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Recovery and Phylogenetic Analysis of *nifH* Sequences from Diazotrophic Bacteria Associated with Dead Aboveground Biomass of *Spartina alterniflora*

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DNA was extracted from dry standing dead *Spartina alterniflora* stalks as well as dry *Spartina* wrack from the North Inlet (South Carolina) and Sapelo Island (Georgia) salt marshes. Partial *nifH* sequences were PCR amplified, the products were separated by denaturing gradient gel electrophoresis (DGGE), and the prominent DGGE bands were sequenced. Most sequences (109 of 121) clustered with those from α-*Proteobacteria*, and 4 were very similar (>99%) to that of *Azospirillum brasilense*. Seven sequences clustered with those from known γ-*Proteobacteria* and five with those from known anaerobic diazotrophs. The diazotroph assemblages associated with dead *Spartina* biomass in these two salt marshes were very similar, and relatively few major lineages were represented.

Low elevations of salt marshes along the Atlantic and northern Gulf of Mexico coasts of temperate North America are characterized by extensive monoculture stands of the smooth cordgrass *Spartina alterniflora* (35). *Spartina* marshes support high rates of macrophyte primary production and microbially mediated nutrient cycling. Numerous studies indicate that primary production (16, 37) and decomposition (15, 19, 38) in *Spartina* marshes are nitrogen limited. In these systems, diazotrophy (N_2 fixation) is an important source of "new" nitrogen (8, 23, 40).

A significant but often overlooked focus of diazotrophy in salt marshes is dead aboveground *Spartina* biomass, particularly standing dead biomass (19, 33). There are quite large amounts of standing dead biomass at all times of the year, with ratios of dead to live aboveground biomass exceeding 1:1 during the winter and spring months (7, 31). Standing dead biomass is partially mineralized and frequently very dry (17) but supports substantial microbial activity (17–19), which is greatly stimulated when the material becomes wet through tidal action or precipitation (17). Rates of diazotrophy in moist standing dead *Spartina* are among the highest reported for decomposing plant materials (see Table 4 in reference 19).

Relatively little attention has been given to the microorganisms involved in decomposition of and diazotrophy in dead aboveground *Spartina* biomass. Much of the microbial biomass in standing dead materials consists of fungal hyphae (18). Cyanobacteria are present but occur chiefly in clay-rich surface films (18), while diazotrophy occurs primarily on and within the decaying biomass itself (19). It seems that the predominant diazotrophs in this material are heterotrophic bacteria, but the types of organisms present have not been determined.

Recent applications of molecular biological methods have greatly facilitated the study of natural bacterial communities and functionally significant taxa within them (9, 34, 39, 42). In particular, PCR amplification has been used to recover segments of *nifH*, the structural gene encoding the nitrogenase iron protein, from various types of environmental samples, including marine and freshwater plankton (1, 3, 44), termite hindguts (11, 20), microbial mats and aggregates (21, 22, 43), terrestrial soils (28, 29, 32, 41), the rhizoplanes of rice (*Oryza sativa*) (36) and of shoal grass (*Halodule wrightii*) (10), and the rhizosphere of *Spartina* (14). PCR amplification of *nifH* sequences followed by their separation through denaturing gradient gel electrophoresis (DGGE) has been used to examine the complexity and stability of the diazotroph assemblage found in the *Spartina* rhizosphere (25–27), and sequence analysis of the DGGE bands has been used to determine phylogenetic relationships of the diazotrophic organisms represented (14). While such methods have certain inherent limitations and biases (25, 42), they provide an efficient means to profile the diazotrophs associated with dead aboveground *Spartina* biomass and to determine the phylogenetic affiliations of these organisms.

In this study we determined the types of diazotrophic heterotrophic bacteria present in standing dead and loose, recently deposited (wrack) *Spartina* biomass, as defined by recoverable partial *nifH* sequences resolved by DGGE. Our primary objectives were to assess the diversity of these assemblages and to identify the major phylogenetic groups of organisms that are capable of contributing to N_2 fixation in dead aboveground *Spartina* biomass.

Sampling sites. Samples of standing dead *Spartina* stalks and *Spartina* wrack were collected from the short-form *Spartina* zones in two different salt marsh systems. The Crab Haul Creek Basin site in the North Inlet estuary, near Georgetown,

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S.C. (79°12′W, 33°20′N), was located in the intertidal zone approximately 50 m from the nearest tidal creek and was sampled on 31 August 2000. The Doboy Sound site on Sapelo Island, Ga. (31°23′N, 81°17′W), was located in the intertidal zone near Doboy Sound (Georgia Coastal Ecosystems Long Term Ecological Research Site 6) and was sampled on 1 August 2000. The upper, approximately 10-cm lengths of dry standing dead stalks were collected. Dry wrack, which consisted of loose stalks (litter) recently deposited on the sediment surface, was collected from deposits lying near the sampling locations for standing dead stalks. Standing dead stalks and wrack were transferred to sterile Whirl-Pak bags and stored at −70°C pending DNA extraction.

DNA extraction. Standing dead and wrack stalks were broken up into 2-cm fragments. DNA was extracted from the samples using a direct lysis procedure described previously (13, 25). DNA extracts were further purified and concentrated using the Wizard DNA clean-up system following the manufacturer's instructions (Promega, Madison, Wis.). DNA quality and quantity were assessed by agarose gel electrophoresis and fluorometry, respectively.

PCR amplification of *nifH*. PCR was performed using *Taq* DNA polymerase (Qiagen, Valencia, Calif.) in a reaction mixture containing (25-μl reaction volume) 25 ng of template DNA, 1.5 mM $MgCl_2$, 2 μM deoxynucleoside triphosphate (dNTP) mixture, 0.5 pmol of each primer, and 10 μg of bovine serum albumin. The *nifH* primers used were those of Piceno et al. (25) and are specific for heterotrophic diazotrophs. These primers were designed to have low degeneracy, which is needed for DGGE applications, and are not expected to amplify *nifH* sequences from cyanobacteria, *Frankia* spp., and methanogens. Primer design and testing have been described previously (25). Amplification was initiated by a denaturation step at 94°C for 2 min and proceeded in two phases: (i) a 20-cycle touchdown program (94°C for 45 s, 58°C for 30 s, decreasing 0.5°C/cycle, and 70°C for 30 s), and (ii) 20 cycles of a standard amplification program at a 48°C annealing temperature for 30 s. A final extension step at 70°C for 2 min was used. Multiple individual reactions were performed for each sample. PCR products were pooled (200-μl final volume per sample) and stored as alcohol precipitates at −20°C. Prior to DGGE, amplimers were recovered by centrifugation and dissolved in 15 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

DGGE. *nifH* amplimers were electrophoresed on denaturing gradient gels (1-mm thick, 6.5% polyacrylamide, 78 to 89% denaturant, where 100% denaturant contains 7 M urea and 40% formamide) at 48°C for 1,900 V · h using the Bio-Rad (Hercules, Calif.) DCode universal mutation detection system. Gels were stained for 30 min in TE with SYBR Gold (Molecular Probes, Eugene, Oreg.) and documented using the Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, Calif.). Gel plugs were collected from all well-resolved bands in the profiles using wide-bore micropipette tips and stored in 50 μl of distilled water at −20°C. Gel bands were designated homoduplexes or heteroduplexes following previously described methods (25).

Amplimer cloning and identification of different cloned amplimer sequences. Amplimers from DGGE gel plugs were recovered by reamplification and cloned as described previously

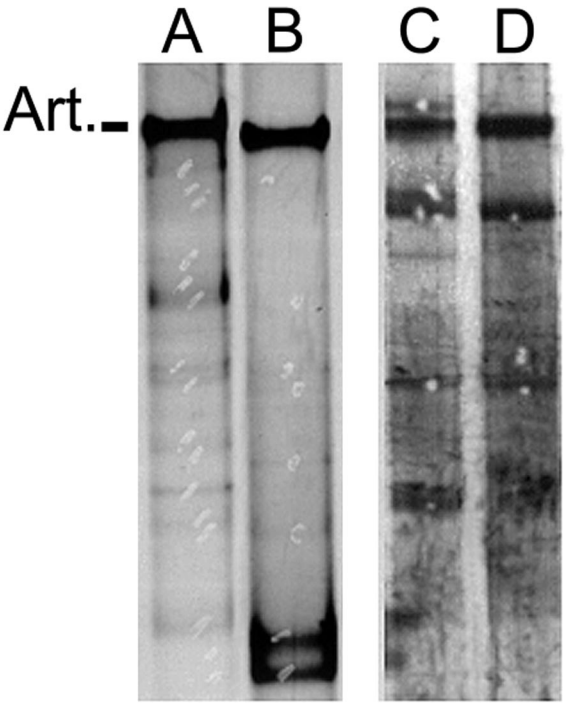

FIG. 1. Denaturing gradient gel images showing *nifH* amplimers from dead aboveground *Spartina* biomass. PCR and DGGE conditions are described in Materials and Methods. Lanes: A, North Inlet standing dead *Spartina*; B, North Inlet *Spartina* wrack; C, Sapelo Island standing dead; D, Sapelo Island wrack; Art., artifact.

(14). Recombinant colonies were maintained on Luria-Bertani agar plates containing 100 μg of ampicillin ml^{-1}. Clones were screened for appropriately sized insert by amplification using primers specific for the SP6 and T7 RNA polymerase binding sites (14). Restriction fragment length polymorphism analysis was employed to assess gel band amplimer composition (i.e., homogeneous or heterogeneous) and to identify different clones for sequencing (14).

DNA sequencing and analysis. Recombinant plasmids were purified from selected clones by using the Qiagen Plasmid Mini Kit. Plasmid concentrations were determined fluorometrically. Sequencing reactions used T7 and Sp6 primers and ABI (Applied Biosystems, Foster City, Calif.) BigDye version 2.0 chemistry. Sequences were determined using an ABI 3100 genetic analyzer. For phylogenetic reconstructions, *nifH* sequences from numerous different known diazotrophs and from various environmental sources were selected using the Blast search feature of the NCBI GenBank database (2). Nucleotide sequences were translated, and the inferred amino acid sequences were aligned (5) and checked by hand for proper alignment of conserved marker residues (14). Neighbor-joining phylogenies (30) were constructed in MEGA version 2.0 (12) using percent dissimilarity distances and pairwise deletion of gaps and missing data. The use of alternative amino acid distance measures (e.g., Poisson and gamma correction for multiple substitutions) or tree construction methods (neighbor joining or Unweighted Pair Group Method Using Arithmetic Averages) had no significant effect on the resulting dendrogram topology (data not shown). NifH amino acid sequences

TABLE 1. Similarities of dead aboveground *Spartina* biomass NifH amino acid sequences to the most similar sequences from known diazotrophic bacterial species[a]

Dead *Spartina* sequence(s)	Organism(s) with most similar sequence(s)	% Similarity
Presumptive α-*Proteobacteria*		
NIS2-1	*Gluconacetobacter diazotrophicus*	95.2
NIS2-2, NIS2-3, NIS4-2, NIS4-4, NIS5-1, NIS5-2, NIS6-1, NIS6-2, NIS7-1, NIS8-1, NIS9-2, NIS10-2, NIS12-3, NIS13-1, NIS13-2, NIW4-1, NIW6-2	*Rhodobacter sphaeroides*	94.4
NIS3-1, NIS6-2, NIS7-3	*Azospirillum brasilense*	94.4
NIS3-2, NIS9-1, NIS10-3, NIS14-1, NIS15-2, NIS15-4, NIS16-3	*Bradyrhizobium japonicum*	97.6
NIS4-1	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	92.8
NIS4-3	*Rhodobacter sphaeroides*	95.2
NIS7-2, NIW2-3, NIW4-2, NIW5-1, NIW8-2, NIW8-3, NIW9-4	*Rhizobium* sp.	94.4
NIS8-2	*Rhodobacter sphaeroides*	92.8
NIS8-3, NIW3-1	*Rhodobacter sphaeroides*	96.0
NIS10-1, NIS13-3	*Rhodobacter sphaeroides*	96.8
NIS10-4	*Azospirillum brasilense*	88.0
NIS11-1	*Rhodobacter capsulatus, Rhodobacter sphaeroides*	97.6
NIS11-2	*Azospirillum brasilense*	92.0
NIS11-3	*Rhodobacter sphaeroides*	98.4
NIS12-1	*Bradyrhizobium japonicum, Rhizobium leguminosarum* biovar phaseoli	95.2
NIS12-2	*Rhodobacter sphaeroides*	93.6
NIS13-4	*Herbaspirillum seropedicae, Rhizobium* sp.	92.0
NIS14-3, NIS14-4, NIS15-3, NIS16-4	*Rhodobacter sphaeroides*	97.6
NIS15-1	*Azospirillum brasilense*	91.2
NIS16-2, NIW8-1, NIW9-1	*Rhizobium* sp.	92.8
NIW2-2, NIW6-3, SIS5-1	*Herbaspirillum seropedicae, Rhizobium* sp.	92.8
NIW2-4	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	96.8
NIW3-2, NIW6-1	*Herbaspirillum seropedicae, Rhizobium* sp.	92.0
NIW3-3, NIW4-3, NIW5-2, NIW6-3, NIW7-1, NIW9-2, NIW9-3	*Rhizobium* sp.	93.6
NIW4-4	*Azospirillum brasilense*	98.4
NIW5-3	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	91.2
SIS1-1, SIS4-4	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	94.4
SIS2-1, SIS4-2	*Azospirillum brasilense*	100.0
SIS2-2, SIW1-2	*Gluconacetobacter diazotrophicus*	94.4
SIS2-3	*Gluconacetobacter diazotrophicus*	92.8
SIS2-5	*Azospirillum brasilense*	92.8
SIS2-6	*Gluconacetobacter diazotrophicus*	86.4
SIS3-1	*Azospirillum brasilense*	95.2
SIS3-2, SIW1-7	*Gluconacetobacter diazotrophicus*	97.6
SIS3-3, SIS3-6	*Azospirillum brasilense*	93.6
SIS3-4	*Azospirillum brasilense, Bradyrhizobium japonicum, Gluconacetobacter diazotrophicus, Rhodobacter sphaeroides*	93.6
SIS3-5	*Gluconacetobacter diazotrophicus, Rhizobium* sp.	83.2
SIS3-7	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	92.0
SIS3-8, SIW2-3, SIW2-5	*Gluconacetobacter diazotrophicus*	93.6
SIS4-1, SIW2-6	*Herbaspirillum seropedicae, Rhizobium* sp.	93.6
SIS4-3	*Azospirillum brasilense*	99.2
SIW1-5	*Azospirillum brasilense, Bradyrhizobium japonicum, Gluconacetobacter diazotrophicus, Rhizobium leguminosarum* biovar phaseoli	94.4
SIW1-6	*Azospirillum brasilense, Gluconacetobacter diazotrophicus*	93.6
SIW2-1	*Rhodobacter sphaeroides*	96.0
SIW2-2	*Azospirillum brasilense*	96.0
SIW2-4, SIW4-2	*Herbaspirillum seropedicae*	92.8
SIW3-1	*Gluconacetobacter diazotrophicus*	96.0
SIW4-1	*Gluconacetobacter diazotrophicus*	91.2
SIW4-3	*Rhizobium* sp.	89.6
SW4-4	*Azospirillum brasilense*	99.2
SW4-5	*Azospirillum brasilense*	97.6

Continued on following page

TABLE 1—*Continued*

Dead *Spartina* sequence(s)	Organism(s) with most similar sequence(s)	% Similarity
Presumptive γ-*Proteobacteria*		
NIS1-2	*Azomonas agilis, Azotobacter chroococcum*	95.2
NIS1-3	*Azomonas agilis, Azotobacter chroococcum*	99.2
NIS1-4	*Pseudomonas stutzeri*	98.2
NIS11-4	*Azotobacter chroococcum, Azotobacter vinelandii*	84.9
SIS2-4	*Vibrio diazotrophicus*	86.1
SIS2-7	*Vibrio diazotrophicus*	84.2
SIW1-4	*Azomonas agilis*	96.8
Presumptive anaerobes		
NIS1-1	*Desulfovibrio salexigens*	84.2
NIS3-3	*Desulfonema limicola*	84.8
NIS14-2	*Desulfonema limicola*	82.8
NIS16-1	*Desulfovibrio salexigens*	88.1
SIW1-1	*Desulfovibrio salexigens*	85.1

[a] Percent similarities are from the distance matrix constructed in MEGA version 2.0. *Spartina* sequences are listed by the DGGE gel bands from which they were recovered (see Materials and Methods) and grouped into the major sequence clusters shown in Fig. 2 to 4. Note that these clusters are named for the predominant types of organisms that they contain, but some sequences from other phylogenetic groupings can occur.

from *Methanobacterium thermoautotrophicum* (accession no. AE00916) and *Methanosarcina barkeri* (X56072) were used as outgroup taxa. Bootstrapping (6) was used to estimate the reliability of phylogenetic reconstructions (500 replicates).

All dead aboveground *Spartina* biomass samples yielded strong amplification products, although the North Inlet samples produced substantially stronger amplification than the Sapelo Island samples (Fig. 1). DGGE yielded 16 well-resolved *nifH* amplimer bands from North Inlet standing dead biomass and 9 bands from wrack. Due to somewhat lower yields of amplification products from the Sapelo Island samples, only five robust bands were recovered from the standing dead material and four bands from wrack. All of these bands were successfully sampled, and the amplimers were cloned and screened for unique sequences. An artifact band, which was described previously (25), was also observed in each sample lane. A total of 54 different partial *nifH* sequences were recovered from standing dead *Spartina* and 24 from *Spartina* wrack collected at North Inlet. Twenty-one different sequences were recovered from the standing dead material and 20 from the wrack from Sapelo Island.

The partial *nifH* sequences were initially translated and examined for key, highly conserved amino acid residues that are important in nitrogenase iron protein structure and function (4, 24). Within the segments analyzed, 11 amino acids, including (*Klebsiella pneumoniae* [J01740] numbering) Lys15 and Ser16 (within the MgATP binding domain), Arg100 (the ADP-ribosylation site), Asp125 (possibly involved in protein conformation changes), Asp129 (involved in ATP hydrolysis), Arg140 and Lys143 (contribute to salt bridge formation), and four conserved Cys residues (numbers 38, 85, 97, and 132, two of which coordinate the Fe_4S_4 cluster), were used as markers for determining sequence accuracy. Four sequences had one substitution each: NIS2-1 and SIW2-6, Cys85 replaced by Arg85; NIS3-2, Ser16 replaced by Trp16; and NIW8-1, Cys97 replaced by Arg97. All sequences were used in subsequent analyses.

The sequences from dead aboveground *Spartina* biomass and their closest affiliations with known diazotrophs on the basis of sequence similarity are listed in Table 1. As has been reported in numerous previous studies of environmental *nifH* sequences (1, 3, 14, 29, 36, 41, 43, 44), these sequences fell into three major clusters. The overwhelming majority of dead *Spartina nifH* sequences were affiliated with a cluster defined by sequences from α-*Proteobacteria* and well supported by bootstrapping (Fig. 2). Forty-six of 54 sequences (85%) from the North Inlet standing dead sample, all 24 of the sequences from North Inlet wrack (100%), 19 of 21 sequences (90%) from the Sapelo Island standing dead sample, and 17 of 20 sequences (85%) from Sapelo Island wrack were from presumptive α-*Proteobacteria*. These sequences were further subdivided into a number of smaller clusters, some of which contained *nifH* sequences from known diazotrophs.

Several sequences from North Inlet standing dead *Spartina* biomass were closely affiliated with purple nonsulfur bacteria, and seven had substantial similarity (≥96%) to the *Rhodobacter sphaeroides* NifH sequence (Table 1). Several sequences from all sample types were over 95% similar to the *Gluconacetobacter diazotrophicus* sequence. Another group of eight sequences from North Inlet samples were substantially similar (≥95%) to NifH sequences from rhizobia. Seven of these were over 97% similar to a *Bradyrhizobium japonicum* sequence. Another substantial sequence grouping, predominantly from the Sapelo Island standing dead sample, was strongly affiliated with the NifH sequence from *Azospirillum brasilense*. Four of these had 99% or greater similarity to the *A. brasilense* sequence, and two were identical to it. The NifH sequences of *A. brasilense* and *Azospirillum lipoferum* are 99.3% similar, so at least four of the dead *Spartina* biomass NifH sequences were very likely from *Azospirillum* species and two seemingly from a strain of *A. brasilense*. This finding confirms the prediction of Newell et al. (19) that *Azospirillum*-like organisms may be involved in degradation of standing dead *Spartina* biomass. There were also many sequences that were not closely affiliated with any known diazotrophs among the α-*Proteobacteria*. Blast searches of the NCBI GenBank database revealed only a few sequences from other types of environmental samples that had meaningful similarity to any sequences from dead aboveground *Spartina* biomass. Among these were four sequences recovered from the *Spartina* rhizosphere (14).

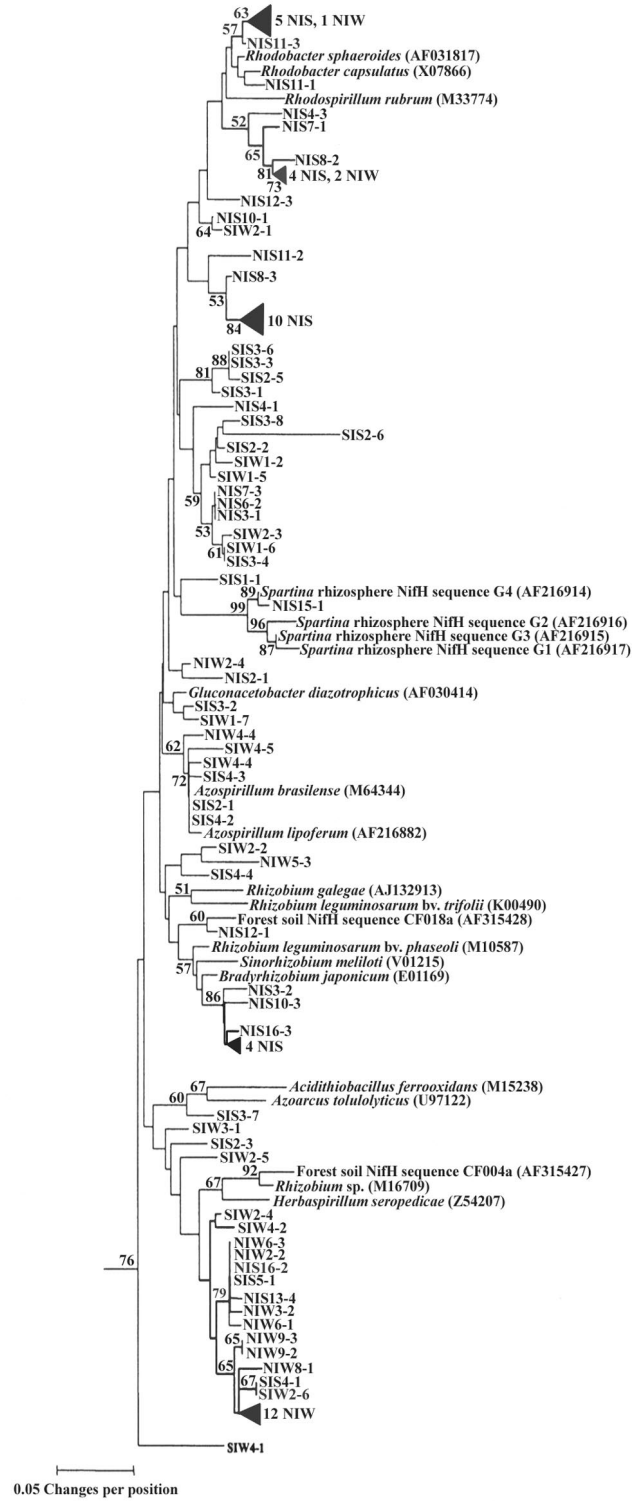

FIG. 2. Phylogenetic analysis of dead aboveground *Spartina* biomass NifH amino acid sequences from presumptive α-*Proteobacteria*, from various known α-*Proteobacteria*, and from selected unknown, presumptive α-*Proteobacteria* from other sources. NIS, North Inlet standing dead *Spartina*; NIW, North Inlet *Spartina* wrack; SIS, Sapelo Island standing dead *Spartina*; SIW, Sapelo Island *Spartina* wrack. The percentage of 500 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

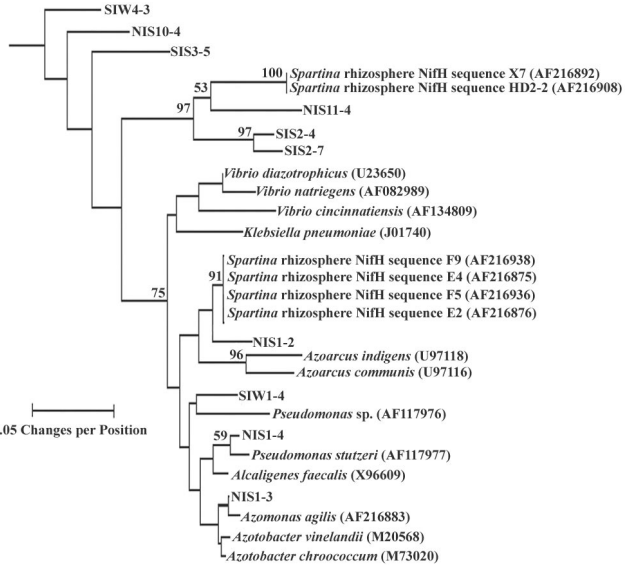

FIG. 3. Phylogenetic analysis of dead aboveground *Spartina* biomass NifH amino acid sequences from presumptive γ-*Proteobacteria*, from various known γ-*Proteobacteria*, and from selected unknown, presumptive γ-*Proteobacteria* from other sources. NIS, North Inlet standing dead *Spartina*; NIW, North Inlet *Spartina* wrack; SIS, Sapelo Island standing dead *Spartina*; SIW, Sapelo Island *Spartina* wrack. The percentage of 500 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

The second major cluster containing sequences from dead aboveground *Spartina* biomass was characterized by sequences from γ-*Proteobacteria* (Fig. 3). This cluster contained three sequences from standing dead *Spartina* whose positions were ambiguous. When these three sequences were omitted and the tree was reconstructed, the γ-*Proteobacteria* cluster was strongly supported by bootstrap analysis (60% for the cluster as a whole, 96 and 91% for the two major subclusters). Omitting these sequences also raised the bootstrap score for the α-*Proteobacteria* cluster from 76 to 99%. One of the remaining seven presumptive γ-*Proteobacteria* sequences was very strongly affiliated with the *Azotobacteriaceae*, with over 99% similarity to the NifH sequences of *Azomonas agilis* and *Azotobacter chroococcum* (Table 1). For comparison, the NifH sequences of *A. chroococcum* and *Azotobacter vinelandii* are 99.3% similar. Two other sequences also had substantial similarity (≥95%) to sequences from azotobacteria. The only other sequence with strong similarity to a sequence from a known organism was a North Inlet standing dead sequence that was >98% similar to the sequence from *Pseudomonas stutzeri*. As was the case for sequences from presumptive α-*Proteobacteria*, few environmental sequences were substantially similar to those from dead aboveground *Spartina* biomass, and these were from the *Spartina* rhizosphere.

Only five sequences recovered from dead aboveground *Spartina* biomass were affiliated with the remaining major NifH sequence cluster, the anaerobes (Fig. 4). While the closest affiliations of these sequences to any from known organisms were all with sulfate-reducing bacteria, none of the dead *Spartina* NifH sequences were sufficiently similar to any sequence from a known diazotroph to permit even presumptive identi-

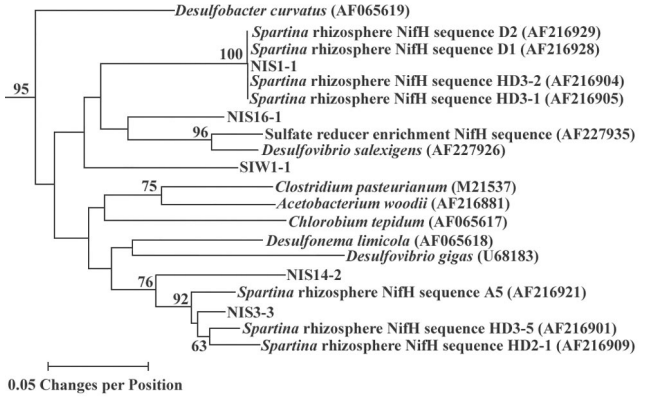

FIG. 4. Phylogenetic analysis of dead aboveground *Spartina* biomass NifH amino acid sequences from unknown, presumptive anaerobic bacterial sequences, from various known anaerobic bacteria, and from selected unknown, presumptive anaerobic bacteria from other sources. NIS, North Inlet standing dead *Spartina*; NIW, North Inlet *Spartina* wrack; SIS, Sapelo Island standing dead *Spartina*; SIW, Sapelo Island *Spartina* wrack. The percentage of 500 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

fication. Two sequences from the North Inlet standing dead sample were similar to sequences from *Spartina* rhizosphere, but since the rhizosphere core samples contained both roots and sediments, and the dead aboveground biomass samples all carried a small amount of sediment, these sequences may be from sediment diazotrophs rather than species closely affiliated with the decomposing plant materials.

NifH sequences recovered from dead *Spartina* biomass reflected a diazotroph assemblage of very limited diversity, consisting almost exclusively of α-*Proteobacteria*. Some of these organisms are apparently related to known diazotrophs, including *Azospirillum brasilense*, *Bradyrhizobium japonicum*, *Gluconacetobacter diazotrophicus*, and *Rhodobacter sphaeroides*. Very few sequences from dead *Spartina* biomass were affiliated with the other major NifH sequence groups. However, it is noteworthy that among the few sequences recovered from presumptive γ-*Proteobacteria*, several were quite similar to those from known diazotrophs, including *Azomonas agilis*, *Azotobacter chroococcum*, and *Pseudomonas stutzeri*. While it is certainly possible that some sequences were lost due to PCR biases or other artifacts (25, 42), identical methods have yielded much more diverse sequence collections from other sample types (14; Lovell et al., unpublished data). It appears that while the relatively harsh (partially mineralized and frequently dry) microenvironments represented by dead aboveground *Spartina* biomass can support impressive rates of diazotrophy (19) when wet, they pose a substantial challenge for many diazotrophic biota and consequently harbor a very restricted range of organisms.

Nucleotide sequence accession numbers. The *nifH* sequences determined in this study are available in the GenBank database under accession numbers AF389702 to AF389823.

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