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Linking Microbial Community Function to Phylogeny of Sulfate-Reducing *Deltaproteobacteria* in Marine Sediments by Combining Stable Isotope Probing with Magnetic-Bead Capture Hybridization of 16S rRNA▽

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We further developed the stable isotope probing, magnetic-bead capture method to make it applicable for linking microbial community function to phylogeny at the class and family levels. The main improvements were a substantial decrease in the protocol blank and an approximately 10-fold increase in the detection limit by using a micro-elemental analyzer coupled to isotope ratio mass spectrometry to determine ^{13}C labeling of isolated 16S rRNA. We demonstrated the method by studying substrate utilization by *Desulfobacteraceae*, a dominant group of complete oxidizing sulfate-reducing *Deltaproteobacteria* in marine sediments. Stable-isotope-labeled [^{13}C]glucose, [^{13}C]propionate, or [^{13}C]acetate was fed into an anoxic intertidal sediment. We applied a nested set of three biotin-labeled oligonucleotide probes to capture *Bacteria*, *Deltaproteobacteria*, and finally *Desulfobacteraceae* rRNA by using hydrophobic streptavidin-coated paramagnetic beads. The target specificities of the probes were examined with pure cultures of target and nontarget species and by determining the phylogenetic composition of the captured sediment rRNA. The specificity of the final protocol was generally very good, as more than 90% of the captured 16S rRNA belonged to the target range of the probes. Our results indicated that *Desulfobacteraceae* were important consumers of propionate but not of glucose. However, the results for acetate utilization were less conclusive due to lower and more variable labeling levels in captured rRNA. The main advantage of the method in this study over other nucleic acid-based stable isotope probing methods is that ^{13}C labeling can be much lower, to the extent that $\delta^{13}C$ ratios can be studied even at their natural abundances.

Linking microbial phylogeny to community function provides us with insights into the roles that microorganisms play in global elemental cycling. In recent years, stable isotope-tracing approaches combined with biomarkers have been widely applied to environmental studies (8, 27, 40). Tracking stable- or radioisotope-labeled atoms from particular substrates into components of microbial cells (biomarkers) can reveal which organisms are involved in the consumption of the substrate and also yield information on rates of specific biogeochemical transformation (8).

Dissimilatory sulfate reduction is a major pathway for organic carbon mineralization in coastal marine sediments, accounting for, on average, 50% of the total carbon mineralization (18, 36). Sulfate-reducing prokaryotes are a diverse and ubiquitous component of the bacterial community. The diversity of sulfate-reducing bacteria (SRB) in marine sediments has been investigated by using clone libraries of 16S rRNA (38) and dissimilatory sulfite reductase genes (11) and by fluorescence in situ hybridization-related techniques (33, 41). *Desulfobacteraceae*, a group of complete-oxidizing SRB belonging to the *Deltaproteobacteria*, have generally been found to be a major group of SRB in marine sediments.

Phospholipid-derived fatty acids (PLFA) were the first type of biomarkers to be used in combination with stable isotope probing (SIP) (8). PLFA-SIP provides high sensitivity in terms of the amount of ^{13}C label needed, but the phylogenetic resolution offered is low and requires reference signatures of closely related culturable relatives (8). The main advantage of DNA- and RNA-SIP is that they offer improved phylogenetic resolution (27, 40). These two methods are based on the separation of the "heavier" ^{13}C-labeled nucleic acid from unlabeled nucleic acid by density centrifugation. Subsequently, organisms incorporating the greatest proportion of label into their DNA or RNA are identified by various molecular-fingerprinting techniques or by constructing clone libraries. RNA has a higher turnover rate than DNA, resulting in faster labeling, and incubation times can therefore be substantially shortened (27, 42). RNA is also more likely to reflect the phylogenetic composition of the metabolically active community, since it is highly susceptible to chemical and enzymatic degradation, and its cellular levels are often tightly regulated (19, 32), although some prokaryotes maintain high numbers of ribosomes during starvation (13).

MacGregor et al. (25, 26) developed a related approach, SIP combined with magnetic-bead capture hybridization (here called Mag-SIP), which is based on the isolation of small sub-

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unit rRNA from particular phylogenetic groups and the detection of ^{13}C-labeling levels by isotope ratio mass spectrometry (IRMS). rRNA is captured by hybridization with specific biotin-labeled oligonucleotide probes, followed by retrieval of hybridized target rRNA using streptavidin-coated magnetic beads. The main advantage of Mag-SIP over other nucleic acid-based SIP methods is that in principle much lower labeling levels can be applied (about 0.001% versus >10% ^{13}C, respectively), as label detection is based on IRMS methods. For instance, it has been shown that the method can be used to study the effects of oil pollution on natural $\delta^{13}C$ ratios of bacterial communities in sediments (37). Moreover, Mag-SIP is not based on PCR, as the isotope ratio of the target rRNA is directly measured without amplification of nucleic acid, avoiding possible PCR artifacts. However, the large amounts (1 to 10 μg) of RNA needed for an accurate isotope ratio analysis by traditional elemental-analyzer (EA)-IRMS has limited the use of Mag-SIP to general domain-specific probes (25, 26). Recently, several methods, such as liquid chromatography (LC) combined with IRMS and spooling-wire microcombustion combined with IRMS, have been introduced that allow isotopic analysis of much smaller samples than with the traditional EA-IRMS systems (20, 43).

In this study, we used the wet-oxidation interface of LC-IRMS as a micro-EA (μEA)-IRMS (20). The use of μEA-IRMS substantially lowers the detection limit of isotope ratio measurements in terms of the amount of rRNA needed for an analysis but also calls for modifications of the Mag-SIP protocol in order to decrease protocol blanks (carbon from materials and reagents used in the protocol). We tested a nested set of three biotin-labeled oligonucleotide probes to capture 16S rRNA derived from *Bacteria*, *Deltaproteobacteria*, and finally *Desulfobacteraceae*. The target specificities and stringencies of these probes were tested against pure cultures of both target and nontarget organisms. Moreover, phylogenetic analysis of captured 16S rRNA from environmental samples was done to check specificity and, where necessary, to adjust probe stringency. Finally, we demonstrated Mag-SIP with a study of in situ substrate use by sulfate-reducing *Deltaproteobacteria* in intertidal anoxic marine sediment. A generalized scheme for Mag-SIP is shown in Fig. 1.

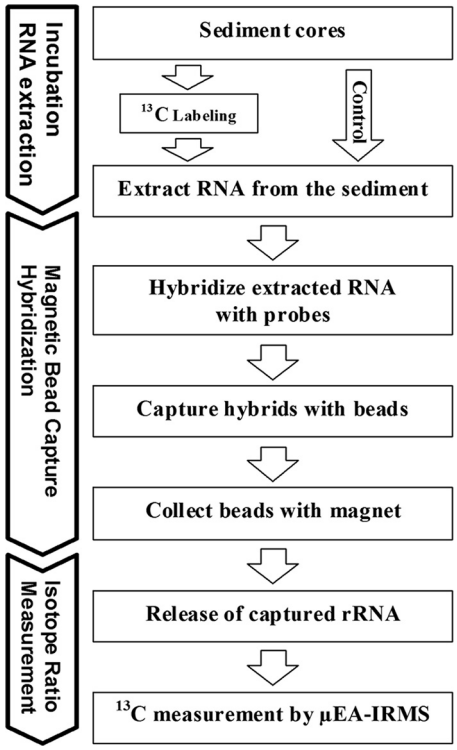

FIG. 1. A generalized scheme for Mag-SIP. The control was sediment incubated without substrate.

MATERIALS AND METHODS

Probes. Biotin-labeled oligonucleotide probes and unlabeled helper and competitor probes (Table 1) were purchased from Isogen Life Science (De Meern, The Netherlands). EUB338 and DELTA495a are commonly used probes for *Bacteria* and *Deltaproteobacteria*, respectively, even though they do not target all the genera in the domain and DELTA495a also targets most *Gemmatimonadetes* (22). The DELTA495a probe was used in combination with a competitor probe (cDELTA495a) to avoid capture of *Gammaproteobacteria*, which have only one mismatch in a target region of DELTA495a. DSS658 is a commonly used probe for *Desulfobacteraceae* (28) but also has only a single mismatch to many *Gammaproteobacteria*, resulting in poor specificity with the Mag-SIP protocol (results not shown). We therefore designed another specific probe (Dbact653) targeting a similar range of *Desulfobacteraceae* using the ARB software (23), which has less probability of capturing *Gammaproteobacteria*. In order to increase the yield, unlabeled 21-mer helper probes (14, 26) complementary to the consensus sequences upstream and downstream of the Dbact653 probe target sites were also designed. The stringencies and specificities of the probes were examined as described below.

Pure cultures. *Desulfococcus multivorans* strain DSM 2059^T, a sulfate-reducing deltaproteobacterium, was grown in DSM medium 197 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *D. multivorans* is a target organism for all the probes used in this study. *Desulfovibrio* sp. strain SB1, a target organism for the EUB338 and DELTA495a probes, was grown in DSM medium 63. *Escherichia coli* DH10B was grown in LB broth (Difco BD Biosciences, San Jose, CA). *E. coli* DH10B is a target for EUB338, but not for the other probes. However, the DELTA495a probe has only a single mismatch with *E. coli* in the target site.

Sampling and ^{13}C labeling. Sediment samples were collected at an intertidal flat in the Rattekaai area of the Oosterschelde estuary (The Netherlands) in May 2008. Undisturbed sediment cores (diameter, 5.2 cm) were transported immediately back to the laboratory. Equimolar ^{13}C amounts of uniformly labeled [^{13}C]glucose, [^{13}C]propionate, or [^{13}C]acetate (50, 100, and 150 mM, respectively; 99% ^{13}C; Cambridge Isotope Laboratories, Andover, MA) were injected (19 injections of 17 μl each) into the top 5 cm of the sediment cores by the line injection method (17). A total of four cores for each substrate were incubated for 24 h in the dark at in situ temperature (14°C). Sediment from the black, anoxic zone (2- to 5-cm depth) of cores was sectioned and stored at −80°C. Unlabeled control cores, incubated without labeled substrate, were also processed.

RNA isolation and electrophoresis. In order to extract total-community RNA from the sediment, a frozen sediment sample (20 to 25 g [wet weight]) was transferred into a sterile 80-ml glass bottle containing 7 g of sterile glass beads (5 g of 1-mm diameter and 2 g of 0.1-mm diameter; Sartorius, Göttingen, Germany), 20 ml of analysis grade phenol, and 10 ml of extraction buffer (250 mM sodium acetate, 50 mM EDTA, 2.5% sodium dodecyl sulfate [SDS], pH 5.1). The two sizes of glass beads were used in the amount and concentration that gave the highest yield of 16S rRNA (data not shown). The samples were vigorously agitated in an MSK-Zellhomogenisator (B. Braun Biotech International, Melsungen, Germany). For cultures, cells were collected by centrifugation and added to 1.5-ml centrifuge tubes with 0.5 g sterile glass beads (0.1-mm diameter; Sartorius), 800 μl of phenol, and 200 μl of extraction buffer (250 mM sodium acetate, 50 mM EDTA, 2.5% SDS, pH 5.1), followed by bead beating with Vortex-Genie 2 (Scientific Industries, Bohemia, NY). RNA preparations from both sediment samples and pure cultures were purified by phenol-chloroform extraction and isopropanol precipitation (25, 45). Total-community RNAs from several sediment extractions were combined and used for later magnetic-bead capture hybridization. All treatments were analyzed in duplicate. The total RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). In order to estimate the amount of 16S rRNA in the

TABLE 1. 16S rRNA-targeted probes used in this study

Probe	Sequence (5′–3′)	%FA[a]	Specificity	Reference
EUB338	GCT GCC TCC CGT AGG AGT	25	Most *Bacteria*	2
DELTA495a	AGT TAG CCG GTG CTT CCT	45	Most *Deltaproteobacteria* and most *Gemmatimonadetes*	21
cDELTA495a	AGT TAG CCG GTG CTT CTT	45	Competitor of DELTA495a	24
Dbact653	TTC CCT CTC CCA TAC TCA	25	Most *Desulfobacteraceae*	This study
Dbact653_up_help	CCC CGG AAG TGC AYT TGA WAC	25	Helper probe for Dbact653	This study
Dbact653_down_help	GTG GAA TTC CTG GTG TAG AGG	25	Helper probe for Dbact653	This study

[a] Percent formamide (FA) in hybridization buffer for hybridizations at 20°C.

total-community RNA, RNA extracts were visualized on a 5% polyacrylamide gel stained with ethidium bromide. The proportion of 16S rRNA among the RNA extracts was analyzed by comparing band intensities with known amounts of capture-isolated 16S rRNA by using ImageJ software (http://rsb.info.nih.gov/ij/).

Magnetic-bead capture hybridization of pure-culture RNA. The capture protocol was first tested with pure cultures. The optimal hybridization stringency was determined for each probe by varying the formamide concentration in the hybridization buffer between 0 and 60%. The formamide concentration that gave about half of the 16S rRNA band intensity at 0% formamide was used further in the protocol. Both target and nontarget cultures were tested at this formamide concentration to determine the specificity of the assay, and where necessary, the formamide concentration was adapted.

For the assay, 10 microliters of pure-culture RNA extract (1 to 2 μg RNA) and 90 μl of hybridization buffer (5× saline-sodium citrate [SSC] buffer [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% *N*-laurylsarcosine, 0.1% NaCl, 0.02% SDS) containing the appropriate concentration of formamide were mixed in a 1.5-ml centrifuge tube. The hybridization mixture was incubated for 10 min at 70°C and then for 30 min at room temperature (30). The probe (10 to 25 pmol) was added to the hybridization mixture at five times the molar concentration of the target 16S rRNA and incubated overnight at 20°C on a rotator (Stuart SB3; Dynalab, Rochester, NY). This ratio between probe and target gave the highest recovery (data not shown). Two types of beads with either a hydrophilic (Dynabeads M-280 Streptavidin; Invitrogen, Carlsbad, CA) or a hydrophobic (Dynabeads MyOne Streptavidin T1; Invitrogen) surface were tested. Aliquots (25 μl per reaction tube) of beads were rinsed three times with an equal volume of 0.5× SSC using a magnetic-particle concentrator (Dynal MPC-S; Invitrogen). The rinsed beads were resuspended in 0.1% blocking-reagent solution (Roche Applied Science, Mannheim, Germany) and incubated for 1 h on a rotator. The blocking-reagent solution was removed using a magnetic-particle concentrator. The beads were resuspended in 0.5× SSC, and 25 μl was dispensed per reaction tube and incubated with probe-target hybrid at room temperature on a rotator for 2 h (3, 26). The concentration of the beads was 10 mg/ml, and the binding capacity of the beads was 400 pmol/mg (according to the manufacturer), which was approximately four times more than the amount of probes added in the protocol. The magnetic-particle concentrator was used to collect the beads on the tube wall, and the hybridization mixture containing the RNA not bound to the beads was removed. The beads were rinsed three times with 7.5× SSC, and the captured 16S rRNA was eluted in Milli-Q water (Millipore, Billerica, MA) at 90°C for 3 min (25). The eluted 16S rRNA was separated from the beads with the magnetic-particle concentrator. The isolated 16S rRNA was precipitated with 1 volume of isopropanol and 0.2 volume of 7.5 M ammonium acetate and finally dissolved in Milli-Q water. The captured 16S rRNA was visualized and quantified as described previously.

Magnetic-bead capture hybridization of total-community RNA from sediment. Magnetic-bead capture was performed as described above, except that different amounts of RNA extract and beads were used and the blocking-reagent treatment and 16S rRNA precipitation steps were modified to minimize protocol blanks (see Results). Total-community RNA extract (40 μl containing 20 to 40 μg RNA) and 360 μl of hybridization buffer (5× SSC, 0.1% *N*-laurylsarcosine, 0.1% NaCl, 0.02% SDS) containing the appropriate formamide concentration (Table 1) were mixed per 1.5-ml centrifuge tube and then incubated for 10 min at 70°C and for 30 min at room temperature. After overnight hybridization with the probes (30 to 50 pmol) at 20°C on a rotator, 100 μl of beads that had been rinsed two times after blocking-reagent treatment were dispensed into each reaction tube and incubated for 2 h. The probe was added at five times the molar concentration of the target 16S rRNA, which was estimated from the amount of the total-community RNA and the proportion of target 16S rRNA clones among the total-community clones. Eluted 16S rRNA was precipitated two times by 1 volume of isopropanol and 0.2 volume of 3 M NaCl and finally dissolved in Milli-Q water that was not treated with diethyl pyrocarbonate (DEPC). DEPC resulted in high protocol blanks. Between 300 and 600 ng C of captured 16S rRNA was pooled for isotope ratio measurement. Captured 16S rRNA was freeze-dried and dissolved in freshly prepared Milli-Q water shortly before analysis by μEA-IRMS. Protocol blanks with no RNA extract were also prepared by the same protocol. A small fraction of the captured 16S rRNA was used for phylogenetic analysis.

^{13}C analysis of captured 16S rRNA. Isotope ratio analysis was performed by μEA-IRMS consisting of a wet-oxidation interface (LC IsoLink; Thermo Fisher Scientific) coupled on-line to an isotope ratio mass spectrometer (Delta V Advantage; Thermo Fisher Scientific, Bremen, Germany) (20). Samples (50 μl containing 300 to 600 ng C of RNA) were directly injected into this μEA-IRMS operating in bulk injection mode. Standard curves were made with phthalic acid ranging from 0 to 1,000 ng of carbon. Linearity for ^{13}C-enriched materials was previously tested (7). Stable carbon isotope ratios were expressed as δ^{13}C values calibrated against the international standard Vienna Pee Dee Belemnite. The delta notation is defined as follows:

$$\delta^{13}C_{sample}(‰) = [(R_s/R_{st}) - 1] \times 1{,}000 \quad (1)$$

where R_s is the ratio of ^{13}C in the sample and R_{st} is the ratio of the international standard VPDB (0.0111797). The measured δ^{13}C values were corrected for the protocol blank as follows:

$$\delta^{13}C_{RNA}(‰) = \left[\frac{(\delta^{13}C_{sample} \times C_{sample}) - (\delta^{13}C_{blank} \times C_{blank})}{C_{sample} - C_{blank}}\right] \quad (2)$$

where $\delta^{13}C_{sample}$ is the δ^{13}C value of the sample, C_{sample} is the amount of carbon in the sample, $\delta^{13}C_{blank}$ is the δ^{13}C value of the blank, and C_{blank} is the amount of carbon in the blank (5).

Clone libraries of total and captured 16S rRNA. To check the specificity of the capture protocol for each probe, aliquots (100 to 200 ng) of captured 16S rRNA from unlabeled sediment samples and total-community RNA were reverse transcribed with reverse primer DXR518 (5′-CGTATTACCGCGGCTGCTGG-3′) (35) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The cDNA was amplified with 10 cycles of PCR using PCR primers 27F and DXR518 (29, 31). The PCR products were quantified and ligated into the pGEM T-easy vector and transformed into *E. coli* JM109 competent cells (Promega, Madison, WI). Positive clones were reamplified with M13 primers and sequenced with the 27F primer on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence chromatographs were analyzed using ChromasPro software version 1.3.3.0 (http://www.technelysium.com/au/chromas.html). Phylogenetic analysis was performed by using the fast aligner and treeing tools implemented in the ARB program package (23). Phylogenetic relationships were determined by inserting sequences from this study into an ARB tree composed of the Greengenes database (http://greengenes.lbl.gov/) to which additional *Deltaproteobacteria* clone sequences from marine environments had been added. The final bootstrapped neighbor-joining tree with 1,000 samplings was created in MEGA4 (46) using the Jukes-Cantor model.

Nucleotide sequence accession numbers. Nucleotide sequences obtained from total-community, EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNAs have been deposited in the GenBank/DDBJ/EMBL database under accession numbers FJ787045 to FJ787299.

RESULTS

Modification of the Mag-SIP protocol for μEA-IRMS. The detection limit for ^{13}C analysis is mainly determined by the

sensitivity of the equipment used for isotopic analysis and the amount of blank carbon added during sample preparation (protocol blank). The sensitivity of the ^{13}C measurements in terms of the amount of carbon needed for accurate analysis was examined with a standard curve of phthalic acid. Approximately 50 ng C was required for an accurate isotope measurement using a μEA-IRMS, which is similar to other studies (20). However, this low detection limit also meant that the capturing protocol blank had to be decreased substantially. The previously published assay gave a high protocol blank of approximately 300 ng of carbon. In order to lower blanks from the magnetic-bead capture protocol, we examined the carbon carryover and its $\delta^{13}C$ values for the different steps in the protocol. We concluded that the blocking reagent, the ammonium acetate and alcohol in the final RNA precipitation step, DEPC treatment of Milli-Q water, and the type of vial used for the final step in the protocol all contributed to the protocol blank. Glass vials generally resulted in high protocol blanks, as did several types of plastic centrifuge tubes (70 to 150 ng C). Standard 1.5-ml centrifuge tubes from Greiner Bio-One (Frickenhausen, Germany) gave the lowest protocol blank (approximately 15 ng C) and were used throughout the protocol. Protocol blanks were also lowered by approximately 200 ng C by including an additional rinsing step of blocking-reagent-treated beads with 0.5× SSC, by use of sodium chloride as the salt for RNA precipitation instead of ammonium acetate, by an additional rinse of RNA pellets with 70% ethanol, and by subsequently freeze-drying the RNA pellets. Finally, freshly prepared Milli-Q water not treated with DEPC was used to dissolve the captured 16S rRNA because DEPC treatment contributed approximately 20 ng C of protocol blank (26). The exclusion of DEPC might have led to some rRNA fragmentation, but this was not evident from the gels, and μEA-IRMS signals would not be influenced by fragmentation. Milli-Q water without DEPC treatment was added shortly before ^{13}C analysis, which also decreased the blank from air CO_2 dissolved in the sample (3 to 9 ng C after 1 day). The standard protocol for removing CO_2 by acidification and purging with helium could not be used because the rRNA was precipitated by the acid (data not shown). As a result of these modifications, the protocol blank dropped to 40 to 50 ng C per 50-μl injection, which is close to the detection limit of the μEA-IRMS. A minimum of approximately 50 ng C of captured 16S rRNA was required for isotope ratio measurements. Consequently, Mag-SIP using μEA-IRMS required about 10 times less 16S rRNA for isotope ratio measurements than Mag-SIP using standard EA-IRMS. In the present study, this improvement in sensitivity enabled us to use Mag-SIP for phylogenetic groups contributing 20% of the cDNA library, but in our experience we can go down to as little as 2 to 5% for similar types of samples.

Testing probe stringency and specificity. The binding strength of each probe was examined by varying the formamide concentration between 0 and 60%. From the result of hybridization with 16S rRNA of *D. multivorans*, a target of all the probes, the initial formamide concentrations for EUB338, DELTA495a, and Dbact653 were determined to be 25, 40, and 25%, respectively. Subsequently, specificity at these formamide concentrations was tested against *Desulfovibrio* sp. strain SB1, which is a target of EUB338 and DELTA495a but not Dbact653. 16S rRNA of *Desulfovibrio* sp. strain SB1 was captured by EUB338 and DELTA495a at the relative band intensities of 13.1 and 9.3%, respectively, but not by Dbact653 (0%) (Fig. 2A). Moreover, initial formamide concentrations were also tested against *E. coli*, which is targeted by EUB338 but not by the other probes. 16S rRNA of *E. coli* was captured by EUB338 but not by Dbact653. The DELTA495a probe has only one mismatch in the target region of *E. coli*, and a faint band of DELTA495a-captured material was recognized on the gel at 40% formamide. The DELTA495a probe was further tested against *E. coli* at 40, 45, and 50% formamide. Captured material was recognized at 40%, but not at 45 and 50% (Fig. 2B). Hence, the formamide concentration for DELTA495a was increased to 45%.

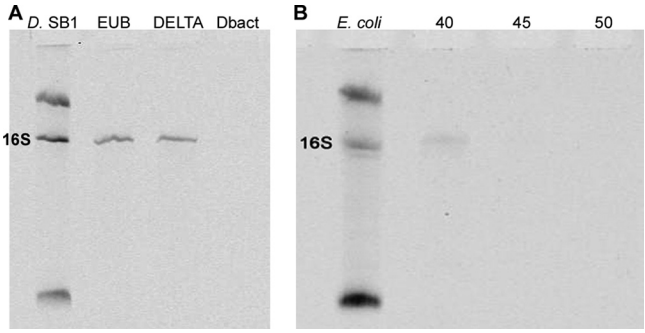

FIG. 2. Relative band intensities of the probe-captured 16S rRNA from pure cultures. (A) *Desulfovibrio* sp. strain SB1 16S rRNA captured by EUB338, DELTA495a, and Dbact653 (lanes EUB, DELTA, and Dbact, respectively) on a 5% acrylamide gel. Lane *D.* SB1 contained total RNA extracted from *Desulfovibrio* sp. strain SB1 pure culture. The relative capture efficiencies were 13.1, 9.3, and 0%, respectively. Note that *Desulfovibrio* sp. strain SB1 is not a target of Dbact633. (B) *E. coli* 16S rRNA captured by DELTA495a (one mismatch) on a 5% acrylamide gel. The formamide concentration was tested at 40, 45, and 50% (lanes 40, 45, and 50, respectively). Lane *E. coli* contained total RNA extracted from *E. coli* pure culture.

In order to further check the target specificity of the probes for sediment rRNA extracts, clone libraries derived from the total-community RNA and the 16S rRNA captured by each probe were examined. Although the result may not be quantitative, this provided a final check of capture specificity for real samples containing a variety of rRNAs. Initially we used hydrophilic magnetic beads as described in the original protocol. However, cyanobacterium/chloroplast-like clones were frequently observed in clones of Dbact653-captured rRNA (11 to 14% of the clones). The capturing of these cyanobacterium/chloroplast-like clones was unexpected, as they had four to five mismatches in the target region of the probe, suggesting that there may be nonspecific binding of rRNA directly to the bead surface. We therefore tested beads with a hydrophobic surface, which also have a higher specific binding capacity, as the capturing efficiency is in general less than with hydrophilic beads. With these hydrophobic beads, the frequency of the cyanobacterium/chloroplast-like clones was decreased to less than 2% of the clones in Dbact653-captured rRNA. Hence, hydrophobic beads were used in further experiments.

For the total-community RNA, *Desulfobacteraceae* accounted for approximately 20% of the clones and other *Deltaproteobacteria* were relatively rare (3%) (Fig. 3). Cyanobacteria/chloroplast and *Gammaproteobacteria* clones were also major groups. The

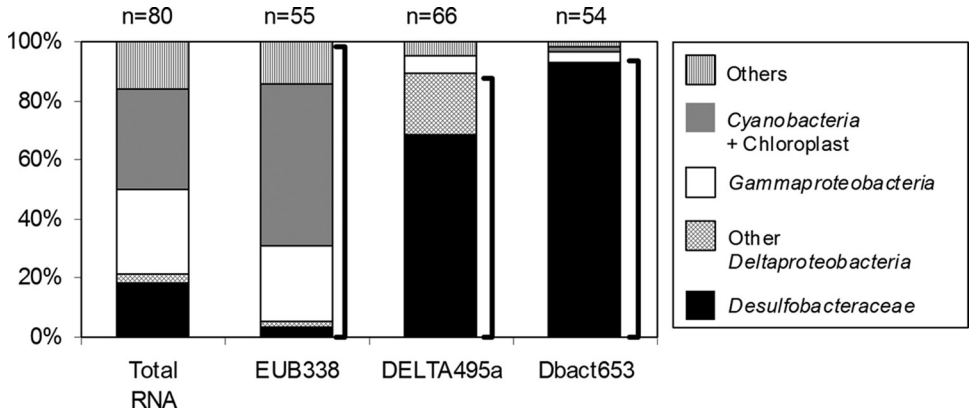

FIG. 3. Frequencies of clones obtained from total-community RNA and 16S rRNA captured with specific probes. The total numbers of clones sequenced are indicated as n. The bracket beside each probe bar indicates the expected target range of the probe. DELTA495a was used in combination with its competitor, and helper probes were used with Dbact653.

frequencies of *Desulfobacteraceae* and other *Deltaproteobacteria* in EUB338-captured rRNA appeared to be lower than in total-community RNA, which may be due to the limited number of clones sequenced. The specificity of the DELTA495a probe (in combination with the competitor probe) was very good, with 90% of the clones belonging to the target group. Most clones captured by DELTA495a were affiliated with the *Desulfobacteraceae*, and the remainder mainly belonged to a variety of other *Deltaproteobacteria* (Fig. 3 and 4). More than 93% of the clones captured by Dbact653 in combination with helper probes were indeed affiliated with the *Desulfobacteraceae* (Fig. 3 and 4). These results showed that specificity should be evaluated on actual samples, as well as pure cultures, and that the final protocol was highly specific.

^{13}C incorporation into captured 16S rRNA. To examine label incorporation into 16S rRNA from different labeled substrates, the δ^{13}C values of captured 16S rRNA were measured by μEA-IRMS. Sediment samples were incubated for 24 h with labeled [^{13}C]glucose, [^{13}C]propionate, or [^{13}C]acetate. Unlabeled control cores were also incubated under the same conditions. Approximately 10 μg of total-community RNA was extracted from 1 g (dry weight) sediment. In order to get sufficient amounts of captured 16S rRNA, 5 to 10 g (dry weight) sediment per assay was used for EUB338 captures and 15 to 20 g for other probes. Between 300 and 600 ng C of captured 16S rRNA was used per isotope ratio measurement, well above the detection limit of μEA-IRMS.

Figure 5 illustrates the increase in the δ^{13}C ratios between labeled sediments and unlabeled control sediments ($\Delta\delta^{13}$C) for the different captured 16S rRNA fractions. Unlabeled controls had δ^{13}C values between −15 and −20‰, within the typical range for marine heterotrophic bacteria (6, 9). Labeling with glucose was higher than with propionate, and labeling decreased going from *Bacteria* to *Desulfobacteraceae* 16S rRNA. In contrast, ^{13}C incorporation of propionate was about two times higher in *Desulfobacteraceae* than in all *Bacteria*. Together, these results suggest that *Desulfobacteraceae* incorporated more propionate than other members of the community and that they were relatively less important for glucose incorporation. Incorporation with acetate was even lower than with propionate, and there were no significant differences in acetate incorporation among the probes.

DISCUSSION

Several culture-independent approaches have been developed to identify the major microbial groups responsible for environmental processes. SIP based on PLFA, DNA, or RNA (8, 27, 40) has become an attractive method in recent years. However, each of these SIP methods has inherent limitations in terms of phylogenetic resolution or ^{13}C sensitivity. The major advantage of Mag-SIP is that it combines excellent phylogenetic resolution through specific probes with the highest possible ^{13}C sensitivity through IRMS analysis. It has been shown that Mag-SIP can be applied to study carbon sources used by bacteria by studying small variations in natural ^{13}C abundance (this paper and reference 37). Another advantage of Mag-SIP is its independence from PCR bias (1, 39). In this paper, we improved the Mag-SIP protocol by substantially lowering the amount of rRNA needed through μEA-IRMS analysis and by lowering the carbon blank from the sample preparation protocol. As a consequence of these modifications, the protocol blank dropped to 40 to 50 ng C per sample and about 50 ng C of captured 16S rRNA was sufficient for isotope ratio analysis. This detection limit was an order of magnitude lower than in previous Mag-SIP studies that used traditional EA-IRMS (25, 26). Similar sensitivities were obtained by Pearson et al. (37), who used a spooling-wire interface to determine natural stable isotope ratios in total bacterial 16S rRNA. These improvements enabled us for the first time to apply Mag-SIP at family level resolution, and we demonstrated the method in an experiment on substrate utilization by sulfate-reducing *Deltaproteobacteria* in anoxic marine sediment.

We tested a nested set of three biotin-labeled oligonucleotide probes with a stepwise narrowing of the target from *Bacteria* to *Deltaproteobacteria* and further to *Desulfobacteraceae*. By changing the hybridization stringency and including unlabeled helper and competitor probes, the protocol enabled the isolation of target rRNA at a high specificity of 90% or more from complex environmental samples. An advantage of the probe capture technique not shared by many other probe-

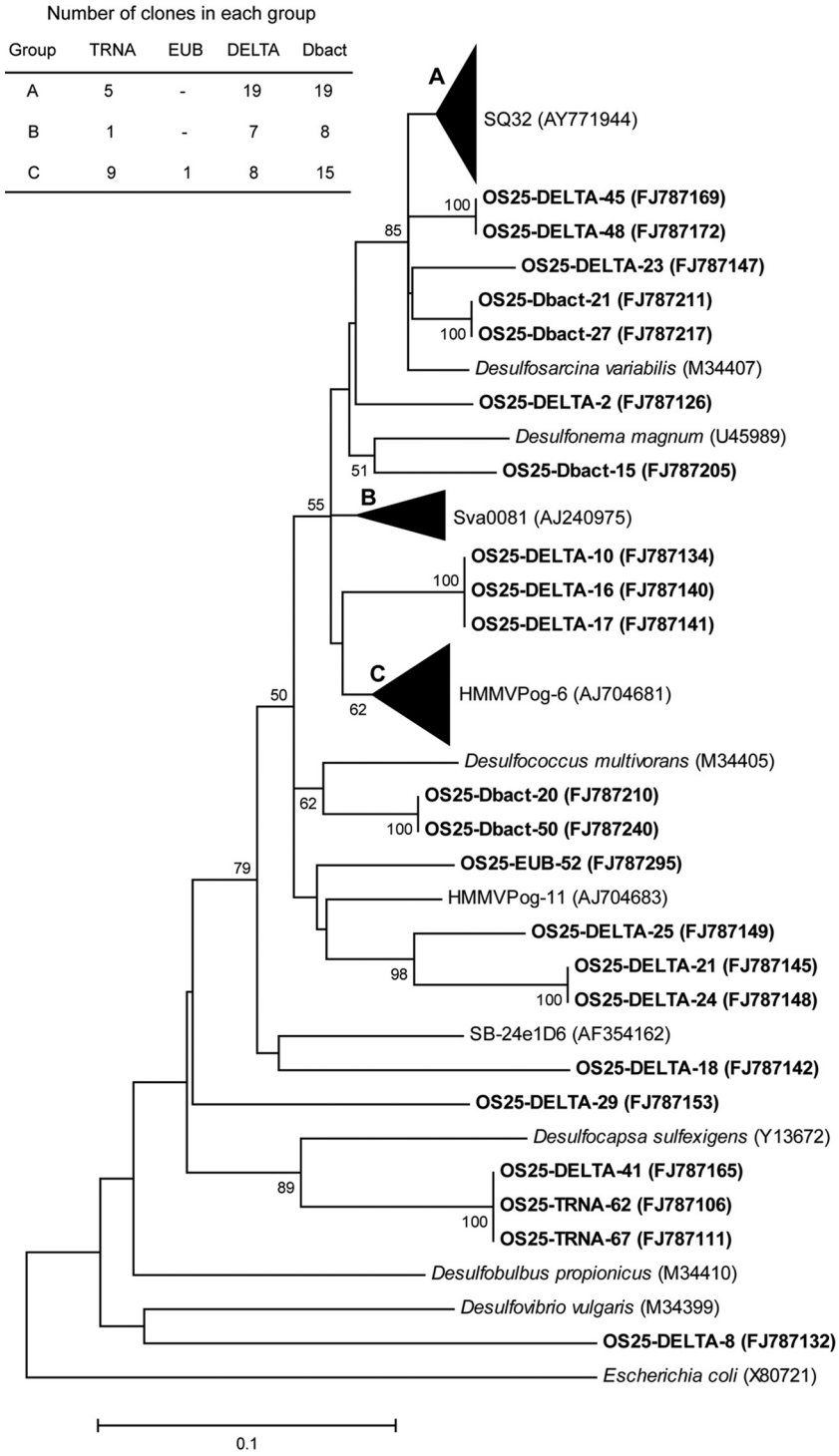

FIG. 4. Neighbor-joining tree showing the affiliations of clones with selected sequences of the *Deltaproteobacteria*. The bootstrap values represent 1,000 replicates, and only values greater than 50% are reported. Clone sequences from this study are in boldface. Clones with designations containing TRNA, EUB, DELTA, and Dbact are derived from total-community RNA and EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNA, respectively. The numbers in parentheses are accession numbers. *E. coli* was used as the outgroup. The scale bar indicates 10% estimated phylogenetic divergence. For each group, a representative environmental clone is indicated. The table lists the numbers of clones derived from total-community rRNA and EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNA affiliating with each group.

based methods is that the phylogenetic composition of the isolated rRNA can be further studied. In general, the cDNA clone libraries of rRNA captured with the more specific probes were a representative subset of those captured with the more general probes (Fig. 4). However, the composition of the clone library from the EUB338-captured material appeared to be different from the total RNA clone library. Some of these differences were expected, as the EUB338 probe does not

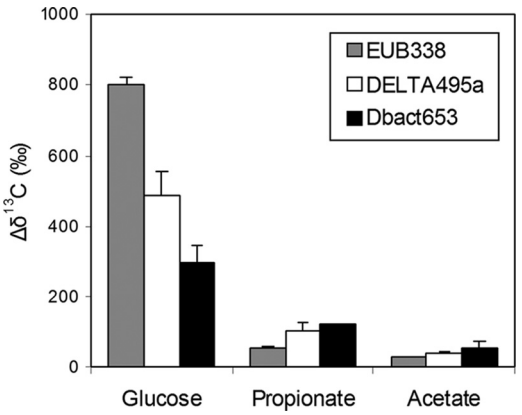

FIG. 5. $\Delta\delta^{13}C$ ratios of captured 16S rRNA from sediments incubated with [^{13}C]glucose, [^{13}C]propionate, or [^{13}C]acetate. Total-community RNA was captured using EUB338, DELTA495a, and Dbact653 probes and analyzed by μEA-IRMS. DELTA495a was used in combination with its competitor, and helper probes were used with Dbact653. Note that differences in $\delta^{13}C$ values between duplicate analyses of unlabeled controls were less than 2‰ and therefore all treatments were significantly labeled.

target *Planctomycetes* (34), which were present in the cDNA library but not in the EUB338 library. This could be circumvented by including other versions of the EUB338 probe that target these groups (10). The abundance of *Deltaproteobacteria* in the EUB338-captured clones was lower than expected from the cDNA library (Fig. 3 and 4). Although most *Deltaproteobacteria* are targeted by the EUB338 probe, this effect could be due to either differences in accessibility of the probe to the binding site or to PCR and cloning artifacts (1, 39), including a stochastic effect, as a relatively low number of clones were sequenced for each clone library. For the Mag-SIP protocol, the newly developed *Desulfobacteraceae* probe Dbact653 showed much better specificity than the commonly used DSS658 probe, which targets a similar range of organisms (28). The basic idea behind the application of this nested set of probes was that an increase in labeling ($\Delta\delta^{13}C$ ratios) with decreasing target range would indicate that the target belonged to a ^{13}C substrate-utilizing member of the microbial community.

To demonstrate the Mag-SIP protocol, we applied it to a study of substrate utilization by SRB in marine sediment. SRB play an important role in the final degradation steps during anaerobic organic matter mineralization in marine sediments (18). They are thought to mainly use fermentation products, such as propionate and acetate, produced by fermenting bacteria, whereas direct consumption of more complex substrates, such as glucose, is generally very limited (47). The three labeled substrates tested, namely, [^{13}C]glucose, [^{13}C]propionate, and [^{13}C]acetate, indeed resulted in differential labeling among rRNAs captured by the three nested probes, suggesting that these substrates were mostly specifically utilized by subgroups within the microbial community. The incorporation was mainly compared within a substrate, as it was not known if yields were the same for each substrate or if all substrate had been consumed completely. With [^{13}C]glucose, the decreasing trend in labeling levels with decreasing target range suggested that *Deltaproteobacteria*, and especially *Desulfobacteraceae*, were not the main consumers of glucose. Some labeling was, however, detected in *Desulfobacteraceae*. Although we cannot directly reject the possibility that some members of the *Desulfobacteraceae* were actively using glucose, this labeling was most likely due to uptake of fermentation products produced by other glucose-utilizing species. We cannot completely disregard the less likely possibility that some of the detected labeling was due to nonspecific rRNA binding (<10% of the total captured). This potential problem may be circumvented by further increasing the stringency at the cost of rRNA recovery or by using a more complete probe set targeting all main groups in the community. In contrast to glucose, the increase in labeling from total bacterial rRNA to *Desulfobacteraceae* with propionate, and to a lesser degree with acetate, showed that *Desulfobacteraceae* were the major players in the consumption of these fermentation products. Both propionate and acetate are considered important substrates for SRB, including the *Desulfobacteraceae* in marine sediments (38, 44). Several dominant clades among the *Desulfobacteraceae* related to *Desulfosarcina* are typically detected in sediment clone libraries (Fig. 4) (33, 41). By using Mag-SIP with specific probes for these clades, it should be possible to further determine whether they play different roles in carbon mineralization. In general, the results of the labeling study are in agreement with the predicted roles of sulfate-reducing *Deltaproteobacteria* in marine sediments.

We demonstrated the utility of Mag-SIP using μEA-IRMS to link microbial community function to phylogeny at the family level. While Mag-SIP is a target-based approach developed to study the substrate ranges of important environmental phylogenetic groups, the approach can also be used to study the roles of different groups by applying a nested design of probes, as in this study. Although the improvement of the protocol in this study substantially lowered the amount of sample needed, Mag-SIP is still best suited for high-biomass samples, such as active sediments, soils, and bioreactors. Improving the sensitivity of the Mag-SIP protocol further, both in terms of the amount of rRNA and the amount of ^{13}C label needed, will be difficult, as a certain amount of carbon is needed for an accurate ^{13}C analysis by IRMS and the transfer of carbon from the sample to the IRMS is already highly efficient (20). However, an LC-IRMS-based detection method for rRNA or its constituents instead of the bulk analysis performed in this study may further decrease protocol blanks, as rRNA will be separated from contaminants by chromatography (Application Note 30055; Thermo Scientific, Bremen, Germany). Further improvement of the method would be in the development of a more comprehensive probe set to target a wider range of dominant bacteria within microbial communities, and the 23S rRNA with its higher phylogenetic resolution may also provide interesting options (16). Other interesting possibilities lie in the combination of Mag-SIP with novel single-cell methods, such as fluorescence in situ hybridization coupled with Raman microscopy (15) or with nanometer scale secondary-ion mass spectrometry (4). Although the proportion of rRNA from particular organisms among total-community RNA may not always represent their actual population sizes (12, 19), this method may allow us to identify active groups in microbial communities and to perform food web studies with limited disturbance of the community structure.

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