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Resolving Genetic Functions within Microbial Populations: In Situ Analyses Using rRNA and mRNA Stable Isotope Probing Coupled with Single-Cell Raman-Fluorescence In Situ Hybridization▽†

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Prokaryotes represent one-half of the living biomass on Earth, with the vast majority remaining elusive to culture and study within the laboratory. As a result, we lack a basic understanding of the functions that many species perform in the natural world. To address this issue, we developed complementary population and single-cell stable isotope (^{13}C)-linked analyses to determine microbial identity and function in situ. We demonstrated that the use of rRNA/mRNA stable isotope probing (SIP) recovered the key phylogenetic and functional RNAs. This was followed by single-cell physiological analyses of these populations to determine and quantify in situ functions within an aerobic naphthalene-degrading groundwater microbial community. Using these culture-independent approaches, we identified three prokaryote species capable of naphthalene biodegradation within the groundwater system: two taxa were isolated in the laboratory (*Pseudomonas fluorescens* and *Pseudomonas putida*), whereas the third eluded culture (an *Acidovorax* sp.). Using parallel population and single-cell stable isotope technologies, we were able to identify an unculturable *Acidovorax* sp. which played the key role in naphthalene biodegradation in situ, rather than the culturable naphthalene-biodegrading *Pseudomonas* sp. isolated from the same groundwater. The *Pseudomonas* isolates actively degraded naphthalene only at naphthalene concentrations higher than 30 μM. This study demonstrated that unculturable microorganisms could play important roles in biodegradation in the ecosystem. It also showed that the combined RNA SIP–Raman-fluorescence in situ hybridization approach may be a significant tool in resolving ecology, functionality, and niche specialization within the unculturable fraction of organisms residing in the natural environment.

Prokaryotes account for about one-half of the total global carbon biomass, providing the foundation of the biosphere and biogeochemical (e.g., carbon and nitrogen) cycles (34, 42). However, the last 2 decades have brought a realization that the vast majority of prokaryotes (probably >99%) in the natural environment have not been cultured in the laboratory using standard culturing methods (1, 8, 37, 39, 40). It is therefore clear that naturally occurring prokaryotes are highly diverse and harbor an enormous genetic reservoir and that the difficulty in culturing and studying many organisms ex situ is hampering our ability to resolve in situ microbial functions.

In last decade we have witnessed a significant increase in linking molecular biological detection strategies with functional tracers, such as stable or radioactive isotopes, in order to circumvent the issues of culturability when examining processes such as natural xenobiotic degradation. Recently, there has been the development of stable isotope probing (SIP), in which stable-isotope-labeled compounds are pulsed into a microbial community, causing the active microbial cells, which are able to utilize the substrate, to become heavily labeled with isotopes such as ^{13}C (31), ^{15}N (4, 5), or ^{18}O (35). The heavy isotopically labeled macromolecules (e.g., phospholipid fatty acid) can subsequently be analyzed by isotope ratio mass spectrometry or recovered by differential ultracentrifugation (i.e., DNA and RNA) for molecular characterization of phylogenetic or functional signatures associated with active diversity (7, 17, 23, 27, 29, 32). SIP is therefore a powerful community level tool, which can reveal the bacterial species in mixed consortia that have roles in processing natural and xenobiotic substrates, such as phenol (23), methane (31), and carbon dioxide (12, 21).

More recently, single-cell technologies which complement the stable isotope analysis of whole communities by focusing the resolution on the single-cell uptake of stable-isotope-labeled compounds, primarily ^{13}C-labeled compounds, have been developed; these currently include technologies such as single-cell Raman microspectroscopy (13) and nano-secondary ion mass spectrometry (30). For Raman microspectroscopy, we have recently demonstrated Raman spectrum acquisition from a single bacterial cell and have found that the Raman spectrum of a ^{13}C-labeled cell has significant "red shifts" (Raman spec-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
pGEM-T	TA cloning vector	Promega
pDTG1	Naphthalene degradation plasmid of *Pseudomonas putida* NCIB9816	9
Acinetobacter baylyi ADPWH_lux	*sal* operon was inserted into *luxCDABE*; salicylate biosensor	15
Pseudomonas putida UWC1	Mutant of *Pseudomonas putida* KT2440; Rip^r	26
Pseudomonas putida NCIB9816	Naphthalene degradation bacterium with plasmid pDTG1	6
Ralstonia sp. strain U2	Naphthalene degradation genes are carried on plasmid	45
Pseudomonas putida G7	Naphthalene degradation bacterium with plasmid NAH7	36
Rhodococcus sp. strain NCIMB 12038	Gram-positive naphthalene degradation strain	19

tral bands move toward longer wavelengths or lower wave numbers due to the stable-isotope-labeled chemical bonds) in comparison with ^{12}C-labeled native cells (13). This allowed the unambiguous detection of single cells which had taken up labeled substrates. Further, we combined the Raman approach with fluorescence in situ hybridization (Raman-FISH), which now enables us to examine single cells and validate rates of uptake of ^{13}C into phylogenetically delimited populations in situ (14).

The approach taken in this study was to combine population rRNA and mRNA analyses with single-cell physiology analyses, all linked by ^{13}C SIP, to provide a fully culture-independent "tool box" for environmental analysis. We applied this combined approach to investigate groundwater microbial communities experiencing polycyclic aromatic hydrocarbon (mainly naphthalene) contamination at a former manufactured gas plant (FMGP) in the United Kingdom. We revealed a hidden population which was unculturable but played a key role in naphthalene degradation in situ. This suggested that unculturable microorganisms could play important roles in the carbon cycle in the ecosystem.

MATERIALS AND METHODS

Chemicals. All chemicals were from Sigma (United Kingdom) except where specified. Uniformly ^{13}C-labeled naphthalene (>99% ^{13}C) was purchased from Isotec (OH) (Sigma catalogue number 579653).

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Site and SEREBAR. A sequential reactive barrier (SEREBAR) was installed to aerobically treat the complex polycyclic aromatic hydrocarbon mixture plume within the groundwater, and this study addressed the microbial population at the inlet to the SEREBAR (10). Groundwater used in this study was sampled from the inlet to the SEREBAR in presterilized, nitrogen-filled bottles (10, 11). The samples were taken from groundwater that converged at the inlet before entering the SEREBAR barrier. The samples were collected in bottles without headspace and were immediately sealed and stored at 4°C before use; although they were not anaerobic, their condition was the nearest approximation to the in situ condition. The primary groundwater contaminant throughout the site was naphthalene, with an average concentration of 3.8 μM. The groundwater temperature was consistently 14 ± 2°C (10, 11).

Total nucleic acid extractions. Twenty or 250 milliliters of the groundwater sample (10^5 to 10^6 cells/ml) was filtered through 47-mm-diameter Sterifil aseptic system filters with a 0.22-μm pore size (Millipore Inc.). The filters were placed into Bio-101 tubes (Q-biogene), 1 ml of DNA extraction buffer was added (44) (100 mM Tris-HCl [pH 8.0], 100 mM sodium-EDTA [pH 8.0], 100 mM phosphate buffer [pH 8.0], 1.5 M NaCl, 1% cetyltrimethylammonium bromide), and the tubes were incubated in a water bath at 65°C for 30 min. The tubes were subsequently placed in a FastPrep FP120 bead beating system (Bio-101, Vista, CA), where the cells were lysed for 30 s at a speed of 5.5 m s^{-1}. The tubes were then centrifuged at 14,000 × *g* for 5 min, and the aqueous phase was transferred into a microcentrifuge tube to which an equal volume of chloroform-isoamyl alcohol (25:24:1) was added and mixed well. Centrifugation was then performed to separate water and chloroform phases at 14,000 × *g* for 5 min. The aqueous phase was then transferred into a new microcentrifuge tube, and 0.6 volume of isopropanol was added to precipitate the DNA. The tubes were incubated at room temperature for 1 to 2 h and then centrifuged at 14,000 × *g* for 10 min. After the supernatant was discarded, 200 μl of 70% (vol/vol) ethanol was added to wash the DNA/RNA pellet. Tubes were finally centrifuged at 14,000 × *g* for 10 min. After the ethanol was discarded, the DNA/RNA pellets were dried under vacuum (Eppendorf; Concentrator 5301) prior to resuspension in 50 μl Tris-EDTA buffer or water. To obtain purified RNA, 20 μl of extracted RNA/DNA was added to 1 μl RNase-free DNase I (New England BioLabs Inc.), incubated at 37°C for 15 min, and then heated at 75°C for 10 min to inactivate the DNase I.

Naphthalene degradation kinetics and substrate pulsing of groundwater. (i) In-situ [^{13}C]naphthalene degradation. A 30 mM stock solution of fully ^{13}C-labeled naphthalene and control [^{12}C]naphthalene was prepared in dimethylformamide. Four treatments, all prepared in triplicate, were initiated to determine naphthalene degradation at ambient levels of naphthalene: 0.22-μm-filtered groundwater control, 0.22-μm-filtered groundwater with added 3.8 μM [^{12}C]naphthalene, whole groundwater with 3.8 μM uniformly ^{13}C-labeled naphthalene, and whole groundwater with 3.8 μM [^{12}C]naphthalene. Treatments were incubated in the dark at 14°C for 72 h, during which time 20-ml aliquots were removed at each of the following time points: 0, 25, 36, 46, 53, and 72 h; the total DNA and RNA were then extracted. An additional 1-ml aliquot was sampled at 0, 5, 25, 29, 46, 53, and 72 h for analysis of naphthalene removal by high-performance liquid chromatography (see detailed methods in the supplemental material).

(ii) Pulsing groundwater with different [^{12}C]- and [^{13}C]naphthalene concentrations. Fully ^{13}C-labeled naphthalene and control [^{12}C]naphthalene were introduced into groundwater to provide final concentrations of 0, 3.8, and 300 μM. Each treatment contained 2 liters groundwater and was prepared in triplicate. After incubation at 14°C for 0, 36, and 72 h, 500 ml groundwater from each treatment was filtered, as previously described, and then each sample was split; one half was used for RNA and DNA extraction, and the other half was used to capture the microbial community for FISH. The filters were placed into 50-ml Fisherbrand tubes (Fisher Scientific, United Kingdom) containing 2 ml phosphate buffer solution (PBS) and vortexed for 5 min. Cells were recovered by centrifugation at 4,000 × *g* for 10 min. The pelleted cells were used for the FISH assay, discussed below.

(iii) Biodegradation of different concentrations of naphthalene. Unlabeled naphthalene was introduced into 250 ml groundwater to final concentrations of 0, 3.8, 30, 60, 300, and 600 μM in triplicate. The RNA and DNA from the groundwater microbial community were extracted, as described earlier, after incubation at 14°C for 74 h. The 16S rRNA gene was amplified by PCR using the primer pair GC338F and 530R (Table 2), and the PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) (see below).

RNA SIP, centrifugation, and fractionation. Equilibrium density gradient centrifugation was performed as previously described (23) with slight modifications. Briefly, 1.8 g/ml RNA gradient medium was prepared by mixing 1.77 ml of 1.99 g/ml cesium trifluoroacetate (Amersham Pharmacia Biotech), 350 μl RNase-free water, and 75 μl of deionized formamide (JT Baker) in a 2-ml microcentrifuge tube. Six microliters of RNA (from [^{13}C]- or [^{12}C]naphthalene pulsing experiments) or deionized water (gradient control) was added to the gradient medium and mixed. The mixture was loaded into a polyallomer bell top quick-seal centrifuge tube (11 by 32 mm; Beckman Coulter) and sealed with a tube sealer (Beckman Coulter). Centrifugation (Optima TLX ultracentrifuge; Beckman Coulter) was performed at 64,000 rpm in a TLA120.2 rotor for 42 h at 20°C. For fractionation of the gradient tube, ca. 100-μl fractions were collected with a Beckman fraction recovery system (Beckman Coulter) by top displacement with water pumped through a syringe pump (model 99E; Razel Scientific Instrument

TABLE 2. Sequences of primers and FISH probes used in this study

Primer or probe name	Sequence (5′→3′)	Reference or source
Primers		
GC338F	CGC CCG CCG CGC CCC CGC CCC GGC CCG CCG CCC CCG CCC ACT CCT ACG GGA GGC AGC	23
338F	ACT CCT ACG GGA GGC AGC	23
530R	GTA TTA CCG CGG CTG CTG	23
63F	CAG GCC TAA CAC ATG CAA GTC	24
1387R	GGG CGG WGT GTA CAA GGC	24
PSE1_F	AAA AGA GCT GTA TGG CGA GT	28
PSE1_R	CCG ATA GAA GCC ACG ATA ACT	28
COM1_F	AAA AGA GTT GTA CGG CGA TG	28
COM1_R	ACG GTA GAA TCC GCG ATA GC	28
RHO1_F	CAA GGA TGC GTA TGG CAA TC	28
RHO1_R	TTC GAG GAA GTT GCG CTG GT	28
FISH probes		
EUB I	Cy5-GCT GCC TCC CGT AGG AGT	14
PSM-G	Fluos-CCT TCC TCC CAA CTT	14
Band2_prob2	Cy3-GAC GCG TGT AGC CCC ACC	This study

Inc.) at a flow rate of 3.3 μl/s. In total, 20 fractions were collected, and the buoyant densities of the fractionated gradients were determined by weighing all fractions to three figures on a milligram balance. RNA was precipitated within each fraction with 0.6 volume of isopropanol, washed with 200 μl 70% (vol/vol) ethanol, and resuspended in 20 μl of RNase-free water.

RT-PCR and DGGE. Purified RNA from the equilibrium density gradient fractions was reverse-transcribed with avian myeloblastosis virus reverse transcriptase (Promega Co., United Kingdom) and reverse primer 530R (Table 2). The cDNA was amplified with GC-clamped forward primer GC338F and reverse primer 530R (Table 2). The PCR products were loaded on a DGGE gel for analysis.

To recover a large fragment of the 16S rRNA (>1.3 kb) from the *Acidovorax* sp., reverse transcription-PCR (RT-PCR) was performed on the sixth fraction of the density gradient extracted, taken at the 36-h time point from the 3.8 μM [^{13}C]naphthalene-pulsed sample using the 63F and 1387R primers (Table 2). A 16S rRNA clone library was obtained by cloning these RT-PCR products into pGEM-T plasmids, which were then transferred into *Escherichia coli* JM109. Plasmids from 16 *E. coli* transformants cloned with 16S rRNA were extracted. PCR was performed using each extracted plasmid as the DNA template and the primer pair GC338F/530R (Table 2). The PCR products were loaded on a DGGE gel, along with GC338F/530R RT-PCR products of the ^{13}C-labeled fraction of the microbial community. After analysis of the DGGE gel and comparison of the bands, the plasmid containing a correct insert of the 16S rRNA gene was sequenced.

For the detection of heavily labeled mRNA associated with naphthalene dioxygenase (NDO), RNA samples from [^{13}C]RNA fractions were prepared, as previously described for RT-PCR, using reverse primers for three types of NDOs: PSE1_R, COM1_R, and RHO1_R (28) (Table 2). The cDNA for the three NDOs (28) was further amplified with the following three primer pairs: PSE1_F/PSE1_R (*Pseudomonas* type), COM1_F/COM1_R (*Comamonas* type), and RHO1_F/RHO1_R (*Rhodococcus* type) (28).

All PCRs were performed with the *Taq* PCR kit (Sigma, United Kingdom). The RT-PCR products were purified with the Qiagen PCR purification kit and prepared for cloning and sequencing.

FISH-Raman microspectroscopy. (i) Instrumentation. FISH-Raman microscopy was performed using a LabRAM 800 confocal Raman microscope (Horiba Jobin Yvon Ltd., United Kingdom), which was modified and equipped with a fluorescence system for FISH (14). The Raman microscope was equipped with an integrated Olympus microscope (model BX41). The Raman scattering was excited by a frequency-doubled 532-nm Nd-doped yttrium aluminum garnet laser, and the incident laser power was typically adjusted to around 5 to 8 mW to ensure that no sample damage occurred, while still maintaining spectral sensitivity. The detector used was a Peltier air-cooled charge-coupled device detector (open electrode format) with a pinhole of 100 μm and slit size of 100 μm, which enabled a spatial resolution of approximately 1 μm to be obtained. The system was calibrated prior to analyses and monitored using a silicon Raman band (520 cm^{-1}) reference.

(ii) Cells and slide treatment. Quartz slides (Agar Scientific, United Kingdom) employed for all Raman measurements were cleaned and coated in accordance with previous FISH hybridization protocols. Briefly, slides were immersed in acidic ethanol (1% [vol/vol] HCl in 70% [vol/vol] ethanol) for 5 min and then treated with 0.01% poly-L-lysine for 5 min at room temperature. The slides were ready for hybridization after drying for 1 h at 46°C.

(iii) FISH. For FISH analyses, cells were initially fixed by adding 1 volume of cell sample from a treatment to 3 volumes of 4% paraformaldehyde in PBS. Cells were fixed at 4°C overnight, centrifuged, and washed three times with 1 ml of PBS prior to addition of 100 μl of ethanol-PBS (50:50, vol/vol) and subsequent storage at −20°C until hybridization.

For hybridization, the probes employed in this study, EUB I, PSM-G, and Band2_prob2, were targeted to the domain *Bacteria*, *Pseudomonas* sp., and *Acidovorax* sp., respectively (Table 2). For triple-FISH hybridization, 20 μl of fixed cells (see above) was pelleted at 14,000 × *g* and resuspended in 100 μl ethanol for dehydration for 10 min. After 10 min the cells were recentrifuged for 30 min, the ethanol was removed, and the tube was dried prior to the addition of 100 μl of probe solution. Probe solution consisted of 1 μl of each of the FISH probes (300 ng/μl stock in Milli-Q water) mixed with 97 μl of hybridization solution (900 mM NaCl, 20 mM Tris-HCl, pH 7.4, 35% formamide, 0.01% sodium dodecyl sulfate). Hybridization was performed at 46°C for 90 min and was followed by centrifugation at 14,000 × *g* for 30 min, removal of hybridization buffer, and addition of 100 μl of prewarmed washing buffer (48°C). Washing buffer consisted of 80 mM NaCl, 20 mM Tris-HCl, and 5 mM EDTA. Cells were washed for 15 min at 48°C to maintain stringency. After being washed, cell pellets were resuspended in 20 μl phosphate-buffered saline. Subsequently, 1 μl of cell suspension was spotted and dried onto a poly-L-lysine-coated quartz slide prior to immersing the slide for 2 s in ice-cold Milli-Q water and final rapid drying with compressed air.

(iv) Raman measurement. For each measurement, a single bacterial cell was selected on the basis of appropriate FISH signals and focused by using a 100×/0.9-numerical-aperture air objective (Olympus; Mplan). The laser beam was targeted on the cell using a charge-coupled device camera monitor and a motorized XY stage (0.1-μm step). The Raman signal was optimized by adjusting the laser focus with a real-time readout; the spectrum was then acquired between 2,160 cm^{-1} and 550 cm^{-1}, with 1,022 data points (~1.5 cm^{-1} per point). The accumulation time for one spectrum was typically 30 to 60 s. Spectra were processed for baseline correction and normalization by Labspec software (Horiba Jobin Yvon Ltd., United Kingdom).

DNA sequencing. All PCR products and plasmid DNA were sequenced using dye terminator sequencing on an Applied Biosystems 3730 DNA analyzer according to the manufacturer's instructions. DNA sequence analysis was carried out using BlastN from NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) for confirmation of sequence homology, and these data were aligned and edited using BioEdit (Tom Hall, Department of Microbiology, North Carolina State University) to confirm correct insertions. The accession numbers of 16S rRNA gene sequences of isolated naphthalene degraders *P. fluorescens* WH2 and *P. putida* WH1 and WH3 are EF413073, EF413072, and EF413074, respectively.

Nucleotide sequence accession number. A 1,349-bp 16S rRNA gene of the *Acidovorax* sp. has been submitted to GenBank, and the accession number is EU202950.

RESULTS

Naphthalene degradation characteristics of groundwater communities within an FMGP site. The naphthalene (3.8 μM) in groundwater can be completely degraded within 72 h at 14°C (Fig. 1). A salicylate bacterial biosensor (15) was used to monitor salicylate appearance and confirm that naphthalene degradation was ongoing. Figure 1 (inset) shows the starting accumulation of salicylate at 36 h, with salicylate reaching a peak at 53 h and completely disappearing within 69 h, which was consistent with overall naphthalene degradation kinetics (Fig. 1).

Nucleic acid extraction recovered high-quality DNA and RNA. Cell counts by flow cytometry revealed that the average cell number in groundwater at the inlet to the SEREBAR

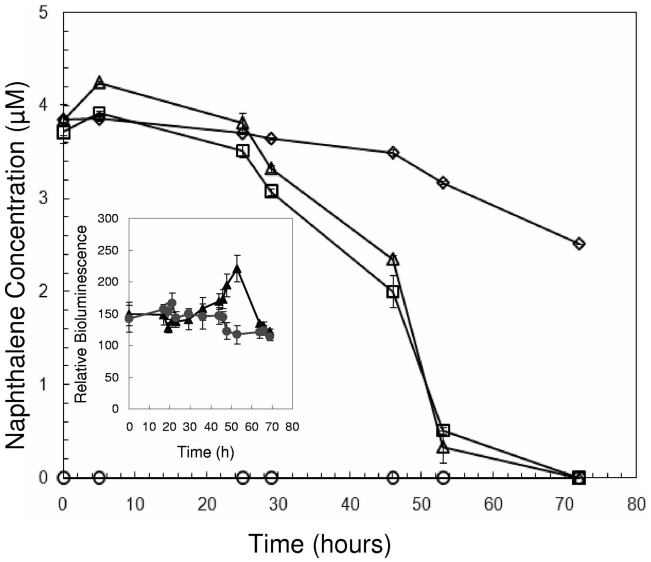

FIG. 1. Degradation of [^{12}C]- and [^{13}C]naphthalene (3.8 μM) in flasks inoculated with groundwater. Open squares and triangles represent [^{12}C]- and [^{13}C]naphthalene degradation, respectively; open diamonds and circles represent filter-sterilized groundwater with and without naphthalene, respectively. Naphthalene was completely degraded within 72 h, as analyzed by gas chromatography-mass spectrometry, and biological confirmation of this was provided by salicylate (metabolic intermediate) accumulation after 40 h, as determined by using a *lux*-based salicylate-specific bacterial biosensor (inset). In the inset, triangles and circles represent the groundwater sample with [^{13}C]naphthalene and filtered sterilized groundwater with naphthalene, respectively.

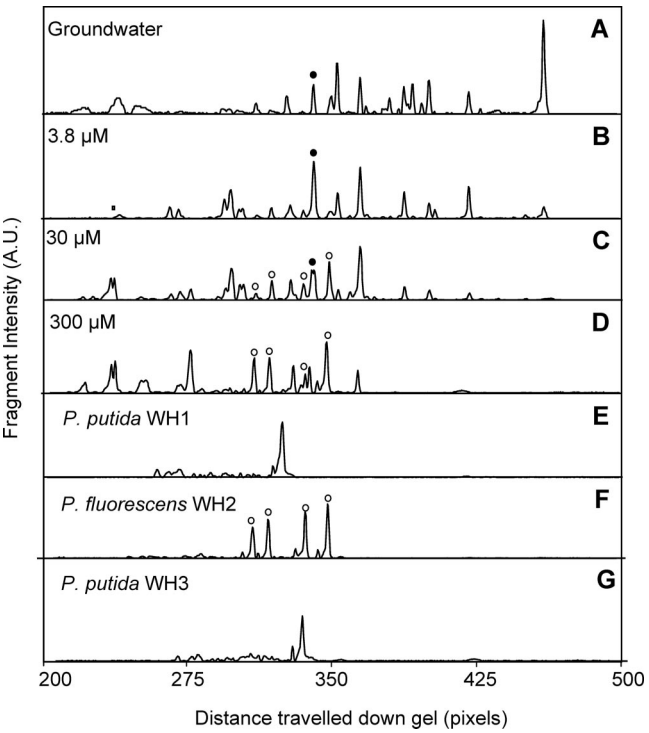

FIG. 2. Digitized DGGE profiles showing the effect of 36 h of exposure to [^{12}C]naphthalene upon 16S rRNA community profiles and comparison to cultured isolates obtained from the incubations. (A) Native groundwater; (B to D) 2-log range of amended naphthalene (μM); (E to G) pure-isolate 16S rRNA profiles for comparison to in situ community structures. For comparisons, open circles represent the band peaks for the key low-affinity degrader *P. fluorescens* WH2 and the filled circles represent the peak obtained from RNA SIP at the ambient concentration of naphthalene and equates to that for the *Acidovorax* sp. high-affinity degrader.

treatment tanks was $(1.5 \pm 0.1) \times 10^6$ cells per ml. The groundwater nucleic acid extraction method employed (see Materials and Methods) in this study can recover large DNA fragments (>10 kb) and sufficient rRNA and mRNA (see Fig. S1 in the supplemental material), which are crucial to the SIP studies.

RNA SIP revealed two distinct naphthalene-degrading functional groups in the FMGP. Three naphthalene-utilizing bacterial strains were isolated directly from the groundwater. Two were identified to be *P. putida* (WH1 and WH3) and one was identified to be *P. fluorescens* (WH2) by analysis of their 16S rRNA sequences. Further confirmation of the presence of these isolates under a range of naphthalene incubations was given by DGGE analyses of 16S rRNA signatures within the enrichments (Fig. 2; see Fig. S2 in the supplemental material). Specifically, after 36 h of exposure to naphthalene, the microbial community with 3.8 μM naphthalene was little changed compared to the communities exposed to higher naphthalene concentrations (>30 μM naphthalene) (Fig. 2; see Fig. S2 in the supplemental material). With increasing naphthalene concentrations, the microbial community underwent significant changes (Fig. 2; see Fig. S2 in the supplemental material) as a result of the isolate *P. fluorescens* WH2 becoming dominant (Fig. 2). Multiple observed peaks for the isolate *P. fluorescens* WH2 indicate that isolates of this species contained different numbers of copies of the 16S rRNA operons. This was further independently confirmed by band excision, by 16S rRNA-based sequencing from the gel, and ultimately by reference to the pure *P. fluorescens* WH2 isolate's band migration patterns.

The SIP DGGE analyses revealed that after 36 h of exposure a novel DGGE band not aligned with those for the recovered cultured isolates was obtained (see Fig. S3 and S4 in the supplemental material). Sequencing of this DGGE fragment indicated alignment within the *Acidovorax* genus (see Fig. S3 to S5 in the supplemental material). To further confirm it, we initially prepared a 16S rRNA clone library from the ^{13}C-labeled fractions of RNA after 36 h of exposure to naphthalene and performed RT-PCR using primers 63F and 1387R (Table 2) and pGEM-T cloning. The plasmids were subsequently extracted and subjected to 16S DNA PCR and DGGE to identify the correct clones (see Materials and Methods). Sequencing of 1,349 bp 16S rRNA of the unculturable degrader and BlastN analysis indicated that this isolate shared 99% homology with *Acidovorax* sp. Calibration of this 16S rRNA signature by DGGE analysis performed on natural communities and enrichments identified the presence of the organism within native groundwater and as a dominant community member when 3.8 μM unlabeled naphthalene was added, as in previous experiments (Fig. 2). These results strongly suggested that the recovered RNA SIP sequence was from a key in situ naph-

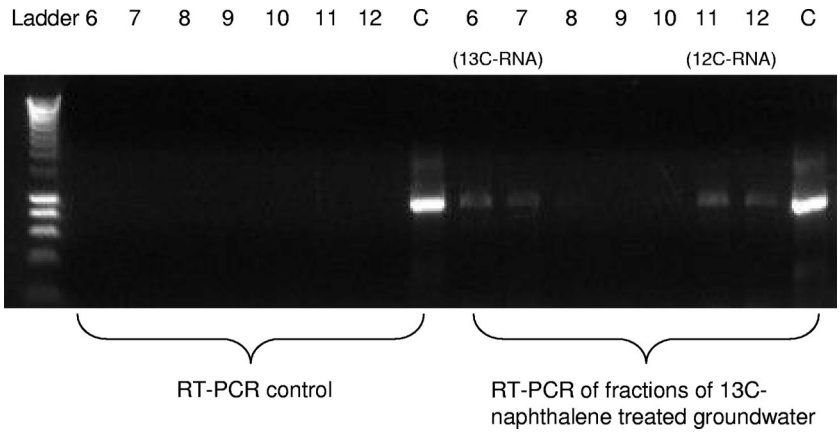

FIG. 3. RT-PCR of a fractionated RNA SIP gradient after exposing groundwater communities to 3.8 μM [$^{13}C_{10}$]naphthalene for 36 h and using *Comamonas*-type NDO gene primers. The RT-PCR control shows that there was no PCR product without reverse transcription. Positive reactions were obtained in both light RNA fractions (fractions 11 and 12), representing unlabeled RNAs, and in heavy fractions (fractions 6 and 7), derived from ^{13}C-labeled mRNA transcripts after substrate metabolism. Fraction 6 represents the lower portion of the centrifuge tube, occupying buoyant densities of around 1.83 g ml^{-1}, while fraction 11 represents the upper portion of the gradient, occupying buoyant densities around 1.79 g ml^{-1}. C represents a positive control for a *Comamonas*-type NDO.

thalene degrader that was distinct from the three *Pseudomonas* isolates previously obtained.

Determining the fate of naphthalene in situ. Three different types of primers, specifically targeting *Pseudomonas*-, *Comamonas*-, or *Rhodococcus*-type NDO genes (28), were employed to recover NDO genes from [^{13}C]mRNA fractions after 36-h incubations on [$^{13}C_{10}$]naphthalene to assess the active transcript pools. The mRNA SIP analysis of density gradient fractions revealed that only *Comamonas*-type NDO gene sequences could be recovered from "light" and "heavy" ^{13}C-labeled fractions at 3.8 μM naphthalene (Fig. 3), while *Pseudomonas*- and *Rhodococcus*-type NDO genes were not recovered (data not shown). Similarly, based upon the hypothesis that the *Comamonas*-type NDO was associated with the heavily labeled *Acidovorax* population under ambient naphthalene concentrations, we further screened total RNA by RT-PCR using the three primer pairs on RNA samples extracted from groundwater incubated with 0, 3.8, 30, 60, 300, and 600 μM naphthalene (Fig. 4). The *Comamonas*-type NDO gene was present in all RNA samples and was identical in sequence to that recovered from mRNA SIP analyses of gradient fractions, suggesting transcript activity across a range of naphthalene concentrations, from ambient through a 2-log increase. However, the only other NDO transcript types detected were those of the *Pseudomonas*-type NDO gene, and these were present only in samples incubated with naphthalene at concentrations greater than 30 μM (Fig. 4). These data suggested that two active degrader pools existed within the population, an *Acidovorax* population utilizing a *Comamonas*-type NDO, capable of relatively high-affinity activity, and a second, low-affinity population, utilizing a range of *Pseudomonas*-type NDOs. In order to investigate this hypothesis, we analyzed the isolated pseudomonads from the naphthalene enrichments and determined the presence of NDO genes aligned to those recovered from the system (Fig. 4). We detected two types of genes, consistent with those recovered from natural samples at naphthalene concentrations above 30 μM. Conjugation mating elucidated that both types of naphthalene degradation genes resided upon two different conjugative plasmids within the isolates. The DNA sequences indicate that the *P. fluorescens* WH2 isolate contained an operon nearly identical to the *Pseudomonas stutzeri* AN10-like Nah operon (2, 3) while the *Pseudomonas*

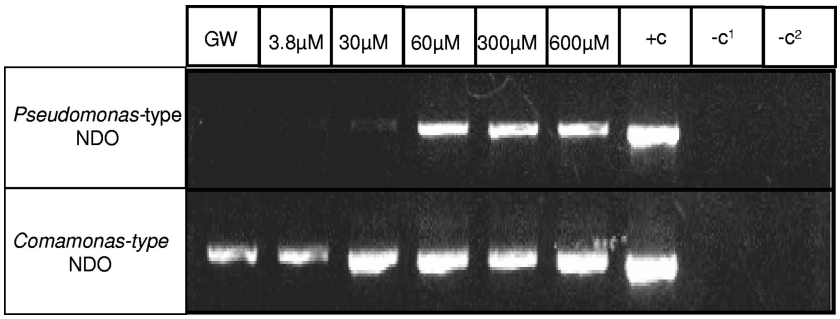

FIG. 4. RT-PCR analysis of NDO mRNA expression in 1 μg total RNA extracted from groundwater incubations with a range of amended naphthalene. *Comamonas*-type NDO expression was consistently detected across all amendments and within native groundwater (GW), whereas *Pseudomonas*-type NDOs were expressed only at higher concentrations of amended naphthalene (30 μM and higher). In each row, +C was an appropriate positive control for each primer set and −C1 and −C2 were RT-PCR-negative controls.

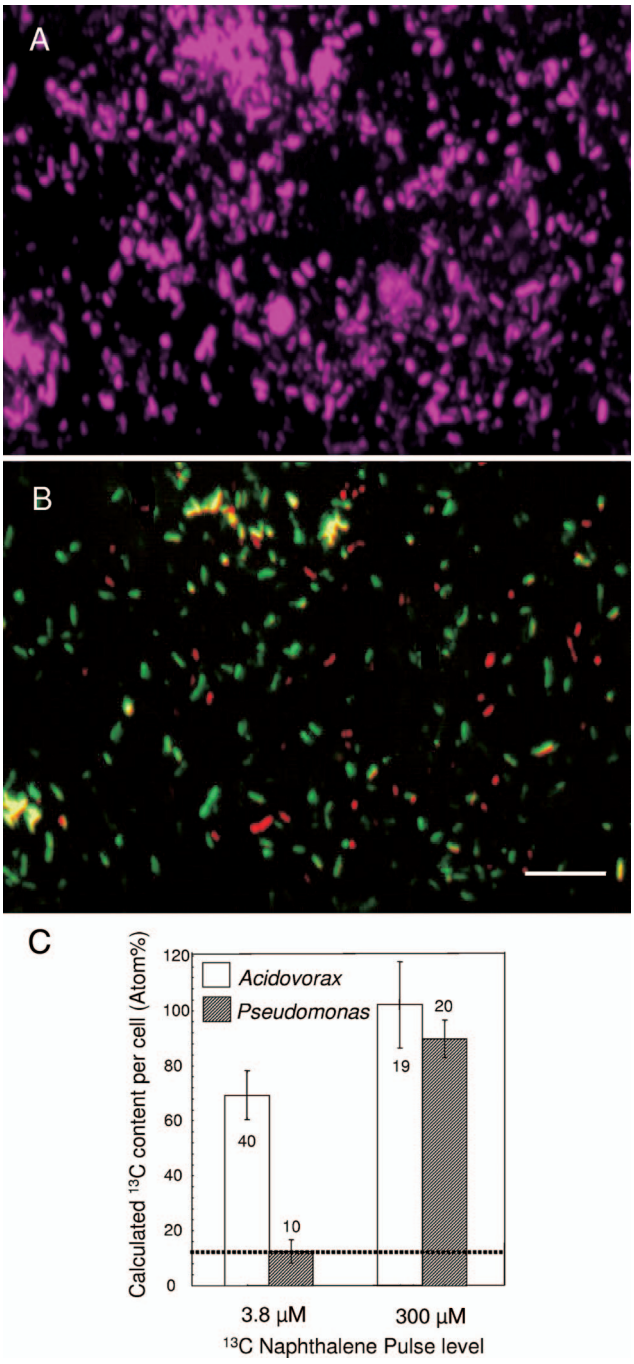

FIG. 5. FISH images of total bacterial cells in groundwater. (A) Cells hybridized with EUB338 (false-colored purple); (B) specifically probed subpopulations. For specific subpopulations *Acidovorax* sp. cells were hybridized with a Cy3-labeled probe (red) and *Pseudomonas* sp. cells were hybridized with a fluorescein isothiocyanate probe (green). Scale bar, 10 μm. (C) Atom% ^{13}C incorporation into individual cells, calculated using Raman peak shift measures (see Fig. S6B in the supplemental material) under two [^{13}C]naphthalene labeling concentrations. The dashed line represents the baseline values derived from [^{12}C]naphthalene calibrations versus spectral shifts performed upon cultured isolates and represents the baseline for the analysis. ^{13}C calibrations were also performed against Raman peak shift values obtained for cultures grown under a range of naphthalene concentrations as in the supplementary information. Numbers indicated within the plots represent the numbers of cells analyzed per treatment for each *Acidovorax* or pseudomonad subpopulation delimited by specific FISH probes.

putida WH1 and WH3 isolates harbored *P. putida* NCIB 9816-type naphthalene degradation genes (9). The details of this work will be reported in another paper (X. H. Yu, A. S. Whiteley, and W. E. Huang, unpublished data).

Raman-FISH analyses to directly confirm the presence and quantify activity of multiple degrader pools in groundwater communities. In order to directly confirm the presence and activity of multiple degrader pools in situ, we performed Raman-FISH (14) upon uniformly [^{13}C]naphthalene-labeled samples (3.8 and 300 μM) using specific probes for the predicted diversity based upon RNA SIP and culture analyses. A specific FISH probe, Band2_prob2 (Cy3 labeled, red) (Table 2), was designed based upon the 1,349-bp 16S rRNA. *Acidovorax* sp. sequence and homology searches of the NCBI GenBank indicated the absolute specificity of this probe for the recovered sequence associated with the environmental *Acidovorax* sp. Probe EUB I (Cy5 labeled, blue) was designed to probe all eubacteria, while probe PSM-G (Fluos labeled, green) was used to probe specifically for *Pseudomonas* sp. Employment of three-color FISH imaging of microbial communities pulsed with 3.8 and 300 μM ^{13}C-labeled naphthalene for 36 h stained the *Acidovorax* sp. red, *Pseudomonas* sp. green, and eubacteria purple (Fig. 5A and B). FISH images indicated the positions of the *Acidovorax* sp. and *Pseudomonas* sp., while the Raman microspectroscope was used to identify individual cells that had incorporated ^{13}C-labeled substrate into cellular biomass. Figure 5C showed that the ^{13}C integration level for the *Acidovorax* sp. was significantly higher than that for the *Pseudomonas* sp. when the microbial community was pulsed with 3.8 μM naphthalene. However, when the microbial community was exposed to a high naphthalene concentration (300 μM), the ^{13}C integration for the *Acidovorax* sp. and that for the *Pseudomonas* sp. were at similar levels (Fig. 5C). This is in a good agreement with RT-PCR results for NDO expression (Fig. 4) and strongly suggested that the key degrader was the *Acidovorax* sp. in groundwater, as it was in the presence of an ambient naphthalene concentration, while *Pseudomonas* sp. became active only in the presence of a high naphthalene concentration.

DISCUSSION

Our previous investigation showed that aerobic naphthalene degradation is more efficient than anaerobic degradation at removing naphthalene in permeable barriers, so this study was focused upon aerobic degradation within the system. Aerobic naphthalene degradation typically yields salicylate as a catabolite (15), a coordinated inducer of naphthalene operon expression in pseudomonads (43). Thus, the presence of salicylate can be used as an indicator of the occurrence of aerobic naphthalene degradation (Fig. 1). The salicylate bacterial biosensor (15) used in this study provided a rapid way to determine the sampling time point and avoided cross-feeding, which is crucial to the SIP experiments. In order to analyze the functional degrading communities without the associated biases of culture, heavily ^{13}C-labeled RNA was recovered from the groundwater communities after pulsing uniformly ^{13}C-labeled naphthalene at 3.8 μM for 72 h and sampled at 36 h, a point indicated to be optimum for RNA SIP analyses by as-

sessment of naphthalene degradation and salicylate production kinetics (Fig. 1).

In order to generate quantitative evidence of the presumptive high- and low-affinity degrader pools, we obtained single-cell Raman spectra (13) for representative cells of each probe-positive population from both ^{13}C-labeled and [^{12}C]naphthalene-supplemented controls. Our previous investigations (13, 14) indicated that bacterial integration of ^{13}C compounds caused a significant "red shift" in key regions of bacterial Raman spectral profiles and that this can be employed to detect ^{13}C incorporation into individual cells (see Fig. S6 in the supplemental material). Using this approach, we measured the ^{13}C contents of individual cells to assess the hypothesis that at a low naphthalene concentration (3.8 μM) the *Acidovorax* cells utilized naphthalene preferentially over pseudomonad degraders. Figure 5C highlights the disproportionate incorporation of the ^{13}C label into the *Acidovorax* sp., compared with incorporation by the *Pseudomonas* sp., in groundwater pulsed with 3.8 μM [^{13}C]naphthalene. However, in the groundwater incubated with 30 μM [^{13}C]naphthalene, both the *Acidovorax* sp. and the *Pseudomonas* sp. became fully ^{13}C labeled, reinforcing the differential NDO expression data and the overall hypothesis of two gene pools within the system.

Ecologically, the naphthalene concentration in the field is often highly variable over space and time. Ambient concentrations of naphthalene throughout the site are around 3.8 μM, but pockets of high naphthalene concentrations also exist (10, 11). This heterogeneity, in the light of these data, has given rise to the evolution of a diverse community of naphthalene degraders. Specifically, a microbial community consisting of niche-specialized high- and low-affinity degrading bacteria is well positioned to exploit the heterogeneous contamination present in such a site (16). We determined that, under low naphthalene concentrations, *Acidovorax* organisms with a *Comamonas*-like NDO system assimilate naphthalene. In contrast, under higher concentrations of naphthalene, fast-growing *Pseudomonas* sp. also begin to exploit the resource, more than likely through dissemination of the low-affinity-*Pseudomonas*-like NDO catabolic genes located on conjugative plasmids, the result being a much more diverse low-affinity degrader population consisting of at least three pseudomonad species. Of further interest is that, at increasing concentrations of naphthalene, coexistence between the two degrader pools is observed, suggesting that, at increasing naphthalene levels, the naphthalene resource may not be the major ecological factor in community structuring (e.g., either through substrate inhibition of the high-affinity *Acidovorax* or through competitive niche exclusion by the pseudomonads). Such observations begin to provide a basic framework to elucidate the main ecological factors which give rise to and maintain these complex in situ degrading communities.

By applying the FISH-Raman-SIP technique, in this study we have established an unequivocal link between an unculturable *Acidovorax* sp. and its ecological role of naphthalene biodegradation, as well as relevant functional genes. In this case, we have successfully used *Comamonas*- and *Pseudomonas*-type primers to recover NDO genes from the ^{13}C-labeled RNA gene pool. This technique enables us to investigate the incorporation of a ^{13}C-labeled substrate at the level of a single cell, but it relies on instruments such as the Raman microspectroscope. However, we are mindful that this technology has much wider applications, for example, in the fields of genomics and wider microbiology. For example, it has been demonstrated that the SIP toolbox can be used for analysis of bacterial species and determination of their ecological roles and can provide critical information about the key "functional" species and their traits. By employing this information in the future, with appropriate technologies such as flow cytometry sorting (22, 38) and single-cell genome amplification (18, 20, 25, 33), we are realistically entering an era of determining a single cell's true in situ ecological function prior to determining the aligned genome sequence as a basis for its ecological function. Alternatively, Raman spectroscopy can be used to directly isolate single cells for downstream analyses through Raman tweezers and microfluidic sorting (41), for molecular analyses of either FISH-labeled or ^{13}C-labeled cells, and for discrimination of live cells based upon ^{13}C incorporation and their subsequent cultivation. In conclusion, the implemented FISH-Raman-SIP approach will be a valuable tool to exploit unculturable bacteria and to link microbial species, their metabolic functions, and ultimately their genomic content within a range of ecosystems. All these factors serve to reveal the secret lives of unculturable microorganisms which are central to the functioning of our natural environment.

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