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Stable Isotope Probing with $^{15}N_2$ Reveals Novel Noncultivated Diazotrophs in Soil[▽]

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Biological nitrogen fixation is a fundamental component of the nitrogen cycle and is the dominant natural process through which fixed nitrogen is made available to the biosphere. While the process of nitrogen fixation has been studied extensively with a limited set of cultivated isolates, examinations of *nifH* gene diversity in natural systems reveal the existence of a wide range of noncultivated diazotrophs. These noncultivated diazotrophs remain uncharacterized, as do their contributions to nitrogen fixation in natural systems. We have employed a novel $^{15}N_2$-DNA stable isotope probing ($^{5}N_2$-DNA-SIP) method to identify free-living diazotrophs in soil that are responsible for nitrogen fixation in situ. Analyses of 16S rRNA genes from ^{15}N-labeled DNA provide evidence for nitrogen fixation by three microbial groups, one of which belongs to the *Rhizobiales* while the other two represent deeply divergent lineages of noncultivated bacteria within the *Betaproteobacteria* and *Actinobacteria*, respectively. Analysis of *nifH* genes from ^{15}N-labeled DNA also revealed three microbial groups, one of which was associated with *Alphaproteobacteria* while the others were associated with two noncultivated groups that are deeply divergent within *nifH* cluster I. These results reveal that noncultivated free-living diazotrophs can mediate nitrogen fixation in soils and that $^{15}N_2$-DNA-SIP can be used to gain access to DNA from these organisms. In addition, this research provides the first evidence for nitrogen fixation by *Actinobacteria* outside of the order *Actinomycetales*.

Nitrogen fixation is an ancient microbial process which evolved early in the history of our planet and is of central importance to the biosphere. All known forms of life require fixed N for biosynthesis, and microbial N fixation provides the largest natural source of fixed N in the biosphere, accounting for the production of 100 to 290 Tg N yr^{-1} in terrestrial systems alone (7). Free-living diazotrophs in soils provide the dominant natural source of fixed N in many of these terrestrial systems (7), and yet we still have much to learn about the ecology and evolution of these organisms. Nitrogenase *nifH* sequences can be divided into 49 different subgroups (49). Twenty-two of these subgroups do not contain any cultivated representatives, and many of the remaining subgroups contain only one or a few members that have been cultivated successfully (49). Surveys of nitrogenase diversity in soil commonly reveal sequence types that correspond to diverse unidentified diazotrophs (6, 32, 33, 40, 45, 47), and these noncultivated diazotrophs, rather than their cultivated cousins, may be the dominant N-fixing organisms in soil systems (13, 33, 38, 44, 47). Currently, noncultivated diazotrophs can be identified only through detection of their *nifH* gene sequences, and since the phylogeny of the *nifH* gene does not consistently correspond with organismal phylogeny (36), *nifH* gene sequences on their own provide limited information with which to identify and characterize novel diazotrophs. $^{15}N_2$-DNA stable isotope probing ($^{15}N_2$-DNA-SIP) can be used to link particular 16S rRNA genes to the process of nitrogen fixation as it occurs in the soil and should provide a valuable technique for characterizing noncultivated diazotrophs in a range of environments. Through $^{15}N_2$-DNA-SIP, it may be possible to link 16S rRNA genes from noncultivated diazotrophs to their corresponding *nifH* genes, and this method may also provide a source of genome fragments that can be used to help to characterize noncultivated diazotrophs, their gene systems, and their ecological significance.

While nucleic acid SIP provides a useful tool for characterizing microbial activity under in situ conditions the method has notable limitations (10, 25, 34, 35). One limitation is the need to add labeled substrates at concentrations that are substantially higher than those typically experienced by cells in situ. Elevated substrate addition is required because cells will assimilate substrates from both native and labeled sources, resulting in the dilution of an isotopic label in the receiving community (34). Another problem encountered when performing SIP experiments is that low in situ growth rates may require prolonged incubations to permit sufficient labeling of nucleic acids. During prolonged incubations, cross-feeding and trophic cascades can result in the movement of an isotopic label into nucleic acids from nontarget functional groups (10, 17, 22, 23, 27, 28, 51). As a result, DNA from isotopically enriched environmental samples can contain a range of isotopic signatures, from 0 to 100% label incorporation. Several strategies have been developed to deal with these issues, and each requires the collection and analysis of gradient fractions and DNA fingerprinting in order to determine the degree of isotope incorporation into DNA from particular microbial groups (21, 24, 25). As a natural consequence of label dilution, the isotopic signature of nucleic acids from organisms involved in cross-feeding or secondary consumption should be less than that of primary consumers unless and until the isotopic label saturates the community (27, 28, 51). Thus, by following the

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incorporation of the isotopic label into the community over time and in comparison to control treatments that receive unlabeled substrates, it is possible to track the movement of the label from a substrate into particular functional groups and then into other components of the soil food web (10, 21).

There are several reasons why $^{15}N_2$-DNA-SIP represents an appealing method for examining nitrogen-fixing organisms. First, incubations can be carried out at realistic substrate concentrations, since atmospheric N_2 can be completely replaced with simulated air containing $^{15}N_2$. Second, since nitrogen fixation is inhibited in the presence of mineral forms of nitrogen (5, 9), isotope dilution is likely to be less of a problem with $^{15}N_2$ than with ^{13}C-labeled substrates. Third, cross-feeding should be less of a problem, since the majority of N fixed by free-living diazotrophs is immobilized in microbial biomass (42), though turnover of microbial biomass may still result in subsequent secondary utilization of ^{15}N-labeled compounds. The challenge associated with ^{15}N-DNA-SIP is that DNA contains less than half as much N as C and thus the change in density associated with ^{15}N-labeled DNA (0.016 g ml^{-1}) (1) is smaller than can be achieved for ^{13}C-labeled DNA. In addition, natural variation in genome G+C content can affect the native buoyant density of DNA and obscure the effects of isotope incorporation (21, 24, 35). The native buoyant density of DNA in CsCl gradients varies by as much as 0.05 g ml^{-1} over the range of genome G+C contents that occurs in complex communities (15, 30, 37). We have developed a method that makes it possible to disentangle the effects of isotope incorporation and genome G+C content on DNA buoyant density in DNA-SIP experiments, and this method makes it possible to perform ^{15}N-DNA-SIP with complex microbial communities (2). The objective in this study was to apply this ^{15}N-DNA-SIP method to nitrogen-fixing communities in soil by using $^{15}N_2$ as a labeled substrate, to demonstrate that ^{15}N-DNA-SIP can be used to study natural communities that possess low rates of nitrogen fixation, and to identify and characterize novel diazotrophs from soil that are engaged in nitrogen fixation in situ.

MATERIALS AND METHODS

Soil microcosm experiment. Soil was collected from a plot on Caldwell Field (Ithaca, NY) that has been maintained as a fallow for more than 30 years. A transect was established across the plot, and three samples were taken at 15-m intervals. Each soil sample consisted of five soil cores (2.5 cm diameter and 5 cm deep) taken within a 1-m^2 area and pooled. Within several hours of sampling, soils were sieved to 4 mm and then a 10-g portion of each sample was placed into 25-ml Balch tubes (Bellco Glass). Tubes were sealed and evacuated, and then the atmosphere was replaced with synthetic air containing 20% O_2 and 80% N_2. All samples were run in duplicate, with half receiving $^{15}N_2$ containing 99.8 atom% ^{15}N (Isotec) and half receiving unlabeled N_2. Tubes were incubated horizontally in the dark at 30°C for 28 days.

Net nitrogen fixation was determined by relating the ^{15}N enrichment of bulk soil for samples receiving $^{15}N_2$ relative that for to parallel controls which received unlabeled N_2. This approach controls for the effects of isotopic fractionation which may occur during incubation as the result of gaseous N loss due to nitrification and denitrification. Soil ^{15}N enrichment was determined by using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation).

DNA was extracted from the soil sample that showed the greatest fixation of $^{15}N_2$ and from its corresponding control. DNA was extracted from four samples of 0.25 g using the UltraClean Soil DNA extraction kit (MoBio, Inc.) as per the manufacturer's instructions, and these DNA extracts were subsequently pooled. DNA was further purified by electrophoresis through a 1% agarose gel to remove DNA fragments smaller than 4 kbp. DNA of greater than 4 kbp was excised from the gel, agarose removed by digestion with agarase (New England Biolabs) as per the manufacturer's instructions, and DNA obtained by ethanol precipitation as described previously (39). A total of 1.8 μg g^{-1} of DNA was obtained for the $^{15}N_2$-enriched soil, and 1.8 μg g^{-1} of DNA was also obtained for the control soil, as determined by analysis of subsamples with the Quant-iT PICO Green dsDNA assay (Invitrogen) as per the manufacturer's instructions.

CsCl density fractionation. CsCl gradient fractionation was carried out as described previously (2). Briefly, primary CsCl gradients were formed by filling 4.7-ml polyallomer Optiseal tubes (Beckman) with 4.3 ml of gradient buffer (15 mM Tris-HCl, 15 mM KCl, 15 mM EDTA, pH 8.0) and 0.45 ml of DNA (1.8 μg) in TE buffer (50 mM Tris-HCl, 15 mM EDTA, pH 8.0) to obtain a homogeneous CsCl density of 1.69 g ml^{-1}. Centrifugation was carried out for 66 h at 55,000 rpm (164,000 × *g* maximum) and 20°C in an Optima Max-E tabletop ultracentrifuge (Beckman-Coulter) equipped with a TLA110 rotor. A fraction recovery system (Beckman) was used to collect 45 fractions of 100 μl from each CsCl gradient, and the density of each fraction was determined by measurement of refractive index using an AR200 digital refractometer (Reichert). DNA of two fractions with buoyant density of 1.727 to 1.733 g ml^{-1} from primary gradients was resolved in secondary CsCl gradients containing bis-benzimide to disentangle the effects of isotope incorporation from genome G+C content as described elsewhere (2). These secondary CsCl gradients were prepared as described above, with the exception that 8 μl of 10-mg ml^{-1} bis-benzimide (Hoechst no. 33258; Sigma-Aldrich) was added to the DNA samples during the preparation of gradient media. Bis-benzimide intercalates into DNA at A-T base pairs, altering the hydration state of DNA and causing a decrease in buoyant density that is inversely proportional to DNA G+C content (15). Thus, secondary gradients containing bis-benzimide cause the separation of unlabeled DNA with high G+C content from isotopically labeled DNA of the same buoyant density (2).

Analysis of CsCl gradient fractions. CsCl was removed from DNA by ethanol precipitation, and DNA was resuspended in 25 μl of 50 mM Tris-HCl, pH 8.0, and stored at −20°C. The distribution of DNA in CsCl gradients was determined by using either the Quant-iT PICO Green dsDNA assay (Invitrogen) or quantitative PCR as described previously (2). Briefly, quantitative PCR was conducted with primers Bact519F (5′-CAG CMG CCG CGG TAA NWC-3′) and Bact907R (5′-CCG TCA ATT CMT TTR AGT T-3′), which target bacterial 16S rRNA genes as described previously (2, 43).

DNA from primary gradient fractions was also characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. Bacterial 16S rRNA genes were amplified by PCR using the primer Bact8F (5′-AGA GTT TGA TCM TGG CTC AG-3′), labeled at the 5′ end with the dye 6-carboxy-fluorescein, and the primer Univ1390R (5′-GAC GGG CGG TGT GTA CAA-3′). Reactions were carried out as described previously (2), PCR products were resuspended in 50 mM Tris-HCl (pH 8.0), 250 to 400 ng of this DNA was digested with MspI (New England Biolabs) in 30-μl reaction volumes as per the manufacturer's instructions, and the enzyme was subsequently inactivated by incubation at 65°C for 20 min. The digested PCR products were desalted and concentrated again and then resolved on an Applied Biosystems Automated 3730 DNA analyzer.

Analysis of 16S rRNA and *nifH* genes. Clone libraries of 16S rRNA and *nifH* genes were constructed with DNA obtained from targeted secondary gradient fractions for both enriched and control samples. PCR of 16S rRNA genes was carried out with primers Bact8F and Univ1390R in 50-μl volumes containing 5 μl of template DNA with each primer at a concentration of 0.3 μM, each deoxynucleoside triphosphate at a concentration of 50 μM, 0.05% Tween 20, 2.5 mM $MgCl_2$, 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 1× PCR buffer (supplied with *Taq* enzyme). Each PCR consisted of a 95°C hold for 5 min, followed by 35 cycles of 45 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final extension for 15 min at 72°C. PCR of *nifH* genes was conducted with primers nifH-b1 (5′-GGC TGC GAT CCC AAG GCT GA-3′) (4) and CDHPnif723R (5′-GAT GTT CGC GCG GCA CGA ADT RNA TSA-3′) (41), and the conditions were as described above except that the annealing temperature used was 60°C. PCR products were cloned into pCR4.0-TOPO using a TOPO-TA cloning kit for sequencing (Invitrogen Corp., Carlsbad, CA). Clones were screened by PCR with primers flanking the cloning site as per the manufacturer's instructions (Invitrogen) to identify inserts of the expected size. Initial sequencing of 16S rRNA genes was carried out with the primer Bact8F, and additional sequencing was carried out with primers for the M13F and M13R priming sites which flank the insertion site on pCR4.0 as per the manufacturer's instructions (Invitrogen). These 16S rRNA gene sequences were screened for the presence of chimeras with the Chimera Check algorithm (8) and by examining base-pair complementarily in 16S rRNA secondary structure.

Phylogenetic analyses were performed by using the programs ARB (2.5 ed.; O.

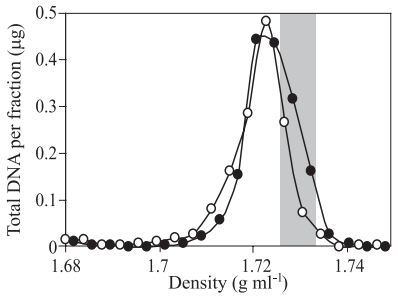

FIG. 1. DNA buoyant density was resolved in primary CsCl gradients, and total DNA was quantified in each gradient fraction. DNA was extracted from soil incubated in the presence of either artificial air (○) or artificial air containing 99.8 atom% $^{15}N_2$ (●). The shaded region corresponds to fractions of buoyant density of 1.727 to 1.733 g ml^{-1} which were selected for further analysis in secondary gradients containing bis-benzimide.

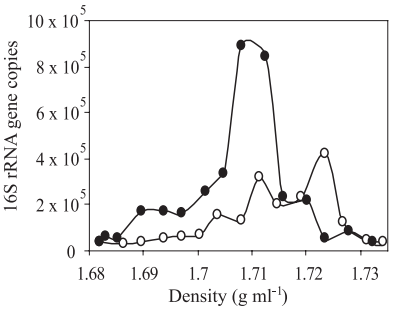

FIG. 2. Secondary CsCl gradients containing bis-benzimide were used to disentangle the effects of ^{15}N incorporation and G+C content on the buoyant density of DNA from targeted primary gradient fractions. The total number of 16S rRNA genes in each gradient fraction was determined by quantitative PCR. The secondary gradients were loaded with DNA that had a buoyant density of 1.727 to 1.733 g ml^{-1} in primary gradients. The symbols correspond to DNA from soil incubated in the presence either of artificial air (○) or of artificial air containing 99.8 atom% $^{15}N_2$ (●).

Strunk and W. Ludwig, Department of Microbiology, Technical University of Munich, Munich, Germany, 1997) and PHYLIP 3.64 (J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, 2005). The 16S rRNA gene sequences were initially aligned by using the ARB automatic aligner and then were verified and corrected manually. The *nifH* sequences were translated and aligned to the Pfam Fer4_nifH amino acid seed alignment (12). All *nifH* gene sequences available in GenBank were downloaded and likewise aligned to facilitate accurate estimation of *nifH* phylogeny relative to existing sequence groups. Regions of ambiguous alignment were identified and excluded from subsequent phylogenetic analyses. A total of 1,334 aligned 16S rRNA gene positions and 105 *nifH* amino acid positions were used in the construction of phylogenetic trees. Phylogenetic trees were generated by performing parsimony (D. L. Swofford, PAUP, 3.0 ed., 1991; Illinois Natural History Survey, Champaign, IL) and maximum-likelihood analyses (31). During tree construction, the sequence composition of trees and outgroups was varied. Bootstrapping was performed using Phylip with 100 randomizations.

Nucleotide sequence accession numbers. The nucleotide sequences of the 95 16S rRNA gene clones described in this study and the 34 *nifH* sequences have been deposited in GenBank under accession numbers EF520944 to EF521038 and EF521039 to EF521072, respectively.

RESULTS

Soil microcosms labeled with $^{15}N_2$. Soil microcosms were incubated in synthetic air with atmospheric N_2 replaced by 99.8% atom-enriched $^{15}N_2$. Nitrogen fixation was assessed after 28 days by determining the change in soil ^{15}N enrichment relative to that for controls that were incubated in parallel. The soil had a $\delta^{15}N$ content of 2.1‰ ± 0.8‰ (mean ± standard deviation [SD]; $n = 3$) prior to incubation, and following incubation the $\delta^{15}N$ content of soil that received $^{15}N_2$ was 9.3‰ ± 8.9‰ (mean ± SD; $n = 3$) and that of control soils was −3.3‰ ± 1.5‰ (mean ± SD; $n = 3$). Thus, incubation conditions resulted in depletion of soil ^{15}N, but soils receiving $^{15}N_2$ were significantly enriched relative to controls ($P < 0.05$, Mann-Whitney U test; 12.6‰ mean difference), indicating significant nitrogen fixation in these samples. The total N content of the soil did not change significantly during the course of incubation and was 2.3 ± 0.6 mg N g^{-1} dry weight (mean ± SD; $n = 9$) across all samples. The sample with the greatest amount of nitrogen fixation had a $\delta^{15}N$ enrichment of 21.2‰ relative to controls, and this soil sample was selected for analysis by SIP. A nitrogen fixation rate of 0.48 nmol N g^{-1} day^{-1} would be required to generate a net $\delta^{15}N$ enrichment of 21.2‰ in this experiment, and when integrated over the top 10 cm of soil, this would represent approximately 0.2 kg N ha^{-1} month^{-1}. In addition, based on the total N content and $\delta^{15}N$ content of soil, it is possible to estimate that there was 203 ng ^{15}N g^{-1} soil, which corresponds to approximately 60 ng ^{15}N-DNA g^{-1} soil (based on the simplifying assumption that all fixed N is present in cell biomass and that a cell on average is composed of 12% N, has a total dry weight of 2.84×10^{-13} g, and contains 10×10^{-15} g DNA (29).

Effect of $^{15}N_2$ on buoyant density of DNA from soil. DNA was extracted from 1 g of the control or enriched microcosm soil, yielding 1.8 μg DNA from each sample. Primary CsCl gradient fractionation of these samples revealed a slight increase in the buoyant density of DNA in the $^{15}N_2$-enriched sample relative to the control sample (Fig. 1). Based on the observed buoyant density in primary gradients, DNA with a buoyant density of 1.727 to 1.733 g ml^{-1} (Fig. 1) was selected for further analysis. DNA in this density range was expected to have a G+C content of 66% to 75% if unlabeled and 51% to 58% if completely ^{15}N labeled (based on the established relationship between DNA G+C content and buoyant density in CsCl gradients and the expectation that the buoyant density of completely ^{15}N-labeled DNA will increase by 0.016 g ml^{-1} [1]). These primary fractions were pooled for each sample, and equal proportions were equilibrated in secondary gradients containing bis-benzimide (representing 190 ng DNA from the $^{15}N_2$-enriched gradient and 135 ng DNA from the control gradient). Following secondary gradient fractionation, the number of 16S rRNA genes present in each gradient fraction was determined by quantitative PCR (Fig. 2). In the presence of bis-benzimide, DNA with a G+C content of 66% to 75% was expected to decrease in buoyant density by 0.013 to 0.007 g ml^{-1} and DNA with a G+C content of 51% to 58% to decrease by 0.024 to 0.018 g ml^{-1} (14, 15). The dominant peak for the control sample occurred at a density of 1.723 g ml^{-1}, corresponding to a reduction of buoyant density by 0.010 to 0.004 g ml^{-1} in response to bis-benzimide (Fig. 2), consistent with unenriched DNA of high G+C content. The majority of DNA in the enriched sample was observed to have a buoyant density of 1.708 to 1.713 g ml^{-1}, which corresponds to a buoy-

ant density decrease of 0.025 to 0.014 g ml^{-1} in response to bis-benzimide (Fig. 2). Thus, the DNA in secondary fractions with densities of 1.708 and 1.713 g ml^{-1} from the enriched sample must be dominated by DNA that is completely labeled with ^{15}N. Based on the total amount of DNA added to secondary gradients and the total number of 16S rRNA genes detected in these gradients, it was possible to estimate 1.9 fg genomic DNA per 16S rRNA gene (which roughly corresponds to an average *rrn* copy number in the range of three to five per *Escherichia coli*-size genome). Using this value, it was possible to estimate that secondary fractions with densities of 1.708 and 1.713 g ml^{-1} contained 82 ng and 25 ng of DNA for $^{15}N_2$-treated and control samples, respectively. The net difference of these values, 57 ng, corresponds to the amount of ^{15}N-labeled DNA present in the secondary gradient.

Analysis of 16S rRNA genes from ^{15}N-labeled fractions. PCR amplification of 16S rRNA genes from the 1.708- to 1.713-g ml^{-1} fractions of secondary gradients resulted in an amplified product of the expected size from the ^{15}N-enriched DNA, but the quantity of the amplified product generated from the control DNA was insufficient to be visualized as a band by gel electrophoresis (data not shown). While a band was not visible in the control, quantitative PCR indicated that 16S rRNA genes were present in this sample (Fig. 2), and it was possible to generate 16S rRNA gene clone libraries from both the ^{15}N-enriched and control DNA (Table 1). The ^{15}N-enriched library contained 51 16S rRNA sequences, while the control library contained 44 sequences. A total of 33 different microbial groups were detected in both libraries (Table 1). Only three groups, the *Rhodoplanes*, the unclassified *Betaproteobacteria*, and the unclassified *Actinobacteria*, were obtained in sufficient numbers that their abundance in the enriched library was unlikely to be due to chance (Fisher's exact test; $P < 0.05$). The significant overrepresentation of these groups in the enriched library would be expected if these organisms were involved in nitrogen fixation in soil.

The cloned 16S rRNA sequences were evaluated to determine the sizes of the terminal restriction fragments (TRFs) they would generate after digestion with the enzyme MspI. The distribution of these TRFs was then evaluated in the primary gradient as a function of gradient density (Fig. 3). The 11 *Rhodoplanes* sequences possessed a total of four TRFs: 134 bp (one sequence), 152 to 154 bp (eight sequences), 166 bp (one sequence), and 439 bp (one sequence). The TRFs of 152 to 154 bp, 166 bp, and 439 bp show secondary peaks in fluorescent intensity at 1.733 g ml^{-1}, which are shifted 0.018 to 0.026 g ml^{-1} relative to the dominant peaks in the unlabeled controls (Fig. 3A, B, and C). It should be noted that since the analytical resolution of gradient fractionation is 0.0025 g ml^{-1} (2), the buoyant density differences observed between the dominant unlabeled TRFs of the control and enriched experiments (i.e., TRF of 439 bp in Fig. 3C has peaks at 1.716 g ml^{-1} and 1.721 g ml^{-1} for the control and enriched samples, respectively) are not significant. The five unclassified betaproteobacterium sequences possessed a total of three TRFs: 142 to 143 bp (two sequences), 432 bp (one sequence), and 477 to 478 bp (two sequences). These TRFs all show secondary peaks in fluorescent intensity that are shifted 0.018 to 0.026 g ml^{-1} relative to the dominant peak in the unlabeled controls (Fig. 3D, E, and F). The five unclassified *Actinobacteria* sequences possessed

TABLE 1. Phylogenetic classification of 16S rRNA genes in clone libraries generated from DNA-SIP of soil incubated with or without $^{15}N_2$

Taxonomic designation	No. of 16S rRNA sequences for soil with[a]:	
	$^{14}N_2$	$^{15}N_2$
Alphaproteobacteria		
Rhodospirillales		
Unclassified *Rhodospirillales*	1	1
Rhizobiales		
Methylocystis	0	1
Bradyrhizobium	3	4
Rhodoplanes	2	11ψ
Labrys	1	0
Unclassified *Alphaproteobacteria*	1	2
Betaproteobacteria		
Burkholderiales		
Massilia	1	0
Ralstonia	2	1
Delftia	2	0
Insertae sedis 5	2	0
Unclassified *Betaproteobacteria*	0	5ψ
Gammaproteobacteria		
Xanthomonadales	1	1
Unclassified *Gammaproteobacteria*	0	1
Deltaproteobacteria		
Myxococcales		
Nannocystaceae	2	1
Desulfuromonales		
Geobacter	2	0
Unclassified *Desulfuromonales*	1	0
Unclassified *Deltaproteobacteria*	2	1
WS3		
Unclassified WS3	0	2
Actinobacteria		
Acidimicrobiales		
Acidimicrobium	2	2
Actinomycetales		
Mycobacterium	0	1
Propionibacterium	1	1
Unclassified *Actinobacteria*	0	5ψ
Firmicutes		
Clostridiales		
Unclassified *Acidaminococcaceae*	0	1
Acidobacteria		
Acidobacteriales		
Acidobacterium	2	2
Unclassified *Acidobacteriaceae*	5	5
Planctomycetes		
Planctomycetales		
Isosphaera	1	0
Pirellula	1	0
Planctomyces	3	0
Unclassified *Planctomycetaceae*	1	0
Verrucomicrobia		
Unclassified *Verrucomicrobia*	2	1
Gemmatimonadetes		
Gemmatimonadales		
Gemmatimonas	1	0
Cyanobacteria		
Subsection 4		
Unclassified family 4.1	0	1
Unclassified *Bacteria*	2	1

[a] ψ, phylogenetic groups for which the number of 16S rRNA sequences recovered in the $^{15}N_2$ library is significantly greater than that observed in the control library (Fisher's exact test; $P < 0.05$).

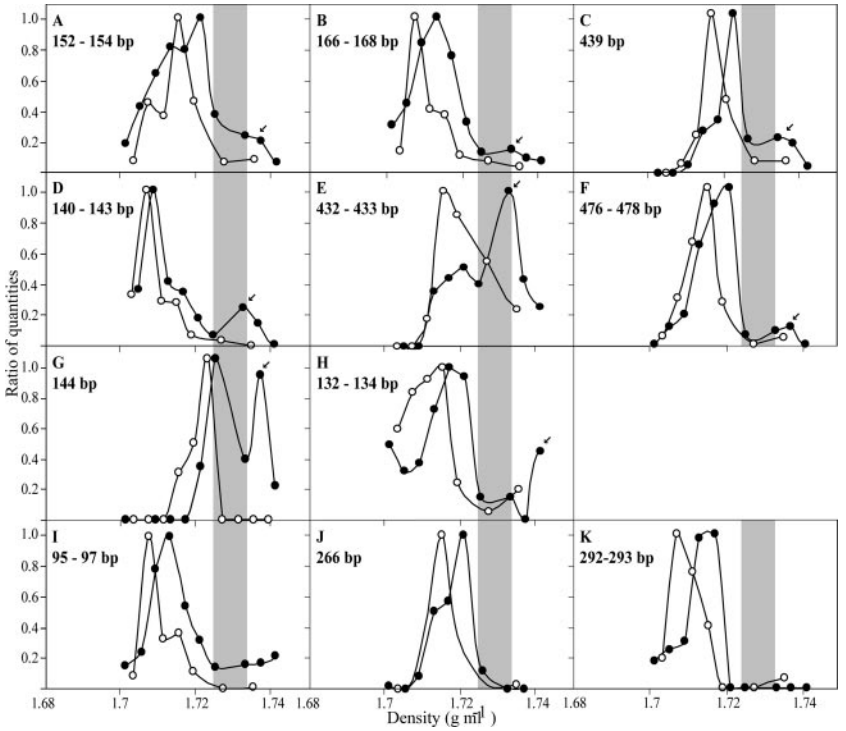

FIG. 3. T-RFLP analysis of 16S rRNA genes was used to examine the buoyant densities of particular TRFs in primary gradient fractions. The TRFs shown are those predicted for cloned 16S rRNA gene sequences described in Table 1 and correspond to *Rhodoplanes* (A, B, and C), *Betaproteobacteria* (D, E, and F), unclassified *Actinobacteria* (G and H), and unclassified *Acidobacteriacea* (I, J, and K). Symbols correspond to DNA from soil with artificial air (○) or artificial air containing 99.8 atom% $^{15}N_2$ (●). TRF peak height was normalized as a function of maximum peak height in each gradient. Arrows are used to indicate putative ^{15}N-labeled DNA, and shading is used to represent DNA from fractions that were added to secondary gradients as described in the legend to Fig. 1.

two TRFs: 132 to 134 bp (four sequences) and 144 bp (one sequence). The 132- to 134-bp TRF has a secondary peak in fluorescent intensity that is shifted 0.026 g ml^{-1} relative to the dominant peak in the unlabeled controls (Fig. 3H), while the shift for the 144-bp TRF is 0.014 g ml^{-1} (Fig. 3G). For comparison, the TRFs of *Acidobacteria* sequences were also determined. Sequences from *Acidobacteria* were found in equal numbers in enriched and control libraries, and thus, their presence in target fractions in not likely due to ^{15}N labeling of DNA. The *Acidobacteria* 16S rRNA sequences possessed a total of six TRFs: 96 bp (two sequences), 148 bp (one sequence), 152 bp (six sequences), 266 bp (two sequences), 293 bp (two sequences), and 402 bp (one sequence). Four of these TRFs were detected in the T-RFLP analysis of 16S rRNA genes in the primary gradient; three of these did not evince a secondary peak in enriched samples, and this is consistent with the absence of N fixation by these organisms (Fig. 3, panels I to K). The final TRF from *Acidobacteria* (152 bp) could not be definitively resolved from the 152- to 154-bp TRF predicted for the *Rhodoplanes*.

Phylogenetic analyses of 16S rRNA genes from the unclassified *Betaproteobacteria* and unclassified *Antinobacteria* revealed that these groups represent previously uncharacterized and deeply divergent lineages within their respective phyla (Fig. 4 and 5). A search of GenBank revealed 88 16S rRNA gene sequences corresponding to the unclassified *Betaproteobacteria* group and 95 16S rRNA gene sequences from the unclassified *Actinobacteria* group, and these sequences were included in subsequent phylogenetic analyses. The unclassified *Actinobacteria* group is most closely related to the order *Rubrobacteriales*, but phylogenetic analyses consistently indicate that it is a monophyletic group and suggest that it is sufficiently divergent from the *Rubrobacteriales* that it may represent a new order within the *Actinobacteria* (Fig. 4). Likewise, the unclassified *Betaproteobacteria* group represents a distinct lineage that may represent a new order within the *Betaproteobacteria* (Fig. 5). Phylogenetic analyses consistently indicated that the unclassified *Betaproteobacteria* group is monophyletic but were unable to demonstrate a consistent affiliation between this group and any of the characterized orders within the *Betaproteobacteria*.

Analysis of *nifH* genes from ^{15}N-labeled fractions. PCR amplification of *nifH* genes from the 1.708- to 1.713-g ml^{-1} fractions of secondary gradients resulted in a product of the expected size from the ^{15}N-enriched sample but no visible band from the control (data not shown). A total of 34 *nifH* clones were identified in the library obtained from the 1.708- to 1.713-g ml^{-1} fraction of the enriched sample, but attempts to recover *nifH* clones from the corresponding fraction of the control were unsuccessful, suggesting that *nifH* genes were either absent or very rare in the control fraction. The 34 *nifH* clones represented 18 different amino acid sequences, and phylogenetic analysis determined that these sequences fell into three different groups within *nifH* cluster I (Fig. 6). One of

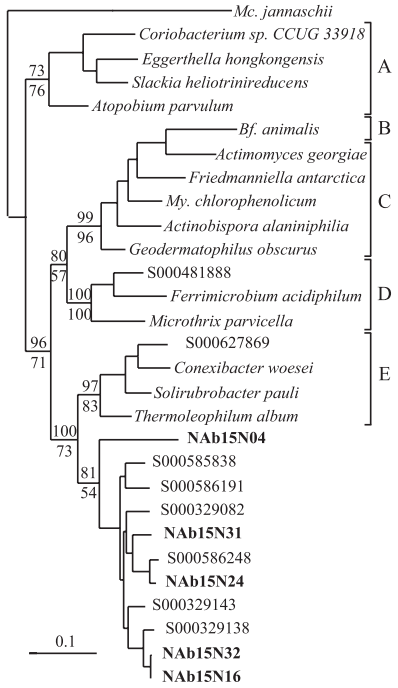

FIG. 4. Phylogenetic tree showing the unclassified *Actinobacteria* group that has been implicated in N fixation in relation to the main orders within *Actinobacteria*. The tree was constructed from 1,343 16S rRNA positions by using maximum-likelihood analysis (PHYLIP 3.64; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, 2005). The numbers listed above and below branches indicate bootstrap values based on 100 replications made using either parsimony (DNApars) or maximum-likelihood (DNAml) analysis, respectively (PHYLIP 3.64). Sequences from this study are in bold type. Designations which begin with S000 are Ribosomal Database Project identifiers representing sequences that are currently unclassified. The scale bar represents a sequence difference of 0.1 nucleotide per position. The letters are used to denote the different orders within the *Actinobacteria* as follows: A, *Coriobacteriales*; B, *Bifidobacteriales*; C, *Actinomycetales*; D, *Acidimicrobiales*; E, *Rubrobacteriales*. The abbreviation *Mc.* is used for *Methanococcus*, *Bf.* for *Bifidobacterium*, and *My.* for *Mycobacterium*.

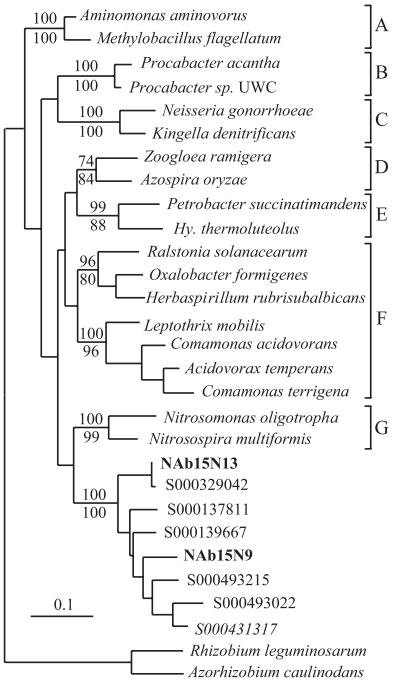

FIG. 5. Phylogenetic tree showing the relationship between the unclassified *Betaproteobacteria* group and the other described orders within the *Betaproteobacteria*. The tree was constructed from 1,334 16S rRNA positions by using maximum-likelihood analysis (PHYLIP 3.64). The numbers listed above and below branches indicate bootstrap values based on 100 replications made using either parsimony (DNApars) or maximum-likelihood (DNAml) analysis, respectively (PHYLIP 3.64). Sequences from this study are in bold type. Designations which begin with S000 are Ribosomal Database Project identifiers representing sequences that are currently unclassified. The scale bar represents a sequence difference of 0.1 nucleotide per position. The letters are used to denote the different orders within the *Betaproteobacteria* as follows: A, *Methylophilales*; B, *Procabacteriales*; C, *Neisseriales*; D, *Rhodocyclales*; E, *Hydrogenophilales*; F, *Burkholderiales*; G, *Nitrosomonadales*. The abbreviation *Hy.* is used for *Hydrogenophilus*.

these *nifH* groups associated with *nifH* sequences from the *Alpha-* and *Betaproteobacteria* and was most closely related to sequences from within the *Rhizobiales*. The other two *nifH* groups represented distinct monophyletic groups within a larger group of uncultivated *nifH* sequences that is deeply divergent within *nifH* cluster I. This uncultivated *nifH* group has been documented previously in a range of soils (3, 4, 11, 18, 48, 50) and is represented by more than 900 *nifH* sequences currently in GenBank. The group appears to contain numerous coherent subgroups, the majority of which are dominated by *nifH* sequences obtained from soil samples (data not shown).

DISCUSSION

The potential for cross-feeding or trophic cascades is a serious concern that must be considered when interpreting results from nucleic acid SIP experiments. The amount of ^{15}N-labeled N mineralized by N-fixing organisms is likely to be small (42) and will be diluted considerably by unlabeled N in the soil. Thus, with certain exceptions, only those organisms that are actively engaged in N fixation should become highly labeled with ^{15}N in response to incubation with $^{15}N_2$. Exceptions are when high N fixation rates are maintained over long incubation periods (i.e., when $^{15}N_2$ fixation makes significant contributions to total N pools in the local environment) or when there is direct transfer of N between organisms (such as during symbiosis). Cross-feeding and trophic effects are unlikely to have resulted in significant ^{15}N labeling of DNA in this experiment, since ^{15}N represented only 0.009 atom% of total soil N at the end of the incubation period. Due to the low proportion of ^{15}N in the total soil N pool, organisms obtaining ^{15}N through cross-feeding or trophic cascades would likely have very low amounts of ^{15}N incorporation due to dilution by unlabeled sources of N. This partially ^{15}N-labeled DNA should easily be discriminated from DNA that is completely ^{15}N labeled by its response to bis-benzimide in secondary gradients. Thus, DNA recovered from secondary gradients in this experiment, due to its response to bis-benzimide (Fig. 2), is likely to be heavily ^{15}N labeled, and this degree of labeling should occur only for organisms involved in N fixation. Another problem associated with nucleic acid SIP experiments involves the potential for isotopic dilution (34). Isotopic dilution is not a

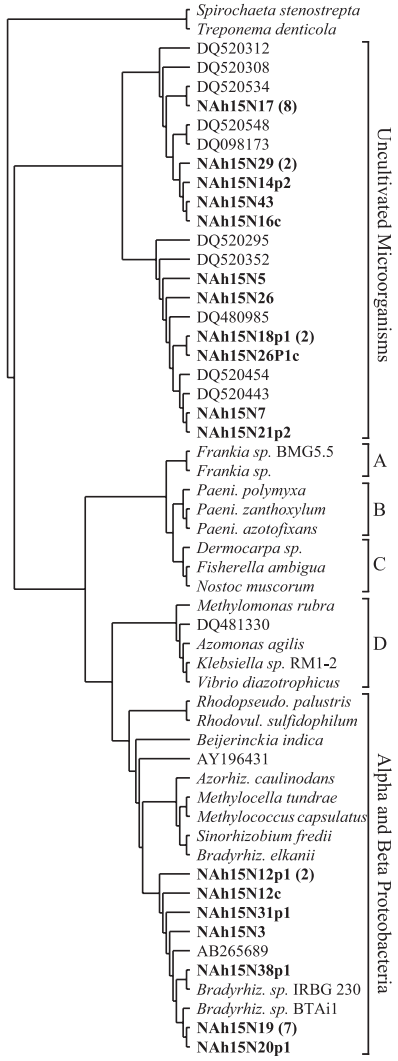

FIG. 6. This phylogenetic tree shows the relationship between the *nifH* sequences identified through $^{15}N_2$-DNA-SIP of soil and other representative sequences from *nifH* cluster I. The tree was constructed from 105 amino acid positions by using maximum-likelihood analysis (PHYLIP 3.64). Unique sequences from this study are in bold type, and the number in parentheses represents the number of times that each sequence was recovered in the clone library. The letters are used to denote the different *nifH* groups as follows: A, *Frankia*; B, *Paenibacillus*; C, *Cyanobacteria*; D, *Gammaproteobacteria*. The abbreviation *Paeni.* is used for *Paenibacillus*, *Rhodopseudo.* for *Rhodopseudomonas*, *Rhodovul.* for *Rhodovulum*, *Azorhiz*. for *Azorhizobium*, and *Bradyrhiz.* for *Bradyrhizobium*.

significant concern with $^{15}N_2$-DNA-SIP, since unlabeled $^{14}N_2$ can be removed completely by evacuation and since significant N fixation occurs only when fixed (and unlabeled) forms of nitrogen are unavailable to the cell (5, 9).

Analysis of 16S rRNA genes from target fractions provided evidence for three groups of organisms involved in N fixation in soil: a group associated with the *Rhodoplanes*, an unclassified group of noncultivated *Betaproteobacteria*, and an unclassified group of noncultivated *Actinobacteria* (Table 1). While N-fixing organisms are widespread within the *Alphaproteobacteria* and within the *Rhizobiales* in particular, N fixation has not previously been attributed to members of the *Rhodoplanes*. One of the *nifH* groups from the target fraction was observed to closely associate with members of the *Rhizobiales* (Fig. 6), which suggests that these *nifH* genes may originate from the *Rhodoplanes* group. Unfortunately, due to the mingling of *nifH* genes from *Alpha-* and *Betaproteobacteria* in the *nifH* phylogeny (possibly as a result of horizontal gene transfer) (36) and the fact that *nifH* genes have yet to be identified in members of the *Rhodoplanes*, it is not yet possible to conclusively link these groups of genes at this time. It is also compelling to note that we observed in the target fraction two groups of unclassified noncultivated 16S rRNA sequences and two groups of unclassified noncultivated *nifH* sequences (Fig. 4, 5, and 6). While it is possible that the two noncultivated *nifH* groups that we identified (Fig. 6) correspond to the novel *Betaproteobacteria* and *Actinobacteria* that we observed (Fig. 4 and 5), it is not possible to provide a conclusive link between these genes at this time.

While nitrogen fixation is widespread in the *Betaproteobacteria*, the 16S rRNA genes that we recovered represent a deeply divergent lineage that may constitute a new order within this group (Fig. 5). More than 88 16S rRNA sequences from GenBank currently fall within this group, none are from cultivated isolates, and more than 75% of these sequences were obtained from soils with samples representing five continents (data not shown). Of even greater interest is our evidence for nitrogen fixation within a deeply divergent lineage of the *Actinobacteria*. Nitrogen fixation is relatively uncommon in the *Actinobacteria*, being found only in *Frankia* and two recently isolated strains of *Actinomycetales* (46). In addition, within the *Actinobacteria*, nitrogen-fixing bacteria have not previously been observed outside of the order *Actinomycetales*. The group that we have identified, while most closely related to the *Rubrobacteriales*, likely represents a new order of *Actinobacteria* (Fig. 4) for which no cultivated isolate is currently available. This group currently encompasses more than 95 16S rRNA sequences in GenBank, 86% of which were obtained from soils originating from six continents (data not shown).

Since this research represents the first attempt to use $^{15}N_2$ in SIP experiments and since few attempts have been made to use ^{15}N isotopes in nucleic acid SIP, it is important to consider the evidence that supports our assertion that the groups identified are involved in nitrogen fixation. While an obvious change in bulk DNA density was not observed in response to $^{15}N_2$ labeling in primary gradients (Fig. 1), such a response would in fact be unlikely in this experiment. The rate of nitrogen fixation in this experiment was low, resulting in a small amount of ^{15}N-labeled DNA relative to unlabeled DNA (an estimated 60 ng ^{15}N target DNA relative to 1,800 ng nontarget DNA). In addition, completely ^{15}N-labeled DNA is expected to change density by only 0.016 g ml^{-1}, while variation of DNA G+C content in soil will cause wide variation in the buoyant density of DNA from soil (1). Thus, secondary gradients containing bis-benzimide were used to resolve ^{15}N-labeled DNA. DNA samples from target fractions in primary gradients were observed to respond to bis-benzimide incorporation in secondary gradients in a manner which is consistent with 100% ^{15}N incorporation into DNA (Fig. 2). Clearly, however, while target fractions from the secondary gradient showed a significant response to $^{15}N_2$ enrichment, some contaminating unlabeled

DNA was still present (Fig. 2 and Table 1). The recovery of unlabeled DNA in target fractions has been observed in previous nucleic acid SIP experiments (20, 22, 25) and could result from failure to completely equilibrate nucleic acids in CsCl gradients, DNA fragmentation, reagent contamination, or carryover of DNA between fractions during fractionation. Thus, it is essential to use proper controls to distinguish isotopically labeled DNA from unlabeled DNA. The 16S rRNA gene libraries constructed from the 1.708- to 1.713-g ml^{-1} fractions of secondary gradients indicate that certain groups occur more frequently in the enriched library than in the control library and that this difference is unlikely to be due to chance (Table 1). This observation is consistent with the conclusion that these groups were involved in $^{15}N_2$ fixation (considering that any PCR bias that may affect the abundance of PCR products should be consistent between enriched and control samples (e.g., see reference 16). Finally, the recovery of *nifH* genes from target secondary fractions (1.708 to 1.713 g ml^{-1}) and failure to obtain *nifH* genes from corresponding unenriched control fractions suggest that the difference in the amount of DNA observed between these fractions (Fig. 2) is likely attributable to N-fixing organisms. This observation is also supported by the fact that the difference in the amount of DNA present in secondary gradient target fractions from $^{15}N_2$-treated and control samples was 57 ng, a value which corresponds with the 60 ng of ^{15}N-DNA estimated per g soil based on measurements of soil ^{15}N enrichment resulting from $^{15}N_2$ fixation.

Independent confirmation that fixation of $^{15}N_2$ caused an increase in the DNA density of these groups is provided by examining the distribution of their 16S rRNA TRFs in primary gradients. When interpreting this evidence, it is important to consider that multiple organisms can possess TRFs of the same size, and this problem can complicate interpretation of T-RFLP patterns (19, 26). This problem is ameliorated to some degree in the current experiment by the fact that organisms that share a TRF but have different genome G+C content will occur in different positions in a CsCl gradient as a function of their DNA buoyant density (2). As a result, however, when conducting nucleic acid SIP experiments, careful comparison is needed between DNA fingerprint profiles of isotopically enriched and control samples in density gradients. For example, TRFs of 476 to 478 bp (Fig. 3F) and 132 to 134 bp (Fig. 3H) have indications of a small TRF peak in the control sample at the same density where ^{15}N-labeled DNA would be expected to occur. This result could occur if organisms with a high G+C content share a TRF with N-fixing organisms. Thus, while the data from these TRFs is consistent with $^{15}N_2$ fixation, this result could also occur if a change in the abundance of certain high-G+C organisms between treatment and control samples took place.

In other cases, examining the distribution of 16S rRNA TRFs in primary gradients provided unambiguous evidence of ^{15}N incorporation into DNA. It was possible to observe TRFs from the *Rhodoplanes* (439 bp) (Fig. 3C), the noncultivated *Betaproteobacteria* group (140 to 143 bp and 432 to 433 bp) (Fig. 3D and E), and the noncultivated *Actinobacteria* group (144 bp) (Fig. 3G) that clearly show an increase in DNA density in enriched samples relative to that for controls. The presence of these heavy peaks in the enriched treatment but not in the control is consistent with ^{15}N incorporation into DNA. The change in the DNA buoyant density of these TRFs in enriched treatments relative to that for controls was greater than 0.014 g ml^{-1}, consistent with 100% ^{15}N incorporation into DNA. In contrast, TRFs from the *Acidobacteria* (Fig. 3I to K), a group detected in control and enriched samples in equal numbers and thus not expected to be involved in N fixation (Table 1), did not demonstrate heavy TRFs, a result that is inconsistent with ^{15}N incorporation into DNA. It is interesting to note that all TRFs from organisms involved in $^{15}N_2$ fixation retained a distinct unlabeled DNA peak (Fig. 3). This observation suggests that nitrogen fixation occurred within certain microsites, while in other microsites, either fixed forms of nitrogen were available or growth did not occur. Such microheterogeneity in soil would result in the simultaneous presence of both completely labeled DNA and unlabeled DNA, as was observed in this experiment. This observation is also consistent with the expectation that organisms that are fixing nitrogen will not simultaneously incorporate unlabeled mineral forms of nitrogen from soil.

This research represents the first application of $^{15}N_2$-DNA-SIP and the first application of nucleic acid ^{15}N-SIP that has been able to identify microorganisms associated with ^{15}N incorporation in the environment. We have identified three microbial groups that we suggest are able to carry out nitrogen fixation in soil. None of these groups has previously been implicated in this process, and two of them, the unclassified *Betaproteobacteria* and the unclassified *Actinobacteria*, remain completely uncharacterized despite being widespread in soils. Our data also suggest the hypothesis that these two 16S rRNA groups may correspond to the two noncultivated groups of *nifH* genes that we observed. Thus, the application of $^{15}N_2$-DNA-SIP may make it possible to identify previously uncharacterized groups of nitrogenase genes. The two groups of *nifH* genes that we identified belong to a larger family of more than 900 *nifH* sequences that are deeply divergent within the nitrogenase cluster I and which currently remains completely uncharacterized due to the lack of any cultivated representatives within this group. Work is currently under way to either isolate these organisms by using the *nifH* or 16S rRNA genes that we have identified as a marker or use DNA from SIP experiments to isolate genome fragments that would allow more-thorough characterization of these novel diazotrophs.

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