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Molecular Analysis of Diazotroph Diversity in the Rhizosphere of the Smooth Cordgrass, *Spartina alterniflora*

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N_2 fixation by diazotrophic bacteria associated with the roots of the smooth cordgrass, *Spartina alterniflora*, is an important source of new nitrogen in many salt marsh ecosystems. However, the diversity and phylogenetic affiliations of these rhizosphere diazotrophs are unknown. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified *nifH* sequence segments was used in previous studies to examine the stability and dynamics of the *Spartina* rhizosphere diazotroph assemblages in the North Inlet salt marsh, near Georgetown, S.C. In this study, plugs were taken from gel bands from representative DGGE gels, the *nifH* amplimers were recovered and cloned, and their sequences were determined. A total of 59 sequences were recovered, and the amino acid sequences predicted from them were aligned with sequences from known and unknown diazotrophs in order to determine the types of organisms present in the *Spartina* rhizosphere. We recovered numerous sequences from diazotrophs in the γ subdivision of the division *Proteobacteria* (γ-*Proteobacteria*) and from various anaerobic diazotrophs. Diazotrophs in the α-*Proteobacteria* were poorly represented. None of the *Spartina* rhizosphere DGGE band sequences were identical to any known or previously recovered environmental *nifH* sequences. The *Spartina* rhizosphere diazotroph assemblage is very diverse and apparently consists mainly of unknown organisms.

Low elevations of salt marsh ecosystems along the Atlantic and northern Gulf coasts of temperate North America are characterized by extensive, typically monoculture stands of the smooth cordgrass, *Spartina alterniflora* (*Spartina* hereafter) (65). *Spartina* marshes support high rates of macrophyte primary production and microbially mediated nutrient cycling, contributing to global carbon (15, 44) and nitrogen (12) budgets. The consensus of numerous studies is that primary production (43, 67) and decomposition (40, 46, 68) in *Spartina* marshes are nitrogen limited. In these systems, diazotrophy (N_2 fixation) is a key source of new nitrogen (27, 52, 71).

The importance of diazotrophy to *Spartina* marsh productivity has led to numerous studies of in situ rates of this process (19, 27, 52). Environmental variables that can influence diazotrophy, including host primary production and root exudation (7, 36, 55, 70) and edaphic physicochemical parameters (51, 54, 71) have also been intensively studied, as have the diazotrophic organisms themselves. Many different physiological types of diazotrophs have been isolated from the *Spartina* rhizoplane and rhizosphere (4, 20, 41, 52), but the true extent of the diversity of these organisms has not been determined. It is reasonable to assume that, as is typical of most types of natural samples (9, 60), only a small fraction of *Spartina* rhizosphere bacteria can be readily isolated into pure culture. It is also likely that many of the organisms that have been isolated, while able to grow rapidly on laboratory culture media, may be relatively unimportant in the natural environment. However, it is clear that the *Spartina* rhizosphere diazotroph assemblage is quite diverse, highly active under most conditions, and that diazotrophy by these organisms can be responsive to both host primary production and several key edaphic environmental variables. It is also clear that the diversity of this assemblage is poorly characterized at present, as is the case for most microbial groups (37).

The application of molecular biological methods has greatly facilitated the study of natural bacterial communities and the identification of functionally significant organisms within them (29, 64, 69, 74). Numerous researchers have employed various PCR primers specific for segments of *nifH*, the structural gene encoding the nitrogenase iron protein, to amplify partial *nifH* sequences from diazotrophic pure cultures (4, 5, 28, 33, 49, 76) and from various environmental samples, including marine plankton (8, 78), termite hindguts (34, 48), microbial mats and aggregates (50, 77), terrestrial soils (57, 72), and the rhizoplanes of rice (*Oryza sativa*) (66) and of shoal grass (*Halodule wrightii*) (33). These studies have yielded a diverse array of *nifH* sequences representing many, mostly unknown, lineages of diazotrophic *Bacteria* and *Archaea*. PCR amplification of *nifH* sequences, followed by their separation through denaturing gradient gel electrophoresis (DGGE), has recently been used to examine the complexity and stability of the diazotroph assemblage found in the *Spartina* rhizosphere (54–56). The *Spartina* rhizosphere diazotroph assemblage was shown to have a consistent species composition over substantial spatial scales, to be composed of a quite diverse array of organisms, and to be stable in composition over a seasonal cycle of host ontogeny and edaphic variability (56). Furthermore, the composition of this assemblage did not change dramatically in response to short-term manipulations of inorganic nutrient levels (54) or host root exudate levels (55). The extent to which the *Spartina* rhizosphere diazotroph assemblage has already been characterized by both classical pure culture methods (4) and molecular biological analyses (3, 4, 54–56; C. E. Bagwell and C. R. Lovell, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. N-215, p. 490, 1999) provides a strong foundation for the determination of the function-specific diversity of these organisms, i.e., the numbers of diazotrophic species and the physiological and phylogenetic groups of these species detectable in the rhizosphere.

In this study, we have determined the diversity of diazotro-

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phic bacteria in the rhizosphere of *Spartina* as defined by recoverable partial *nifH* sequences resolved by DGGE. We also compared the sequences recovered from *Spartina* rhizosphere to those from several other sources in order to identify sequences broadly distributed among plant-associated and/or marine habitats.

MATERIALS AND METHODS

Reference cultures. *Acetobacterium woodii* (ATCC 29683) was provided by Lars Ljungdahl, University of Georgia. *Azomonas agilis* (ATCC 7494) was purchased from the American Type Culture Collection (Rockville, Md.). *Azospirillum lipoferum* Sp 59b was provided by Peter van Berkum, United States Department of Agriculture. Four pure cultures previously isolated from the rhizoplanes of tall and short form *Spartina alterniflora* (TS210, SC16, SG21) and the black needle-rush, *Juncus roemerianus* (JC110) (4), were also used. These rhizoplane isolates are all gram negative and rod shaped, but differ substantially in their substrate utilization patterns and other physiological features (4). SC16 is a facultative anaerobe, SG21 is a microaerophile, and TS210 and JC110 are aerobes. These organisms have not been definitively identified to date, and physiological testing does not establish their placement in any known genus of free-living nitrogen-fixing bacteria. Cultivation conditions and DNA extraction procedures for these organisms have been described previously (4, 13, 38).

***nifH*-specific PCR primer design and specificity.** PCR primer design was based on analysis of *nifH* sequences from the NCBI GenBank database (6) by using the Wisconsin Genetics Computer Group software (18). In order to maximize the specificity of the primers for amplification of free-living diazotroph and rhizobium-like *nifH* sequences and to limit primer degeneracy as much as possible, *nifH* sequences from cyanobacteria, *Frankia*, and methanogens were excluded from our analysis (56). Most of the representatives of these groups have *nifH* sequences so divergent from those of other diazotrophs, that their inclusion results in excessive primer degeneracy. Also, *Frankia* and diazotrophic cyanobacteria, although very important in other environments, would not be expected to be prevalent in the rhizosphere of *Spartina alterniflora*. To further reduce degeneracy, the primers were synthesized with the artificial nucleotides P {6-(β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c-]-[1,2]oxazin-7-one} (35) and K [2-amino-9-(2-deoxy-β-ribofuranosyl)-6-methoxyaminopurine] (Glen Research, Sterling, Va.) (10). P pairs with either purine, and K pairs with either pyrimidine. Duplexes formed with primers containing these bases are more stable than would be the case for comparable primers containing a weakly pairing nucleotide, such as inosine (10, 35). The forward primer [5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′; primer 1] corresponds to *Klebsiella pneumoniae* (GenBank accession no. X13303) *nifH* position 25 to 44. The reverse primer (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG(G/C)ACGATGTAGATPTCCTG-3′; primer 2) sequence (underlined; the balance is the GC clamp) corresponds to *K. pneumoniae* position 436 to 453. Primer degeneracy was eightfold for the forward primer and twofold for the reverse primer. Primers were previously tested against DNA from known diazotrophs and nondiazotrophs (56) to establish their effectiveness and specificity.

***nifH* sequences from natural assemblages of *Spartina alterniflora* diazotrophs.** The *Spartina* rhizosphere *nifH* sequences analyzed in this study were obtained from denaturing gradient gels produced for previous studies of *Spartina* rhizosphere diazotroph assemblages (54–56). Briefly, rhizosphere samples were collected from an intertidal marsh zone on Goat Island in the North Inlet estuary near Georgetown, S.C. (33°20′N: 79°12′W). Sampling transects were established parallel to a small tidal creek within the tall form *Spartina* growth zone (near the creek bank) and the short form *Spartina* growth zone (inland from the tall form zone). Cores (2.4 cm in diameter by approximately 5 cm in length) were collected from the *Spartina* sod along these transects on several sampling dates in 1997. The *Spartina* rhizosphere includes the soil directly influenced by plant roots and rhizomes (see reference 11) and supports elevated levels of many microbial activities, including diazotrophy, relative to unvegetated soils and sediments (17, 73). It should be noted that these core samples contained high levels of live and dead roots and rhizomes (54, 55), as well as sediment and decaying plant-derived organic matter. Acetylene reduction rates measured in intact rhizosphere cores during the sampling period ranged from 0.35 to 2.63 μmol of ethylene produced per liter of sediment day^{-1} (56). Experimental plots for manipulations of inorganic nutrients (54) and host exudates (55) were established in the short-form *Spartina* zone, and cores were collected from these plots as well. DNA was extracted from the cores by using a previously described direct lysis procedure (39, 56).

Specific *nifH* sequence segments were amplified from bacterial *nifH* sequences for DGGE with primers 1 and 2. PCR was performed with rTth DNA polymerase XL (Perkin-Elmer, Foster City, Calif.), which has proofreading capability and high tolerance for various types of contaminants that might occur in DNA preparations (1). The reaction conditions and touchdown thermal cycling program have been described previously, as have the reagents and methods for DGGE (56). See references 55 and 56 for images of the gels sampled for this study. Gel plugs were taken from well-resolved bands from several gels by using wide-orifice micropipette tips. The gel plugs were stored in 100 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at −20°C until used in this study.

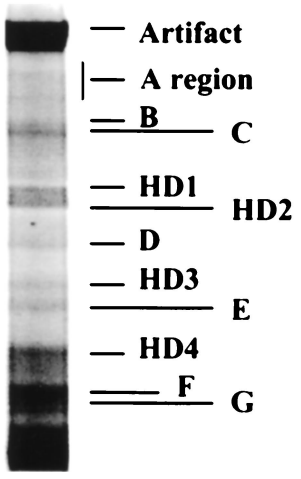


FIG. 1. Selected denaturing gradient gel lane showing relative positions of *nifH* amplimers analyzed in this study. PCR and DGGE conditions are described in Materials and Methods and reference 56.

Homoduplex and heteroduplex bands (Fig. 1) were previously identified by reamplification of 1-μl samples of the TE from each band and resolution of the amplimer(s) by DGGE (56). A homoduplex band is composed of two fully complementary strands and will yield a single DGGE band on reamplification. A heteroduplex band is composed of strands that are not completely complementary (i.e., from different parental sequences) and will yield three DGGE bands, one for each of the partially complementary strands, and one for the heteroduplex (23). Prior to adoption of the PCR and DGGE conditions described above, some reactions were performed with the Advantage GC PCR system (Clontech, Palo Alto, Calif.) and resolved with somewhat different denaturant gradients (75 to 95% denaturant). Since the bands from these gels did not correspond in position to bands from gels obtained with the optimized system, sequences from them were designated separately (X1 to X8 and Y1 to Y4). These sequences were included in our analysis to expand the *nifH* sequence database available for the *Spartina* rhizosphere.

Amplimer cloning and identification of different cloned amplimer sequences. Amplimers from gel plugs taken from DGGE gel bands were recovered by reamplification. The forward primer used in these reamplifications was 5′-GGTAT(C/T)GG(C/T)AA(A/G)TG(G/C)AC(G/C)AC-3′ (primer 3), and the reverse primer was 5′-GACGATGTAGAT(C/T)TCCTG-3′ (primer 4). These primers were selected to provide inexpensive alternatives to the P- and K-containing primers 1 and 2. Primer 3 had lower redundancy than primer 1 would have without P and K substitutions and corresponds to *K. pneumoniae* positions 37 to 56. Primer 4 is colinear with the non-GC clamp portion of primer 2. Reamplification employed the Expand High Fidelity System (error rate of 8.5×10^{-6}) by using the reaction mixture recommended by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.) and 1 μl of TE from each stored band plug. The following thermocycling program was used: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 30 s. This was followed by a final extension step at 70°C for 2 min. The same PCR primers and procedures were also used to amplify partial *nifH* sequences from DNA purified from *A. woodii*, *A. agilis*, and *A. lipoferum* and from four pure culture isolates from the *Spartina* and *Juncus* rhizoplanes (4). The amplimers from these reactions were ligated into the pGEM-T vector (Promega, Madison, Wis.), and the ligation reactions were used to transform competent *Escherichia coli* strain JM109. Recombinant colonies were selected on Luria-Bertani agar plates containing 80 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) ml^{-1}, 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 100 μg ampicillin ml^{-1}. Plates were incubated overnight at 37°C.

Small amounts of growth from selected recombinant colonies were collected with sterile toothpicks and transferred into PCR tubes. PCRs employing primers specific for the T7 and Sp6 RNA polymerase binding sites and Ampli*Taq* (Perkin-Elmer) were used to amplify the cloned sequences in a reaction volume of 25 μl. These reactions employed the same thermocycling program as that used in the DGGE gel band reamplification. Five microliters of each reaction mixture was run on 1.5% agarose–TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) gels to verify amplification of the insert DNA. Restriction digests were set up with 10 μl of PCR product and *Hae*III or *Msp*I in separate reaction mixtures having a final volume of 20 μl. Digests were incubated at 37°C overnight, and restriction fragments were resolved by electrophoresis in 4% NuSeive–3:1 agarose (FMC, Rockland, Maine)–0.5× TAE (1× TAE is 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) gels. Amplimers having the same restriction fragment length polymorphism (RFLP) patterns for both digests were considered to be the same

sequence. Any difference in either digest pattern was taken to indicate different amplimer sequences. All amplimers yielding unique RFLP patterns were sequenced.

DNA sequencing. Recombinant plasmids were purified from selected clones by using the Qiagen Plasmid Mini Kit (Santa Clarita, Calif.). Plasmid concentrations were determined fluorometrically. Sequencing reactions used 0.2 pmol of template DNA, fluorescently tagged T7 and Sp6 primers (LiCor, Lincoln, Nebr.), and the Thermosequenase DYEnamic Direct Cycle Sequencing kit with 7-deaza-GTP (Amersham, Cleveland, Ohio). The thermocycling program used was 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min. After amplification, loading dye was added to the samples, which were then analyzed with a Li-Cor DNA4000LS sequencer. Sequences were determined for both strands of each cloned amplimer.

Sequence analysis. NCBI GenBank *nifH* sequences from a variety of diazotrophs were selected for use in phylogenetic analyses and are listed in Table 1. Sequences from two vanadium nitrogenase iron protein genes were also included. Additional sequences from various *nif*-like genes, including *anfH* (Fe nitrogenase iron protein) and *frxC* and *bchX* (light-independent chlorophyllide reductase) were examined, but had low similarities to all of the *nifH* sequences analyzed and were dropped from further consideration. Environmental *nifH* sequences from the NCBI GenBank database that aligned with known cyanobacteria and *Archaea* were dropped from further analysis (see above), as were sequences belonging to other microbial groups, but having low similarities to *nifH* sequences from the *Spartina* rhizosphere. In all cases, primer sequences were removed prior to sequence analysis.

Nucleotide sequences were aligned by using ClustalW (63) and translated directly into amino acid sequences by using the "export file" options in MEGA (version 1.0; The Pennsylvania State University, University Park, Pa.). Alignments were hand corrected when necessary. Neighbor-joining phylogenies (58) were constructed in PAUP* (version 4.0b2) by 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 nucleotide sequences (first and second positions only) had no significant effect on the resulting dendrogram topology (data not shown). A pronounced G+C bias precluded the use of codon third positions in phylogenetic analyses. NifH amino acid sequences from *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* were used as outgroup taxa. Bootstrapping (22) was used to estimate the reliability of phylogenetic reconstructions (1,000 replicates).

Multiple *nifH* sequences were recovered from some DGGE bands. Analysis of duplex melting profiles of selected sequences from several bands was performed with WinMelt (version 2.0; Bio-Rad, Hercules, Calif.). This software package calculates duplex melting profiles by using the melting temperature of each base pair in the sequence, effects of neighboring nucleotides on those melting temperatures, positions and effects of major sequence domains, and the effects of secondary structure on the melting behavior of the sequence as a whole.

Nucleotide sequence accession number. The *nifH* sequence segments determined in this study are available in the GenBank database under accession no. AF216874 to AF216939.

TABLE 1. Reference *nifH* sequences from the GenBank database used in the phylogenetic analyses

Organism or source	GenBank accession no.
Known diazotrophic bacteria	
Acetobacter diazotrophicus	AF030414
Alcaligenes faecalis	X96609
Anabaena azollae	L34879
Anabaena sp. strain PCC7120	AF012326
Azoarcus communis	U97116
Azoarcus indigens	U97118
Azoarcus tolulolyticus	U97122
Azospirillum brasilense	M64344
Azotobacter chroococcum	M73020
Azotobacter chroococcum vnfH	X51756
Azotobacter vinelandii	M20568
Azotobacter vinelandii vnfH	M32371
Bradyrhizobium japonicum	E01169, K01620
Chlorobium tepidum	AF065617
Clostridium cellobioparum	U59414
Clostridium pasteurianum	M21537
Desulfobacter curvatus	AF065619
Desulfonema limicola	AF065618
Desulfovibrio gigas	U68183
Frankia alni	L41344
Herbaspirillum seropedicae	Z54207
Klebsiella pneumoniae	J01740
Klebsiella sp.	M63691
Marichromatium purpuratum	AF059648
Methanobacterium thermautotrophicum	AE00916
Methanosarcina barkeri	X56072
Pseudomonas sp.	AF117976
Pseudomonas stutzeri	AF117977, AF117978
Rhizobium leguminosarum biovar phaseoli	M10587
Rhizobium leguminosarum biovar trifolii	K00490
Rhizobium sp.	M16709, M26961
Rhodobacter capsulatus	M15270, X07866
Rhodobacter sphaeroides	AF031817
Rhodospirillum rubrum	M33774
Sinorhizobium meliloti	J01781
Thiobacillus ferrooxidans	M15238
Trichodesmium thiebautii	U23507
Vibrio diazotrophicus	U23650
Unnamed cultivated diazotrophs	
Marine microbial mats	AF046827-AF046854, U43438, U43442–U43445
Nonaxenic cyanobacterial cultures	U43436, U43437, U43439
Environmental *nifH* sequences	
Halodule wrightii (shoal grass) rhizoplane	M63688, M63689
Oryza sativa (rice) rhizoplane	D26284-D26306
Douglas Fir forest soil and litter	AF099775–AF099798
Marine microbial mats	U23633–U23646
Marine plankton	AF016592–AF016615, AF016617, AF016618, AF059621–AF059623, AF059629, AF059643–AF059647, U26186
Antarctic diazotrophic microbial consortia	AF049033–AF049040, AF049043

RESULTS AND DISCUSSION

There were usually 11 prominent, well-resolved bands per lane of typical DGGE gels loaded with *nifH* amplimers from field *Spartina alterniflora* rhizosphere samples (Fig. 1) (54–56). This banding pattern was highly reproducible, but the intensity of any given band often varied among replicate sample lanes within a gel. Band intensities also varied among samples from different plant height zones, dates of sample collection, or experimental manipulation. The best-resolved and strongest bands representing those designated in Fig. 1 were sampled from four gels. An additional band, designated band B prime (BP) appeared in some gel lanes from a manipulative experimental study (55), and the best-resolved, strongest example of this band was also sampled. Amplimers from all of these prominent bands were successfully recovered by reamplification and were cloned. It should be noted that additional, typically faint bands were observed sporadically in the DGGE gels, but since these bands were not seen consistently, only two (A2 and A5) were examined in this study.

RFLP analysis of cloned amplimers was employed to differentiate sequences recovered from a given DGGE gel band. In preliminary studies, *nifH* sequences from the same well-resolved DGGE gel band and having identical RFLP patterns were either identical in nucleotide sequence or differed only slightly. None of these minor differences resulted in different amino acid sequences. Some bands contained a single RFLP pattern, but multiple patterns were recovered from others (Table 2). Note that band F was originally thought to be a doublet (56), but RFLP analysis revealed two distinct groups of sequences (bands F and G).

TABLE 2. Percent similarities of *Spartina* rhizosphere NifH amino acid sequences to the most similar sequence(s) from known diazotrophic bacterial species[a]

Spartina sequence	Organism with most similar sequence(s)	% Similarity
Presumed γ-*Proteobacteria*		
A2	*Azotobacter chroococcum*	94.2
B2	*Azoarcus indigens*	97.3
B5	*Azoarcus indigens*	97.3
BP2, HD2-4	*Pseudomonas stutzeri*	97.5
BP3, HD4-1, HD4-2, X1, X3, X5, X8, Y1, Y2	*Pseudomonas stutzeri*	98.3
BP4	*Pseudomonas stutzeri*	96.6
C1	*Azotobacter chroococcum*	96.4
E2, E4, F5, F9	*Azomonas agilis*, *Azotobacter chroococcum*, *Klebsiella* sp., *Marichromatium purpuratum*	93.5
F1	*Azomonas agilis*, *Azotobacter chroococcum*	87.8
F2	*Azoarcus indigens*	95.6
F3	*Klebsiella* sp.	92.5
F4	*Marichromatium purpuratum*	92.5
F6	*Marichromatium purpuratum*	90.7
F7	*Marichromatium purpuratum*	91.6
F8	*Marichromatium purpuratum*	89.7
HD2-2, X7	*Azotobacter chroococcum*, *Azotobacter vinelandii*	85.6
HD2-3	*Azoarcus indigens*	92.9
HD3-6	*Marichromatium purpuratum*	89.7
HD3-8	*Pseudomonas stutzeri*	97.5
X2	*Pseudomonas stutzeri*	98.3
X6	*Pseudomonas stutzeri*	95.8
Y3	*Pseudomonas stutzeri*	94.1
Y4	*Azotobacter chroococcum*	95.0
JC110	*Klebsiella* sp., *Vibrio diazotrophicus*	97.2
SG21	*Azomonas agilis*, *Azotobacter chroococcum*	95.0
TS210	*Pseudomonas stutzeri*	96.6
Presumed α-*Proteobacteria*		
A4	*Azoarcus tolulolyticus*	95.5
E1	*Bradyrhizobium japonicum*	87.0
G1	*Azospirillum brasilense*, *Sinorhizobium meliloti*	90.6
G2	*Azospirillum brasilense*	90.6
G3	*Azospirillum brasilense*, *Sinorhizobium meliloti*	92.0
G4	*Azospirillum brasilense*	92.8
SC16	*Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Rhizobium* sp. M16709	93.5
Presumed anaerobes		
A1	*Desulfonema limicola*	87.6
A3	*Desulfonema limicola*	88.6
A5	*Desulfonema limicola*	85.7
B1	*Desulfonema limicola*	90.5
B3, HD1-1	*Desulfovibrio gigas*	84.2
B4	*Desulfonema limicola*	87.6
D1, D2, HD3-1, HD3-2	*Desulfovibrio gigas*	83.5
HD2-1	*Desulfonema limicola*	85.7
HD3-3	*Desulfonema limicola*	88.6
HD3-4	*Desulfovibrio gigas*	84.1
HD3-5	*Desulfonema limicola*	86.7
HD3-7	*Desulfobacter curvatus*	88.8
X4	*Desulfonema limicola*	90.5

[a] Percent similarities are from the distance matrix constructed in PAUP* (version 4.0b2). *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 they contain, but some sequences from other phylogenetic groupings can occur. Pure culture isolates from the *Spartina* rhizoplane are also included.

Sequences from DGGE gel bands and laboratory cultures were initially translated and examined for key, highly conserved amino acid residues that are important in nitrogenase iron protein structure and function (16, 53). Within the segments analyzed, 11 amino acids including (*Klebsiella pneumoniae* numbering) Lys 15 and Ser 16 (within the Mg ATP binding domain), Arg 100 (the ADP ribosylation site), Asp 125 (possibly involved in protein conformation changes), Asp 129 (involved in ATP hydrolysis), Arg 140 and Lys 143 (contribute to salt bridge formation), and four conserved Cys residues (no. 38, 85, 97, and 132, two of which coordinate the Fe_4S_4 cluster) were used as markers for determining sequence accuracy. Only two sequences (BP1 and E3) had substitutions at more than one of these positions, and in both cases, residues considered essential to protein function (i.e., Cys 132 for BP1 and Arg 100 for E3) were affected. Only two sequences (A2 and G1) had a single substitution each, neither of which would be expected to severely impact protein activity (16). Sequences BP1 and E3

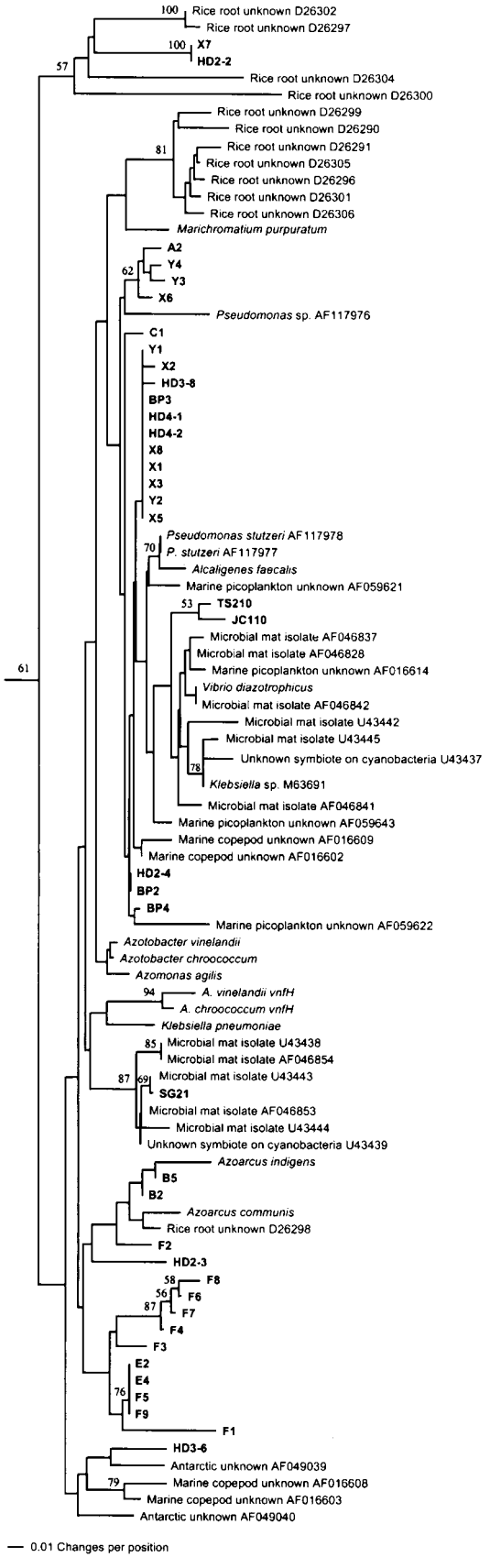
were not included in phylogenetic analyses; sequences A2 and G1 were used.

Due to the pronounced G+C bias in the third positions of codons and the similar outcomes of phylogenetic analyses employing polypeptide sequences or first and second nucleotide position sequences, all phylogenetic analyses reported employed the NifH polypeptide sequences (47, 75). Phylogenetic trees were constructed from *Spartina* rhizosphere sequences (59 in all), sequences from pure culture isolates from the *Spartina* rhizoplane (4 sequences), and sequences from reference strains of known diazotrophs (45 sequences). Relatively few sequences from cyanobacteria or from diazotrophic *Archaea* were included, since the primers used to recover *Spartina* rhizosphere *nifH* sequences for DGGE analysis would be unlikely to amplify sequences from these organisms (56). As has been observed in other studies (8, 48, 66, 74, 77, 78), there are several large clusters of NifH sequences representing important major groups of diazotrophs. The neighbor-joining algorithm yielded a topology that contained three major NifH sequence clusters that contained *Spartina* rhizosphere sequences and were found in greater than 60% of all bootstrap replicates.

The first major cluster was well supported by bootstrapping (61%) and contained sequences from known members of the γ subdivision of the division *Proteobacteria* (γ-*Proteobacteria* [and some β-*Proteobacteria*]), 36 sequences from *Spartina* rhizosphere, and three sequences from rhizoplane pure culture isolates (Fig. 2). This cluster was designated the γ-*Proteobacteria* cluster due to the preponderance of sequences deriving from known organisms in that group. Pairwise similarities among sequences in this cluster averaged 92.1%. Several of the *Spartina* sequences were highly similar to the NifH sequences from *Pseudomonas stutzeri* and may represent species in the genus *Pseudomonas* or closely related genera. However, most sequences segregated into distinct clades to the exclusion of sequences from known species. Among these was a cluster of sequences from bands E and F that had substantial similarity to the sequence from SG21, an unnamed *Spartina* rhizoplane isolate.

Numerous NifH sequences from unknown bacteria inhabiting plant-associated and/or marine environments also fell into the γ-*Proteobacteria* cluster (Fig. 2). Many of these had relatively small similarity scores to sequences from the *Spartina* rhizosphere and are not shown. However, one sequence from the rice rhizoplane (D26298) was placed in a clade containing two band B sequences, and one sequence from an Antarctic diazotrophic consortium (AF049039) was sister to rhizosphere sequence HD3-6 (93.1% similarity). Several sequences from γ-*Proteobacteria* isolated from marine microbial mats were also highly similar to the SG21 sequence. These observations may imply that some of the γ- and/or β-proteobacterial diazotrophs represented by our DGGE gel bands and sequences may be amenable to isolation and laboratory cultivation.

It is interesting in this regard that no *Spartina* rhizosphere NifH sequences were highly similar to NifH sequences from the *Azotobacteriaceae*. Based on pure-culture isolation methods, *Azotobacter*-like organisms have been reported as abundant diazotrophs in vegetated salt marsh sediments (20). Organisms belonging to the *Azotobacteriaceae* can be readily isolated from *Spartina* and *Juncus roemerianus* rhizoplanes (4), and *nifH* sequences from them are efficiently amplified with the primers employed in this study (data not shown). It seems likely from these results that members of the family *Azotobacteriaceae*, while efficiently recovered from vegetated salt marsh sediments and easily cultivated in the laboratory, may not be numerically important diazotrophs in the *Spartina* rhizosphere.



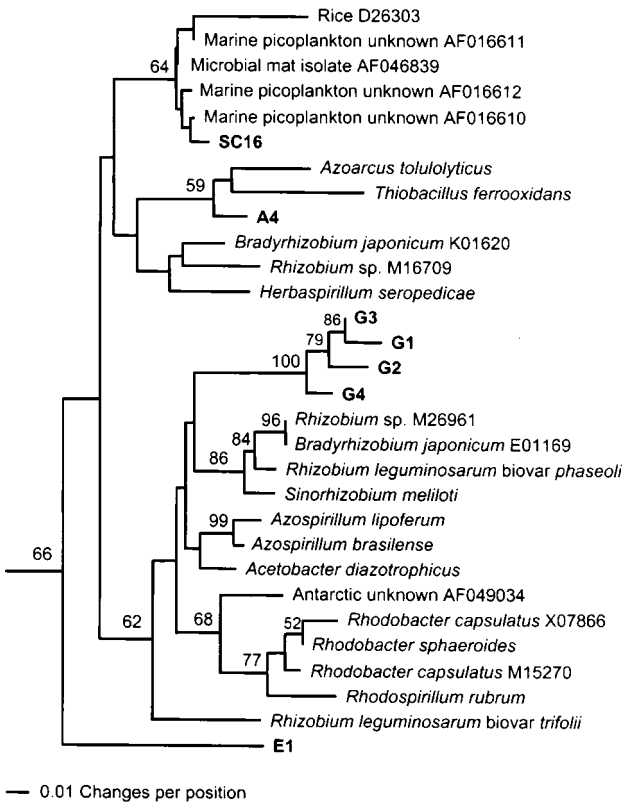


FIG. 3. Phylogenetic analysis of *Spartina* rhizosphere NifH amino acid sequences from presumed α-*Proteobacteria*, from various known α-*Proteobacteria*, and from selected unknown, presumed α-*Proteobacteria* from other plant-associated and/or marine environments. The percentage of 1,000 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

Similarly, *Klebsiella* sp. and their relatives, while readily isolated from rhizoplanes of wetlands plants (4, 14), were not represented by any of the *nifH* sequences we recovered. It is also possible that PCR biases prevented recovery of these sequences from *nifH* sequence mixtures, but quantitative determination of the abundance of *Azotobacter*- and *Klebsiella*-like organisms (Bagwell and Lovell, Abstr. 99th Gen. Meet. Am. Soc. Microbiol.) will be required to resolve this issue.

The second major cluster was found in 66% of 1,000 bootstrap replicates and contained sequences from many familiar diazotrophs, including species of the *Rhizobiaceae* and the purple non-sulfur bacteria (Fig. 3). In addition to known α-*Proteobacteria*, some β-*Proteobacteria* (*Azoarcus tolulolyticus* and *Herbaspirillum seropedicae*) and the γ-proteobacterium *Thiobacillus ferrooxidans* are also represented in this cluster, as well as six sequences from the *Spartina* rhizosphere, and a single sequence from a rhizoplane isolate. Lateral transfer of *nifH* is considered probable among diazotrophs belonging to several subgroups of the α- and β-*Proteobacteria* (21, 28, 31) and may explain the appearance of sequences from β- and γ-*Proteobacteria* in this cluster. This cluster was designated the α-*Proteobacteria* cluster due to the preponderance of sequences derived from known organisms in that group. Overall similarity among sequences in the α-*Proteobacteria* cluster was 89.8%.

Inclusion of unknown environmental NifH sequences from other sample types substantially expanded the α-*Proteobacteria* cluster (Fig. 3). For example, almost all NifH sequences recovered from Douglas Fir forest soil and litter (72) and one sequence from the rice rhizoplane fell into this well-supported cluster. Numerous sequences in this cluster have also been recovered from various planktonic marine environments (78), but few from rhizoplanes (33, 66) or rhizospheres (this study) of wetland plants to date. Most *Spartina* rhizosphere NifH sequences formed a monophyletic group to the exclusion of sequences from unknown, presumed α-*Proteobacteria*. In contrast, the NifH sequence from the rhizoplane isolate SC16 was identical over the length it shared with a sequence from an unknown diazotrophic bacterium in marine picoplankton (AF016612) and from a pure culture isolate from a marine microbial mat (AF046839). It should be noted that the algorithms used to construct dendrograms produce an adjusted "grand average" representation of the various pairwise distance values from the sequence distance matrix. In our analyses, gaps were treated as missing data and the sequences listed as identical (for the sequence stretches they have in common) were of unequal lengths. The software scoring all of the sequences against each other can score otherwise identical sequences differently based on differences in the "missing data" among the sequences that include those data. This can result in horizontal distances in the dendrogram between sequences having 0% dissimilarity in the distance matrix. SC16 is physiologically similar to species of the *Rhizobiaceae* (4), but has not been definitively identified as yet.

The third cluster contained NifH sequences from known obligate anaerobes and 17 sequences from unknown bacteria from the *Spartina* rhizosphere (Fig. 4). Although completely supported by bootstrap analysis (100%), the anaerobe cluster contained many highly divergent lineages with an average similarity of only 85.5%. Most of the *Spartina* rhizosphere sequences formed well-supported monophyletic groups, and none were closely related to any sequence from a known diazotrophic anaerobe. The largest similarity score between any *Spartina* NifH sequence and any sequence from a known anaerobic diazotroph was 90.5%, and was between two DGGE band sequences (B1 and X4) and *Desulfonema limicola*. An additional 10 sequences from rice rhizoplane (66) and 2 sequences from the rhizoplane of shoal grass (*Halodule wrightii*) (33) were included in this cluster. The largest similarity score between any *Spartina* sequence and any NifH sequence from any other type of environmental sample was 93.1% and involved DGGE band sequence HD3-3 and a sequence recovered from an unknown bacterium associated with a marine copepod (AF016595). We view this relationship with caution, however, since the branch representing the copepod-associated unknown sequence is quite long.

The finding of numerous NifH sequences from anaerobes is consistent with the known characteristics of the *Spartina* rhizosphere. While the rhizoplane of *Spartina* and sediments in close proximity to roots receive substantial, transient oxygen input through the aerenchyma system (2, 62), rhizosphere sediments not closely associated with live plant roots are likely to be anoxic (30), and our samples included live and dead roots and rhizomes along with surrounding sediment and organic matter. Even the *Spartina* rhizoplane would be expected to rapidly become suboxic after the onset of darkness. Inhibitor studies of nitrogen fixation associated with *Spartina* roots and rhizosphere sediments have revealed a potentially significant role of diazotrophic sulfate-reducing bacteria (26), and this

FIG. 2. Phylogenetic analysis of *Spartina* rhizosphere NifH amino acid sequences from presumed γ-*Proteobacteria*, from various known γ-*Proteobacteria*, and from selected unknown, presumed γ-*Proteobacteria* from other plant-associated and/or marine environments. The percentage of 1,000 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

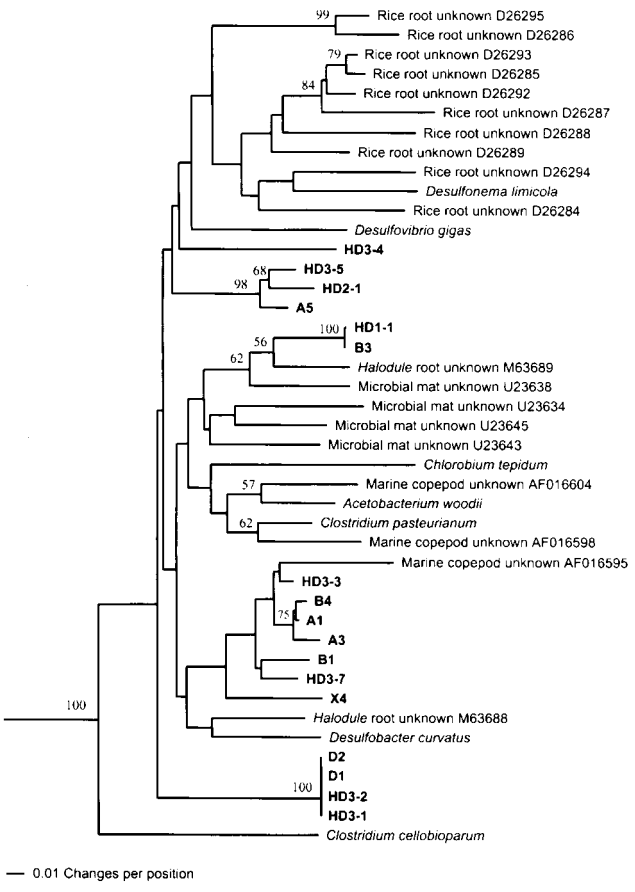


FIG. 4. Phylogenetic analysis of *Spartina* rhizosphere NifH amino acid sequences from unknown, presumed anaerobic bacterial sequences, from various known anaerobic bacteria, and from selected unknown, presumed anaerobic bacteria from other plant-associated and/or marine environments. The percentage of 1,000 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

seems to be supported by our results. While almost all of the *Spartina* anaerobe NifH sequences were less than 90% similar to those from any known anaerobe, the highest similarities were all between *Spartina* anaerobe sequences and sulfate reducer sequences (Table 2). Low representation of organisms very similar to the known clostridia, as reported by Dicker and Smith (20) for surface sediments from various vegetated salt marsh zones, may also be indicated. We have recovered gram-positive bacterial 16S ribosomal DNA sequences from DNA purified from other types of marine sediment samples by using the same direct lysis method used here (G. Matsui and C. R. Lovell, unpublished observations). We have also successfully amplified *nifH* sequences from gram-positive fermentative anaerobes (including *Acetobacterium woodii*, a non-spore-forming gram-positive organism related to *Clostridium* [61]). We surmise that the clostridia were either numerically insignificant in the *Spartina* rhizosphere or that *nifH* sequences from these organisms were recovered too inefficiently to permit their detection on our DGGE gels. Ueda et al. (66), using quite different DNA extraction procedures and PCR primers, also recovered few *nifH* sequences from rice rhizoplane that had substantial similarity to those from the clostridia, while sequences clustering with those from sulfate reducers were common. Anaerobe NifH sequences are highly divergent, even within some defined, monophyletic clusters (e.g., the δ-*Proteobacteria*, the low G+C *Firmicutes*). It is also possible that the membership of some *Spartina* anaerobe sequences in these groups may be obscured by such divergence.

NifH sequences are quite conservative, so similarity values for closely related species, such as *Azotobacter chroococcum* and *A. vinelandii* (99.3%) or *Azospirillum brasilense* and *A. lipoferum* (99.3%), are typically very high. Thus, even the highest similarity scores observed between *Spartina* rhizosphere NifH sequences and those from known diazotrophic bacteria are most likely too low to reflect species-level relationships (Table 2). In addition, many of the *Spartina* NifH sequences were found in distinct, well-supported clades that were monophyletic with respect to known NifH sequences. The largest similarity score between any *Spartina* rhizosphere NifH sequence and any NifH sequence from any known diazotroph was between several sequences (HD4-1, HD4-2, X1 to X3, X5, X8, Y1, and Y2) and *Pseudomonas stutzeri* and amounted to 98.3%. As expected from the primer design, no clustering of *Spartina* rhizosphere NifH sequences with any archaeal, cyanobacterial, or *Frankia* NifH sequences was observed (data not shown).

Numerous NifH sequences from rhizoplanes of other wetlands plants, particularly from rice, also fell into the three major sequence clusters containing the *Spartina* sequences. Like *Spartina*, rice also introduces oxygen into the rhizosphere via aerenchyma transport (32), but the existence of reduced microzones in close proximity to the roots is likely. Clearly, the superficial similarities among the rhizoplane and rhizosphere microenvironments of these wetlands plants did not lead to the development very similar diazotroph assemblages. However, it is noteworthy that the assemblages from the only two wetlands plants from whose rhizosphere diazotroph assemblages have been examined in some detail, *Spartina* (this study) and rice (66), yielded numerous sequences from the γ-*Proteobacteria* and the anaerobes, but few from the α-*Proteobacteria*.

Several bands from the DGGE gels contained heterogeneous sequences. While DGGE can separate sequences differing by as little as a single nucleotide (25), a given band can contain a mixture of sequences (45, 56). This is due at least in part to the fact that the denaturant gradient and gel running conditions are optimized to yield a profile of bands encompassing the total recoverable diversity of sequences from a sample. However, DGGE separates sequences based on their melting profiles (25, 59), and it is possible for nonidentical sequences to have profiles sufficiently similar to allow their near codenaturation in the gel. The five band B sequences had some substantial sequence differences (Table 2), but extremely similar melting profiles (Fig. 5), permitting their effective codenaturation in our DGGE gels. Melting profiles of sequences within more homogeneous bands (bands F and G) were effectively identical (data not shown). The occurrence of DGGE bands containing nonidentical sequences would greatly hinder attempts to interpret changes in organism abundance on the basis of DGGE band intensity. For this reason, Piceno et al. (56) and Piceno and Lovell (54, 55) examined only band numbers and positions in their analyses of natural diazotroph assemblages.

The finding of radiations of highly similar *nifH* sequences in certain DGGE bands also has an interesting implication for the population dynamics and ecological functions of rhizosphere diazotrophs. Bagwell et al. (4) recovered numerous strains of diazotrophs from *Spartina* and *Juncus* rhizoplanes, and in several cases, these strains formed distinct clusters of physiologically similar, but distinguishable organisms. Some closely related strains having high levels of genomic DNA homology differed substantially in key physiological character-

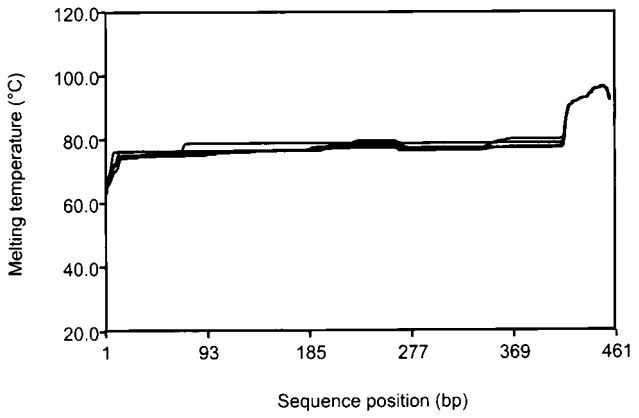


FIG. 5. Melting profiles of the partial *nifH* sequences recovered from denaturing gradient gel band B (B1 to B5) (Table 2). The sequences analyzed included both primers and the GC clamp to reflect their behavior in DGGE gels.

istics (3). The occurrence of groups of strains or very closely related species (i.e., ecotypes) (23, 24), all able to inhabit similar niches (such as different locations on the rhizoplane), may result in a spectrum of organism physiological features that could support diazotrophy at different stages of host plant ontogeny or under different edaphic conditions. This microdiversity (3, 42) could provide an important foundation for functional redundancy in a highly dynamic microenvironment, such as the *Spartina* rhizosphere, where conditions can vary over relatively short time frames.

The micro- and macrodiversity of diazotrophs occurring in the *Spartina* rhizosphere and the dissimilarity of assemblages from presumably comparable habitats underscore the largely undescribed diversity of plant-associated diazotrophic bacteria. The diversity of diazotrophs inhabiting the rhizospheres of wetlands plants clearly reflects a plethora of functioning microniches, and these microenvironments may be much more dynamic than is generally appreciated. This complex array of unknown species and undefined ecotypes is certainly capable of maintaining high levels of nitrogen fixation, the unifying ecological function of the group, across a broad range of host-driven and abiotic environmental variability.

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