross-feeding. While cross-feeding has been extensively mentioned as a shortcoming of the utilization of the SIP technique, it has never been illustrated (12, 21, 22, 24, 30). The use of a time course study with carrier DNA and short incubation times has allowed for the identification of cross-feeding microorganisms at both the Norfolk Harbor site and the LEO-15 site. In both cases, a small number of denitrifying bacteria with the ^{13}C label were detected first. The number of ^{13}C-labeled bacteria then increased over time. This finding implies that there are only a few organisms responsible for the degradation of an environmental pollutant (in this case benzoate), and there is a broader population able to utilize the ^{13}C-labeled molecules after the degradation.

Finally, the loss of ^{13}C-labeled T-RFLP peaks during the incubation suggests that the microorganisms bearing ^{13}C-DNA are being consumed by bactivorous protozoa in the enrichments, accelerating the remineralization of the benzoate during the incubation. Another possibility is that the microorganisms could be utilizing other carbon sources present in the sediment, causing a dilution of the ^{13}C label and lowering the number of peaks seen from the T-RFLP fingerprint. Alternatively, the shifting of T-RFLP peaks could result from DNA turnover and the release of $^{13}CO_2$ during the extended incubation for many of the microorganisms. The increase and the shifting of T-RFLP peaks that are detectable in this anaerobic benzoate degradation experiment from day 0 to day 21 emphasize the need to perform a time course study to ascertain the true benzoate degraders active among the bacteria present in an environmental sample when using stable-isotope probing, as opposed to those gaining the ^{13}C label from cross-feeding. Clearly, the sample complexity within the enrichment can change over time, and the members of the community that are using the ^{13}C-labeled benzoate can also change. Furthermore, the microorganisms responsible for the initial ring cleavage of benzoate under anaerobic conditions can now be discerned by searching for the T-RFLP peaks at the earliest possible time point. In conclusion, the use of carrier DNA in SIP experiments can reduce incubation times and allow for a rapid assessment of the organisms present in a complex environmental sample that are able to utilize a labeled substrate. The incorporation of labeled benzoate into the DNA of organisms at both Norfolk Harbor and LEO-15 could be an important marker in the degradation abilities of the organisms present at these two sites. Since benzoate is a common intermediate in aromatic pollutant degradation (4, 31), the immediate incorporation of benzoate into the DNA of organisms at these sites could prove important in further degradation studies.

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