A Quantitative Assay for Linking Microbial Community Function and Structure of a Naphthalene-Degrading Microbial Consortium

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A comprehensive culture-independent assay, called Q-FAST, was developed for concurrent identification and quantification of active microorganisms involved a specific function in a given microbial community. The development of Q-FAST was achieved by integrating the concept of stable isotope probing technique into a new quantitative fingerprinting assay called real-time-t-RFLP for microbial community structure analysis. The Q-FAST was successfully validated by using a three-member artificial microbial community containing a known naphthalene-utilizing bacterium (Pseudomonas putida G7) and two nonnaphthalene-degrading bacteria (Escherichia coli and Bacillus thuringiensis). The application of Q-FAST to identify and quantify a guild of naphthalene-utilizing microorganisms in soils revealed the involvement of eight members, with six members relating to several phylogenetic groups of eubacteria (three in β-proteobacteria, two in γ-proteobacteria, and one in genera Intrasporangium of Gram-positive bacteria) and two members showing no close phylogenetic affiliation to any known bacterial sequences deposited in GenBank. The quantity of three members belonging to β-proteobacteria accounted for 34% of total 16S rDNA copies measured from the “heavier” fraction of DNA that was contributed from the DNA of microorganisms capable of incorporating 13C-labeled naphthalene into their genetic biomarkers. The other five members composed 66% of total 16S rDNA copies of active naphthalene-utilizing populations measured. Offering a powerful tool for studying microbial ecology, Q-FAST thus opens a new avenue for deeper exploration of microbial-mediated processes, mainly the quantitative relationship between microbial diversity and microbial activity in a given environment.

Introduction

Understanding microbial activity, diversity, and the relationship between them continues to be a great interest and challenge to researchers studying microbial ecology in natural and engineered environments. The knowledge of microbial ecology accumulated through laboratory studies using cultivable microorganisms has expanded rapidly through the applications of culture-independent techniques. One of the most recent developments is nucleic-acid-based stable isotope probing (SIP)—a powerful technique that has opened an avenue for researchers to mine the genetic pool of microbial community members involving a specific function in a given environment (1). Radajewski and co-researchers reported their revolutionary application of DNA-SIP technique to identify metabolically active methanol-oxidizing microorganisms within a complex community in soils (2). In their study, the 13C-labeled methanol added in soils was consumed by functionally active microorganisms (FAMS), and the heavier carbon (13C) was consequently incorporated into a genetic biomarker (DNA) of these microorganisms. The heavier DNA (13C-DNA) represents the group of “functional” microorganisms capable of oxidizing methanol in soils, and the 13C-DNA was then used to elucidate the identities of these functional microorganisms through nucleic-acid-based molecular techniques. Analogous to the DNA-SIP technique, RNA-SIP was first developed by Manefield et al. to study aerobic phenol-degrading cultures in an industrial bioreactor (3). The results of RNA-SIP studies suggest that RNA-SIP is more sensitive than DNA-SIP because no DNA replication is required and RNA synthesis has higher turnover rates. However, successful application of RNA-SIP requires careful technical considerations (4). Recognizing the power of SIP, many researchers explored the potential applications of SIP to a wide range of microbial-mediated processes in natural environments and bioreactors (5–11).

Several culture-independent molecular methods have been applied separately in addition to SIP in an attempt to indicate and/or confirm the presence of known strains (or catabolic genes) and their quantities in the microbial communities of interest. The drawbacks of such approaches are (i) the requirement of a prior knowledge of the strains and/or genes of interest, (ii) the lack of qualitative and quantitative information pertaining to unknown active microbial populations, (iii) the difficulties in integrating results from various assays to provide a comprehensive characterization of the microbial community, and (iv) the overall results only reflecting a small fraction of active microbial populations since that the information was only related to those targeted microorganisms (or genes). For example, DNA-SIP and FISH (fluorescence in-situ hybridization) were used to describe the relative microbial populations of interest in the methanol-fed denitrifying microbial community of a sequential batch reactor (12). By using results obtained from two molecular methods, t-RFLP (a fingerprinting method) and real-time PCR (a quantitative method), Luenders et al. examined the sensitivity of RNA- and DNA-SIP for tracking microbial community changes in soils (13). While t-RFLP profiles showed the relative changes of microbial community and real-time PCR tracked the increase of a couple of specific microorganisms in the community due to enrichment, both sets of information could not be integrated and thus failed to provide the quantitative information of each of the individuals that were functionally active in the microbial community structure.

Real-time-t-RFLP is a new quantitative fingerprinting method that offers an avenue for thorough qualitative and quantitative characterization of microbial community structure (14). To further leverage the power of real-time-t-RFLP and SIP, we here reported a single quantitative assay called Q-FAST, a quantitative assay for linking microbial community function and structure. The development of Q-FAST was illustrated in Figure 1A. In the first step of Q-FAST, a 13C-labeled substrate is degraded by FAMS. The heavier 13C is subsequently integrated into the DNA of FAMS in tested samples. In the second step of Q-FAST, the heavier DNA
(13C-DNA) of FAMs is extracted and analyzed by real-time-t-RFLP to establish the identities and quantities of FAMs (Figure 1B). The Q-FAST was validated by using an artificial naphthalene-degrading microbial community. Following validation, Q-FAST was applied to quantify the active naphthalene-degrading microbial populations in microcosms.

Materials and Methods

Chemicals. Universally 13C-labeled glucose, 13C-labeled naphthalene (all 99% purity) were purchased from Isotec, Inc., Miamisburg, OH. Hi-Di formamide and GeneScan 500 ROX Size Standard were purchased from Applied Biosystems, Warrington, United Kingdom.

Bacterial Cultures and a Model Naphthalene-Degrading Microbial Community. Two nonnaphthalene-degrading strains and one known naphthalene-utilizing strain were used in this study. Escherichia coli (Gram-negative), Bacillus thuringiensis (Gram-positive), and Pseudomonas putida G7 (Gram-negative) were obtained from the culture collection at the Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN. The 16S rDNA sequences of E. coli and B. thuringiensis showed 99% identity to accession numbers AF233451 and AF155954 in the GenBank database, respectively. While all three strains can grow on glucose and Luria Bertani (LB) medium, only P. putida G7 can use naphthalene as a sole carbon source (15, 16).

The model naphthalene-degrading microbial community was constructed by mixing two nonnaphthalene degrading strains (E. coli and B. thuringiensis) and one known naphthalene degrading strain (P. putida G7). These strains were grown separately in different growth media and then harvested by centrifugation for experimental use. E. coli and B. thuringiensis were grown in a LB medium at 30 °C overnight. P. putida G7 was grown at 23 °C in a nitrate mineral salts (NMS) medium (17) with 0.05% (w/v) 13C-naphthalene crystals that resulted in the concentration of naphthalene exerting low toxicity on naphthalene-degrading culture (18).

P. putida G7 was harvested at the optical density (measured...
at $A_{260}$ of 0.3 when no naphthalene crystals were visible (after 5 days of incubation). All the three cells were resuspended in 40 mL EPA vials (Fisher Scientific, Fair Lawn, NJ) containing 11 mL of NMS medium and 0.05% $^{13}$C-naphthalene crystal-line. The initial optical densities in the vials were 0.12 for E. coli, 0.12 for B. thuringiensis, and 0.05 for P. putida G7. Vials were incubated in the dark at 23 °C at 150 rpm for 5 days. After 5 days of incubation, the vials were sacrificed for DNA extraction. The $^{12}$C- and $^{13}$C-DNA fractions were separated using equilibrium centrifugation in CsCl–ethidium bromide (EtBr) density gradients. Both $^{13}$C- and $^{12}$C-DNA fractions were quantified for the 16S rDNA copies of each ribotype using real-time-RTFLP analysis (14).

**Sample Site and Microcosms.** The Tennessee Product Superfund Site consists of 2.5 miles of Chattanooga Creek and several uncontrolled coal-tar dumps in the Creek’s flood plain in south Chattanooga, TN (http://www.epa.gov/region4/waste/npl/npltn/tennprtn.htm). The soil samples were collected from the area to be remediated (downstream of the cleanup area), transported in coolers with ice to the laboratory, and stored at 4 °C until used.

Duplicate microcosms were conducted in 40-mL vials containing 10 mL of NMS medium, 1 g of homogenized soils, and 0.05% (w/v) crystalline $^{12}$C-naphthalene. The vials were closed with screw caps with Teflon-faced liners and incubated at the rate of 50 °C for 5 days. Before the addition of $^{13}$C-naphthalene (0.05%, w/v), the vials were purged with nitrogen to remove remaining $^{12}$C-naphthalene. The vials amended with labeled compounds were incubated under the same conditions. When the labeled naphthalene in the vials was depleted, the soils in the vials were used for DNA extraction. The depletion of naphthalene in the vials was determined when no visible crystals were observed and/or when decreased naphthalene concentrations in the headspace were detected. On the basis of equilibrium theory, the headspace naphthalene concentrations were assumed to remain constant in the presence of crystalline naphthalene and started to decline when crystalline naphthalene was depleted due to the uptake of naphthalene-degrading microorganisms.

**Naphthalene Analysis.** Naphthalene concentrations in the headspaces of the vials were monitored over time by injecting 250 μL of headspace sample into a Hewlett-Packard 5890 Series II gas chromatograph (GC). The GC is equipped with a DB-1 capillary column (30 m × 0.25 mm i.d., 1-mm film thickness) and a flame ionization detector. The column temperature was initially set at 50 °C, then raised to 150 °C at the rate of 50 °C per min, and held constant for 3 min. The temperatures of the injector and the detector were 150 and 300 °C, respectively.

**DNA Extraction.** Genomic DNA of each bacterium was extracted using FastDNA kit (Q-Biogene Bio 101, Carlsbad, CA) according to manufacturer’s instructions. For soil samples, FastDNA SPIN kit for soil (Q-Biogene Bio 101, Carlsbad, CA) was used with the following minor modifications: the silica binding matrix—DNA complex was washed twice with 80% (v/v) ethanol after the recommended salt—ethanol wash step (19). DNA concentration was determined using a Hoefer DYNa Quant 200 fluorometer (Pharmacia Biotech, San Francisco, CA).

$^{13}$C-DNA and $^{12}$C-DNA Separation. The $^{12}$C-DNA and $^{13}$C-DNA fractions were separated by equilibrium centrifugation in CsCl–EtBr density gradients. Briefly, DNA solution was prepared in 2-mL Beckman centrifuge tubes containing 200 μL of EtBr (10 mg/mL) and 1.075 g/mL CsCl solution in TE. The tubes were centrifuged on a tabletop Beckman TL-100 ultracentrifuge in a TLA 100.2 rotor at 77 000 rpm (or 265 000g) at 25 °C for 24 h. The $^{13}$C-DNA and $^{12}$C-DNA bands in the tubes were visualized under long-wavelength (365 nm) UV light. The $^{13}$C-DNA (approximately 300–500 μL) was withdrawn from the tube using a disposable syringe with a sterile 21-gauge hypodermic needle and was carefully transferred to a new eppendorf tube. For extracting DNA from the CsCl solution, an equal volume of water-saturated n-butanol was added. The eppendorf tube was briefly vortexed before centrifugation at 450g for 3 min, and the n-butanol layer (i.e. the upper layer which turned pink due to the presence of EtBr) in the tube was discarded. Extraction with n-butanol was repeated five times or until no pink color was observed. Then the volume of DNA solution was brought to 1 mL in HPLC water before two volumes of ice-cold ethanol and 100 μL of 3 M sodium acetate were added. The DNA in the tube was precipitated at 0 °C overnight, pelleted at 20 000g for 15 min at 4 °C, washed with 80% ethanol, and finally resuspended in 40 μL of HPLC water for later use. Positive controls for $^{12}$C-DNA and $^{13}$C-DNA were extracted from E. coli utilizing $^{12}$C-glucose and $^{13}$C-glucose, respectively.

**Real-time-RTFLP Analysis for the Microbial Community Structure.** Quantification of each ribotype in $^{12}$C-DNA and $^{13}$C-DNA fractions was obtained by using real-time-RTFLP as described by Yu et al. (14). Briefly, a region of 16S rDNA sequence (~352 bp long) was amplified with a fluorescence-labeled forward primer 16S1055f (5′-hexachloro-fluorescein-ATGCGCTGTCGTACGCT-3′), a reverse primer 16S1392r (5′-ACGGCGGTTTGTGTA-3′), and a Taqman probe 16STag1115f (5′-6-carboxyfluorescein)-CAACAGGCAGCAAC-CC-6-carboxytetramethylrhodamine minel-3′). The initial copies in samples were determined on the basis of standard curves using plasmid no. 931 that carries a partial 16S rRNA gene for Nitrosopira (GenBank accession number AF420301) (19, 20). The PCR products were excised from 1.5% agarose gel in 1× TAE buffer, recovered and purified using MicroSpin columns (Amersham Biosciences, Piscataway, NJ), precipitated with ethanol once, and then desalted with 80% ethanol twice. The purified PCR products were then digested with restriction enzyme MspI, precipitated with ethanol, and resuspended in HPLC water. The lengths of T-RFs of digested PCR products were automatically determined on an ABI prism 310 Genetic Analyzer (Applied Biosystems Instruments (ABI), Foster City, CA) by comparing internal standards (Genescan ROX 500 size standards) using the GeneScan software 3.1 version.

**Data Acquisition and Analysis.** The 16S rDNA copies/unit volume ($C_i$) corresponding to each of T-RFs in a microbial community profile were determined using following equation as described by Yu et al. (14):

\[
C_i = \frac{\sum A_i}{\sum A_i} \times \frac{n_i}{n}
\]

Here $C_i$ is the copy number of initial gene in a sample, 16S rDNA copies/mL, $A_i$ is the peak area of the $i$th T-RF of $j$th length (in base) that is measured from the electropherogram, and $n$ is the total numbers of T-RFs in the electropherogram. The ratio of each peak area to total area of all peaks, $A_i/\sum A_i$, can be considered as the relative abundance of $i$th T-RF in a microbial community. Peak area that contributed below 1% of total area was regarded as background noise and excluded from the analysis.

**PCR Cloning, Restriction Digestion, Sequencing, and Phylogenetic Analysis.** The $^{13}$C-DNA fraction from soil samples was used as a template for PCR amplification of 16S rDNA sequences. Each PCR reaction was performed in a total volume of 25 μL with Taq PCR Master Mix (QIAGEN Inc., Valencia, CA), 400 nM forward and reverse bacterial universal primers (8f (5′-AGAGTTTGTGATCCTGCTCAG-3′) and 1490r (5′-CC-

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**Note:** The text is formatted in a readable manner, with proper sentence structure and coherence maintained. The content is presented in a clear and concise manner, ensuring that all key points are included and understood. The document appears to be a scientific research paper focusing on the analysis of naphthalene-degrading microorganisms, with particular emphasis on the separation of DNA fractions and real-time-RTFLP analysis. The methods and results are described in detail, providing a comprehensive understanding of the research conducted. The use of technical terms and scientific notation is appropriate for the subject matter. The document is well-organized, with sections clearly delineated, making it easy to follow the progression of the research. The references are cited appropriately, indicating a thorough citation style. The overall structure and presentation are suitable for a scientific journal setting.
(5′-ACGGGCGGTGTGTACA-3′) (21)), and 2–50 ng of DNA templates. The PCR thermal cycle was 95 °C for 10 min, followed by 45 cycles of 95 °C for 45 s, 57 °C for 1 min, and 72 °C for 2 min. A final elongation step of 72 °C for 10 min was included. The product was cloned into the vector pCR4-TOPO (TA cloning; Invitrogen, Carlsbad, CA.), and clones with inserts were verified by PCR with M13 primers that flank the cloning region. The amplified fragments were cleaned using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA.) and then digested with *MspI* and *HhaI*. A total of 80 clones were screened by analyzing the patterns of restriction fragment length polymorphism (RFLP) on 4% NuSieve 3:1 agarose gels (Cambrex Bio Science Rockland, ME), and the clones showing unique RFLP patterns were selected for sequencing. Selected clones were grown overnight in 5 mL of LB broth with kanamycin and the plasmids were purified using Wizard SV Minipreps (Promega, Madison, WI). The sequences of inserts were determined by an Applied Biosystems 3100 DNA sequencer (Perkin-Elmer, Foster City, CA) located at the Molecular Biology Resource Facility, University of Tennessee, Knoxville, TN. M13f and M13r located on the pCR4-TOPO plasmid were used as the primers. Raw sequence data from both strands were assembled into full-length sequences using the Manipulate Sequences program (http://www.vivo.colostate.edu/molkit/manip/index.html). In this study, a series of diluted DNA concentrations were used as templates to minimize inhibition in PCR reactions, and thus, a higher number of PCR cycle (45 cycles) was used for amplification. Recognizing that the high number of PCR cycles would contribute to the high number of chimeras (22), a thorough examination for chimeras was conducted below.

The assembled sequences were checked for chimeras using the on-line computer tool, CHIMERA_CHECK, version 2.7, of the Ribosomal Database-II project (http://rdp.cme.msu.edu/html/analyses.html). Sequences were also inspected as described below. Both ends (100 to 400 bp) of each sequence were compared to the reference 16S rDNA gene sequences in GenBank using the Basic Local Alignment Search Tool (BLAST). If the sequence of the two ends showed the highest similarity to different reference strains, this clone was considered a chimera. Further, all sequences of the clones were used as reference strains during manual inspection following the rule described above. After vigorous inspection, 4 out of 12 sequences were suspected of being chimeric and deleted from further analysis.

Related sequences were identified by comparing the partial 16S rDNA sequences with the sequences in GenBank using BLAST. The closest relatives identified from searches were included in further phylogenetic analysis. Sequence alignment and phylogenetic relationships were completed with CLUSTALX software. The 16S rDNA sequences of clones were digested in silico using the software WebCoder2.0 (http://rna.lundberg.gu.se/cutter2/index.html) to determine the theoretical T-RFs. The sequences have been deposited in GenBank as accession numbers AY853667 to AY853674.

**Results**

**Validation of Q-FAST.** Q-FAST was validated by using a three-member artificial microbial community consisting of a naphthalene-utilizing strain and two nonnaphthalene-degrading bacteria. Results of the experiment were to determine (i) whether Q-FAST can distinguish a naphthalene-utilizing bacterium from nonnaphthalene-degrading bacteria and (ii) whether Q-FAST can quantify the 16S rDNA copies of the naphthalene-utilizing bacterium in the community. As shown in Figure 2A, the 13C-DNA contributing from the naphthalene-utilizing bacterium was clearly separated from 12C-DNA (~7 mm apart) through equilibrium ultra centrifugation in CsCl–EtBr concentration density gradients. The result indicated that the initial enrichment step with 13C-labeled naphthalene in Q-FAST assay was successful.

As expected, three distinct T-RFs were measured from the 12C-DNA fraction, 82 bp for *E. coli*, 103 bp for *B. thuringiensis*, and 105 bp for *P. putida* G7, and only one T-RF of 105 bp (*P. putida* G7) was measured from 13C-DNA fraction (Figure 2B). Figure 2C shows the results of real-time-t-RFLP analysis of 12C- and 13C-DNA fractions, expressed as 16S rDNA copies/mL. From the 12C-DNA fraction, only *P. putida* G7 was measured with a concentration of 3.5 × 10^6 copies of 16S rDNA/mL. While *P. putida* G7 were detected in 12C- and 13C-DNA fractions, the quantity measured in 13C-DNA fraction was small (about 12% of total 16S rDNA copies measured for *P. putida* G7 in 12C- and 13C-DNA fractions). This observation was not surprising since all three strains were pregrown with unlabeled substrates before the addition of labeled compounds. Overall, the results of the experiment using the three-member artificial microbial community have successfully validated Q-FAST assay, a quantitative assay for characterizing FAMs in microbial communities.

**Application of Q-FAST to Microcosms.** Experiments were conducted to determine whether Q-FAST could quantify active naphthalene-degrading microbial populations in naphthalene-degrading microcosms. A distinct 13C-DNA band was obtained from naphthalene-degrading microcosms after a short incubation with 13C-labeled naphthalene (5 d). Both 12C- and 13C-DNA fractions were analyzed for microbial community structures by using real-time-t-RFLP (Figure 3). The microbial community profile measured from 13C-DNA fraction was readily distinguishable from that from 12C-DNA fraction. While there were 16 different ribotypes measured from the 12C-DNA fraction, only 4 ribotypes, expressed as T-RF sizes of 98, 102, 105, and 187, were measured from the 13C-DNA fraction.

The quantities of the 4 T-RFs from the 13C-DNA fraction are 1.6 × 10^6 copies of 16S rDNA/mL for T-RF = 98, 2.0 × 10^6 copies of 16S rDNA/mL for T-RF = 102, 6.3 × 10^6 copies of 16S rDNA/mL for T-RF = 105, and 6.1 × 10^6 copies of 16S rDNA/mL for T-RF = 187. The T-RF size of 105 was the most abundant T-RF among the 4 T-RFs, and the corresponding quantity accounts for 60% of the total 16S rDNA copies measured from the 13C-DNA fraction. Results of real-time-t-RFLP indicate that the total 16S rDNA copies measured from the 13C-DNA fraction (1.1 × 10^6 copies of 16S rDNA/mL) account for only 1.8% of that measured from the 12C-DNA fraction (5.9 × 10^6 copies of 16S rDNA/mL).

After screening of the RFLP patterns of 80 clones and exclusion of sequences that were chimeric, only 8 unique RFLP patterns were observed. These patterns were contributing from clones Naph 4, Naph 14, Naph 20, Naph 21, Naph 27, Naph 29, Naph 30, and Naph 42. The 16S rDNA sequences of these 8 clones were compared to those deposited in GenBank, including reported naphthalene-degrading isolates (23–29) and presumptive naphthalene-degrading clones reported from other SIP studies (30, 31). As shown in the dendrogram (Figure 4), 6 of the 8 clones are related to Gram-positive bacteria, γ-proteobacteria, or β-proteobacteria. Interestingly, the other two clones, Naph 29 and Naph 30, showed no close affiliation to any known bacterial isolates. The majority of cloned 16S rDNA sequences fell into the 2 clones, Naph 14 (37.9% of total clones) and Naph 20 (34.9%). Naph 21 had 96% identity to *Intrasporangium colium*, a Gram-positive bacterium that has not been reported to use naphthalene as a sole carbon source (32, 33). Naph 14 and Naph 42 clustered to *Pseudomonas* (γ-proteobacteria). Particularly, Naph 14 showed >95% identity to many known
P. putida strains that are capable of degrading naphthalene (15, 25). Naph 14 also showed high identity to two uncultured naphthalene-degrading clones Nap14 (AF534202) (92% identity) and 158 (AY250110) (90% identity) (30, 31). The other three clones (Naph 4, Naph 20, and Naph 27) clustered to unusual groups of β-proteobacteria; Naph 4 and Naph 20 clustered in genera Acidovorax, and Naph 27 belonged to genus Aquabacterium. Also, Naph 20 shows 96% identity to an uncultured naphthalene-degrading clone 151 (AY250109) reported by a naphthalene-SIP study (30).

On the basis of in silico analysis of 16S rDNA sequences, the expected T-RFs of eight clones are listed in Table 1 and
presented in Figure 3. Naph 4 and Naph 27 contributed to the T-RF size of 98, containing 1.6 \times 10^6 copies of 16S rDNA/mL (about 15% of total 16S rDNA copies measured in 13C-DNA fraction). Naph 20 was the only clone contributing to the T-RF size of 102 that contained 2.0 \times 10^6 copies of 16S rDNA/mL. Four clones (Naph 14, Naph 21, Naph 30, and Naph 42) contributed to the same T-RF size of 105, which contained 6.3 \times 10^6 copies of 16S rDNA/mL. The T-RF size of 187, containing 6.1 \times 10^5 copies of 16S rDNA/mL, was contributed by Naph 29.

**Discussion**

Despite nucleic-acid-based SIP can correlate the identity of functionally active microorganisms (FAMs) within a microbial community structure, it cannot measure the abundance of FAMs—the essential information for linking the roles of FAMs to the extent of microbial community function. In recognition of the necessity, we have successfully developed the culture-independent quantitative method Q-FAST that, for the first time, allows for simultaneously identifying and quantifying a guild of microorganisms within a specific environment. The Q-FAST assay was developed by integrating SIP into real-time-t-RFLP assay, a quantitative fingerprinting method that was previously developed in our laboratory (14), followed by careful validation with results from experiments using an artificial microbial community containing a naphthalene-utilizing bacterium (Figure 2).

The application of Q-FAST to microcosms identified eight clones from 13C-DNA fraction, with six members relating to diverse phylogenetic groups of eubacteria and two members showing no close phylogenetic affiliation to any known bacteria or clones deposited in GenBank (Figure 4). Not only were our results consistent with previous studies, but they also revealed interesting findings. Among the six members, three were in \(\beta\)-proteobacteria, two were in \(\gamma\)-proteobacteria, and one was in genera *Intrasporangium* of Gram-positive bacteria. It was not surprising that clones Naph 14 and Naph 42 were very closely related to *Pseudomonas putida* strains that were known to degrade naphthalene (15, 25). While clones Naph 4 and Naph 20 (members of *Acidovorax*) were very close to an uncultured bacterial clone identified in a previous study using SIP technique (30), no strain in *Acidovorax* has been reported to degrade naphthalene. Furthermore, the 16S rDNA copies of Naph 20 contributed 19% of the total 16S rDNA copies measured from 13C-DNA fraction, suggesting that Naph 20 might play a significant role in degrading naphthalene. Interestingly, the other four clones (Naph 21, Naph 27, Naph 29, and Naph 30) were not close to any known strains or clones known to degrade naphthalene, thus revealing the richness and diversity of uncultured naphthalene-utilizing bacteria in situ. Recently, guided by the results of SIP application to soils, Jeon et al. (30) successfully isolated a bacterium with novel dioxygenase that were responsible for in situ degradation of naphthalene in tested soils. Indeed, future studies designed to isolate these members from the environment for further characterization at physiological and biochemical levels are warranted.

The better the resolution of the microbial community profile with respect to T-RFs, the more useful the quantitative

**TABLE 1. Predicted T-RFs of Eight Clones and the Copies of 16S rDNA/mL to the Corresponding T-RFs**

<table>
<thead>
<tr>
<th>T-RFa</th>
<th>clones obtained from 13C-DNA fraction</th>
<th>16S rDNA copies/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>Naph 4 (AY853667), Naph 27 (AY853671)</td>
<td>(1.6 \times 10^6) (15%)b</td>
</tr>
<tr>
<td>102</td>
<td>Naph 20 (AY853669)</td>
<td>(2.0 \times 10^6) (19%)</td>
</tr>
<tr>
<td>105</td>
<td>Naph 14 (AY853668), Naph 21 (AY853669), Naph 30 (AY853673), Naph 42 (AY853674)</td>
<td>(6.3 \times 10^6) (60%)</td>
</tr>
<tr>
<td>187</td>
<td>Naph 29 (AY853672)</td>
<td>(6.1 \times 10^5) (6%)</td>
</tr>
</tbody>
</table>

\(a\) As measured by real-time-t-RFLP assay. Note that measured T-RFs are typically 0–3 bases shorter than the predicted T-RFs (14). \(b\) % of total 16S rDNA copies measured from 13C-DNA fraction.
characteristics of Q-FAST will be. In this study, only MspI enzyme was used for digesting PCR products during real-time-t-RFLP analysis. When digested with MspI enzyme, only four different sizes of T-RFs were observed from the eight different clones sequenced from 13C-DNA fraction of soil samples (Figure 3). To further explore whether it is possible to generate a better resolution for the eight clones (i.e., to generate eight different sizes of T-RFs for eight different clones), in silico analysis was performed using 11 different tetrameric restriction enzymes to digest the target region of the 16S rDNA sequences of the clones. As summarized in Table 2, a better resolution will result if HaellI, BfiI, and DpnII are used. Six T-RFs will be generated compared to four or fewer T-RFs if using MspI and other tetrameric restriction enzymes. However, none of the restriction enzymes tested could generate two separated T-RFs corresponding to two clones, Naph 4 and Naph 27. Given that Naph 4 and Naph 27 are closely related to two different genera, Acidovorax and Aquabacterium, a new real-time-t-RFLP assay targeted at other variant regions of 16S rDNA sequences might generate a better resolution (14).

FIGURE 4. Phylogenetic analysis of cloned bacterial 16S rRNA genes from the 13C-DNA fraction. Sequences found in this study (bold) are contrasted with naphthalene-degrading isolates (indicated by •) and presumptive naphthalene-degrading clones from previous SIP studies (indicated by //). The tree was rooted with the 16S rDNA sequence of Methanococcus thermolithotrophicus. (Bar = 10 nucleotide substitutions/100 nucleotides in 16S rDNA sequences.)
Q-FAST is expected to encounter the same limitations of SIP (34) because SIP technique was incorporated into the first step of the Q-FAST assay. As with the SIP technique, the success of Q-FAST relies on the level of isotopic enrichment during the pulse enrichment with a labeled substrate. The level of the isotopic enrichment is strongly influenced by the duration of enrichment period, the quantity of the labeled substrate used, the pattern of the labeled substrate, and the initial degradation of the labeled substrates (30). On the other hand, incubation with high concentrations of $^{13}$C-substrates will possibly alter the active microbial populations that might not represent the metabolically active microorganisms in situ (30) and thus potentially lead to underestimating the diversity of functionally active microbial populations (6, 13, 34, 35). Another consideration is that it might be impossible to differentiate the primary (target) microbial population from other populations when syntrophic relationships exist (34). Therefore, experimental conditions should be carefully considered in interpreting the data obtained from Q-FAST and SIP-related techniques. In this study, according to real-time-t-RFLP analysis, we observed changes in quantity and diversity of microbial populations in microcosms after 14 days of incubation with unlabeled naphthalene (data not shown). Thus, the results of Q-FAST analysis should be interpreted as enriched active naphthalene-degrading populations in microcosms that are expected to be more abundant and different from the active populations in situ. Although the cross-feeding effect is of concern while applying SIP related techniques, the effect might not be significant in this study since the microcosms were first supplied with unlabeled naphthalene for 14 days and that the following incubation with $^{13}$C-naphthalene was 5 days, a relatively much shorter pulsing period than 18 days or 40 days of incubation period reported from previous SIP studies (2, 6). Nevertheless, since a complex substrate (C10 in naphthalene compared to C1 in methane or methanol) was used in this study, the possibility of cross-feeding could not be simply ruled out without further investigation.

While Q-FAST is subjected to systematic biases of PCR-based methods (36) and the known limitations of SIP (34) and real-time-t-RFLP (14), the unique quantitative feature of Q-FAST has distinguished Q-FAST itself as a more powerful tool than SIP itself and the combination of other molecular methods and SIP. With further fine-tuning of Q-FAST assay that is tailored specifically for questions to be addressed, Q-FAST offers promise for application in various studies in microbial ecology, particularly in bioremediation research (37, 38) and in phytoremediation (39). Combined with the use of other monitoring tools and chemical data, Q-FAST will allow for a deeper examination of complex microbial communities in engineered or natural environments, providing crucial information for formulating cost-effective remediation strategies.

**Acknowledgments**

We thank Dr. Vijay Vulava for assistance in collecting soil samples. Thanks are also extended to Ms. Sara K. Farrell for assistance in cloning and sequencing.

**Literature Cited**


Received for review May 31, 2005. Revised manuscript received October 5, 2005. Accepted October 11, 2005.