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Diversity of Heterotrophic Nitrogen Fixation Genes in a Marine Cyanobacterial Mat

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The diversity of nitrogenase genes in a marine cyanobacterial mat was investigated through amplification of a fragment of *nifH*, which encodes the Fe protein of the nitrogenase complex. The amplified *nifH* products were characterized by DNA sequencing and were compared with the sequences of nitrogenase genes from cultivated organisms. Phylogenetic analysis showed that similar organisms clustered together, with the exception that anaerobic bacteria clustered together, even though they represented firmicutes, δ-proteobacteria, and γ-proteobacteria. Mat *nifH* sequences were most closely related to those of the anaerobes, with a few being most closely related to the cluster of γ-proteobacteria containing *Klebsiella* and *Azotobacter* species. No cyanobacterial *nifH* sequences were found from the mat collected in November when *Microcoleus chthonoplastes* was the dominant cyanobacterium, but sequences closely related to the cyanobacterium *Lyngbya lagerheimeii* were found during summer, when a *Lyngbya* strain was dominant. The results indicate that there is a high diversity of heterotrophic nitrogen-fixing organisms in marine cyanobacterial mats.

Marine benthic cyanobacterial mats are a common feature of nearshore environments (5, 6) and contain metabolically diverse groups of microorganisms that perform critical steps in biogeochemical cycles. Biological nitrogen (N_2) fixation is an important source of nitrogen for cyanobacterial mats (2), as well as for the marine environment (4). The measurement of nitrogen fixation rates provides no information on the diversity or types of organisms which fix nitrogen in the environment. Experimental manipulations, such as incubation under light or dark conditions, are often used to infer whether N_2 fixation is carried out by heterotrophic or photosynthetic organisms. The results of such experiments can be interpreted in multiple ways, since interactions and nutrient exchange between microorganisms can cause N_2 fixation to be light stimulated, even if the N_2 fixer is not itself photosynthetic. Identification of N_2-fixing organisms in microbial assemblages has been dependent on culturing microorganisms (24), but <10% of diazotrophs are believed to be cultivable from the marine environment (11).

It has frequently been assumed that N_2 fixation in cyanobacterial mats is due to the visually prominent cyanobacteria themselves (1, 21) although the mats are composed of a rich assemblage of prokaryotic organisms, any of which could potentially fix N_2 (20). A few N_2-fixing cyanobacterial isolates have been obtained from mats (21, 26). Nitrogenase genes, which encode the proteins that catalyze the fixation of nitrogen, are widely dispersed among bacterial and archaeal genera (22, 27, 30) such that taxonomic information alone cannot be used to predict N_2 fixation capabilities. Thus, many different types of organisms are responsible for N_2 fixation in nature.

The genes for nitrogenase are highly conserved (22), yet previous studies indicated that the DNA sequence of nitrogenase genes contains useful taxonomic information (3). Degenerate oligonucleotides that encode the sequence for two very highly conserved amino acid sequences within *nifH* have been shown to be effective in amplification of *nifH* from diverse microorganisms (3, 16). Sequences of a 325-bp fragment of *nifH*, the gene encoding dinitrogenase reductase (component II) of nitrogenase, were amplified from cyanobacterial mat DNA obtained from the Rachel Carson Research Reserve in coastal North Carolina. A diverse group of *nifH* sequences was obtained from the cyanobacterial mat, but no cyanobacterial *nifH* genes were detected. These results suggest that heterotrophic N_2-fixing microorganisms may be particularly abundant, at least at certain times during the year, and more importantly that there are many unidentified and uncharacterized N_2-fixing organisms in the marine environment.

MATERIALS AND METHODS

The microbial mat investigated was located in an intertidal lagoonal region of Bird Shoal, a dredge spoil island which is part of the Rachel Carson National Estuarine Research Reserve (NOAA/NERRS), near Beaufort, N.C. While severe storm activity (tropical storms and hurricanes) can remove mats or cover them with sand, mats proved to be a permanent feature of the lagoon throughout the study period (no major storms transited the area). While mats persisted on a year-round basis, their development (i.e., thickness) varied seasonally, ranging from approximately 5 mm in wintertime to >10 mm in summertime. The upper 3 to 5 mm of the mat "fabric" was composed of a mixture of filamentous cyanobacteria (*Microcoleus chthonoplastes* and *Lyngbya aestuarii*) and diatoms. The cyanobacteria accounted for approximately 75% of the microalgal biomass. All cyanobacteria species were present year-round, but *M. chthonoplastes* was the overwhelmingly dominant species from late fall through winter, while *L. aestuarii* assumed greater importance (in biomass) during summer months. Minor amounts of *Oscillatoria* sp. and *Synechocystis* sp. were present year-round. Distinct vertical biogeochemical zonation (ranging from fully oxic to anoxic) was present all year but was more pronounced with the development of a thicker mat in summertime. This indicated a potential for supporting a wide range of diazotrophs (ranging from oxygenic phototrophs like cyanobacteria to anoxygenic phototrophs [photosynthetic bacteria] and microaerophilic and anaerobic microheterotrophs). Previous and ongoing work (1, 20a) indicates that mat primary production (CO_2 fixation) during summer months is 3 to 10 times higher than in wintertime. Summertime N_2 fixation (acetylene reduction) rates are two to five times higher than wintertime rates.

The cyanobacterial mat was collected in November 1993, when the cyanobacterial component was dominated by aggregated filaments of *M. chthonoplastes*. The mat slice (0.5 cm thick) was sectioned into two layers, and DNA was

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extracted from the upper oxic zone (dominated by cyanobacteria) and the lower more reduced portions of the mat, using a modification of the DNA extraction protocol of Giovannoni et al. (9). In order to obtain sequences representing groups not represented in the *nifH* databases, DNA was also extracted from pure cultures of *Vibrio diazotrophicus* (ATCC 33466), *Bacillus azotofixans* (ATCC 35681), and *Methanobrevibacter arboriphilicus* DC (DSM 1536; gift of T. Miller, New York State Department of Health, Wadsworth Center for Laboratories and Research, Albany). Following lysis, the lysates were extracted with an equal volume of phenol and then with an equal volume of chloroform. The extracts were ethanol precipitated, dried, and resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0). The *nifH* fragments were amplified from the community DNA with degenerate primers (16, 31). The amplified fragments were gel purified and cloned into a plasmid vector (pT7Blue; Novagen, Madison, Wis.). Clones containing the amplified *nifH* fragment were sequenced by the dideoxynucleotide termination method (25). The inserts in 14 clones containing different *nifH* fragments from the upper and lower fractions of the mat were completely sequenced on both strands. The DNA sequences were translated, and the amino acid sequences were aligned manually, using GDE software (S. Smith, Millipore Corp.). Identical sequences were only obtained four times, and 19 additional sequences, most closely related to the mat sequences, were cloned but only partially sequenced. Fourteen mat *nifH* sequences were compared with 50 *nifH* sequences (Table 1). Phylogenetic trees were constructed from the deduced amino acid sequences by both parsimony and distance (maximum likelihood option of neighbor-joining program in PHYLIP) methods (7).

RESULTS

Multiple unique sequences were obtained from the cyanobacterial mat (Fig. 1). None of the sequences that were obtained are closely related to cyanobacterial sequences (Table 1; Fig. 1). Identical sequences were only obtained four times. The sequences obtained are related to sequences from one of two groups: the γ-proteobacteria or anaerobes containing sulfate-reducing bacteria, clostridia, and purple sulfur bacteria (Fig. 1). Thirteen of 19 additional clones, which were only partially sequenced, are most closely related to *Chromatium buderi.*

The *nifH* phylogenetic tree is largely consistent with the 16S rRNA phylogenetic tree (19, 29) with respect to clusters of organisms, but the higher-order branching is less well defined (see bootstrap values, Fig. 1). Heterocystous cyanobacterial *nifH* sequences form a tight cluster nested within the nonheterocystous sequences (Fig. 1). N_2-fixing species in the gamma subdivision of the proteobacteria, *V. diazotrophicus*, *Azotobacter vinelandii*, and *Klebsiella pneumoniae*, also group together on the basis of *nifH* sequences (Fig. 1), as do the α-proteobacteria *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhizobium meliloti*, and *Azospirillum brasilense*. The second alternative nitrogenase gene sequence from *Azotobacter* clusters closely with the alternative *nifH* gene from *Rhodobacter capsulatus* and the *Clostridium pasteurianum nifH*3 sequence (Fig. 1). One cluster of *nifH* sequences is not consistent with 16S rRNA phylogeny. Sequences of *nifH* from *C. pasteurianum*, *Chromatium buderi*, and *Desulfovibrio gigas* cluster together, even though they represent firmicutes, γ-proteobacteria, and δ-proteobacteria, respectively (Fig. 1).

The cyanobacterial mat *nifH* sequences obtained from the North Carolina mat are clearly not cyanobacterial *nifH* genes (Fig. 1). A few sequences cluster within a branch containing *Azotobacter* and *Klebsiella* spp., but most group within clusters containing *Chromatium* (purple sulfur bacteria), *Desulfovibrio* (sulfate reducers), or *Clostridium* (low-G+C firmicutes, strict anaerobes) spp. (Table 2 and Fig. 1). Some of the sequences obtained from the mats are closely related to sequences that were previously obtained from marine sea grass roots (DNA extracted from *Halodule* roots) (16). The cluster of *nifH* sequences that contained mat, *Klebsiella*, and *Azotobacter* sequences also contains the sequence from a marine heterotroph isolated from planktonic *Trichodesmium* aggregates (M1) (16).

Although the anaerobe and mat *nifH* sequences cluster by phylogenetic analysis (Fig. 1), the sequences are not very similar at the DNA level (Table 2). The deduced amino acid sequence similarities are surprisingly high among the anaerobes *C. pasteurianum*, *D. gigas*, and *Chromatium buderi* (82 to 84%) given the relatively low DNA sequence similarities (60 to 74%; Table 1). The most closely related anaerobes are *D. gigas* and *Chromatium buderi* (74% similar at the DNA level).

DISCUSSION

The results demonstrated that there are diverse groups of noncyanobacterial N_2-fixing microorganisms within the mat, and repeated attempts to find a cyanobacterial *nifH* gene from the November mat were unsuccessful. In contrast, when DNA sequences of *nifH* genes were amplified from *L. aestuarii*-dominated mats collected from this location during summer, the dominant *nifH* sequence was most closely related to the cyanobacterium *L. lagerheimii nifH* sequence (87% identical at the DNA level [unpublished data]). A strain of *L. aestuarii* that does express nitrogenase has previously been isolated from these mats (21). Therefore, cyanobacterial *nifH* sequences are detected when N_2-fixing cyanobacteria are present and abundant. These results indicate that the *M. chthonoplastes* strain present in the winter mat does not fix nitrogen or that the *M. chthonoplastes* cells are greatly outnumbered by heterotrophic N_2-fixing microorganisms at that time of year. The genetic potential for N_2 fixation appears to shift from cyanobacterial diazotroph dominance in summer to bacterial diazotroph dominance in fall and winter, which is consistent with conclusions based on experimental evidence (1). The relative importance of cyanobacteria and bacteria in carbon and nitrogen dynamics apparently changes in winter when environmental conditions are suboptimal for cyanobacterial growth.

Identification of the species from which the noncyanobacterial *nifH* genes were derived is dependent on the ability to classify *nifH* genes. Previous studies indicated that N_2-fixing organisms may be identified by analysis of a *nifH* fragment amplified with a pair of degenerate oligonucleotide primers (3, 16). This is possible since the phylogeny of *nifH* appears to be largely similar to 16S rRNA phylogeny (12, 30), although some discrepancies have been noted (13, 18). The phylogeny of cyanobacterial *nifH* genes is consistent with the phylogeny of cyanobacterial 16S rRNA genes (10) (Fig. 1). *nifH* sequences for *Bacillus* spp. and vibrios were not previously available. The *nifH* sequence for *V. diazotrophicus* clusters with other γ-proteobacteria, as expected. The *M. arboriphilicus nifH* sequence is most closely related to other methanogen *nifH* sequences. These data demonstrate that the *nifH* fragment can be amplified from diverse organisms and that the sequence of the amplified fragment of *nifH* can be used to determine the taxonomic identity of unknown N_2-fixing organisms. This was also the conclusion of a similar study of rice-root-associated, nitrogen-fixing microorganisms (28).

The *B. azotofixans nifH* sequence did not cluster well with any other group, which is also true of *Frankia nifH* sequences. Both genera are gram positive, but if *nifH* phylogenies were completely consistent with 16S rRNA phylogenies, the *B. azotofixans nifH* sequence would group with the *C. pasteurianum nifH* sequence. Instead, *C. pasteurianum nifH* seems more closely related to anaerobe *nifH* sequences from some γ- and δ-proteobacteria (*Chromatium buderi* and *D. gigas*) (Fig. 1).

It is not clear why the *nifH* sequences from anaerobes of diverse groups cluster. Obviously, the similarity of *nifH* sequences could be due to lateral transfer of the *nif* genes (18, 23), but it could also be explained on the basis of environmental selection. Regardless of the explanation for the clustering of anaerobic organisms, the *nifH* sequence can be used to identify

TABLE 1. Species used for phylogenetic analysis of mat *nifH* sequences, including species description and GenBank accession numbers for *nifH* sequences

Source description	GenBank accession no.	Group
Lyngbya lagerheimii	L15550	Nonheterocystous cyanobacteria
Plectonema boryanum	L15552	Nonheterocystous cyanobacteria
Gloeothece sp.	L15554	Nonheterocystous cyanobacteria
Trichodesmium sp.	L00688	Nonheterocystous cyanobacteria
Trichodesmium thiebautii	M29707	Nonheterocystous cyanobacteria
Trichodesmium erythraeum	L00689	Nonheterocystous cyanobacteria
Anabaena sp. strain Z	L15553	Heterocystous cyanobacteria
Anabaena sp. strain L31	L04499	Heterocystous cyanobacteria
Nostoc sp. strain PCC 6720	Z31716	Heterocystous cyanobacteria
Anabaena (*Nostoc*) sp. strain 7120	J01538	Heterocystous cyanobacteria
Anabaena oscillarioides ASOC1	M63686	Heterocystous cyanobacteria
Anabaena oscillarioides ASOC2	M63687	Heterocystous cyanobacteria
Nostoc muscorum	V04054	Heterocystous cyanobacteria
Nostoc sp.	L15551	Heterocystous cyanobacteria
Frankia sp. strain ArI3	M21132	High-G+C firmicute
Frankia alni HRN18a	X17522	High-G+C firmicute
Rhizobium meliloti	V01215	α-proteobacteria
Rhizobium meliloti 41	J01781	α-proteobacteria
Rhizobium phaseoli (clone pCQ15)	M10587	α-proteobacteria
Rhizobium trifolii	K00490	α-proteobacteria
Rhizobium sp. strain ANU289	K00487	α-proteobacteria
Rhizobium japonicum	K01620	α-proteobacteria
Rhizobium sp. strain ORS571	M16709	α-proteobacteria
Thiobacillus ferrooxidans	M15238	β- or γ-proteobacteria
Azospirillum brasilense	X51500	α-proteobacteria
Rhodobacter capsulatus	M15270	α-proteobacteria
Rhodospirillum rubrum	M33774	α-proteobacteria
Klebsiella pneumoniae	J01740	γ-proteobacteria
Azotobacter vinelandii (*vnfH*)	M36688	γ-proteobacteria
Azotobacter vinelandii	M20568	γ-proteobacteria
Azotobacter chroococcum	M73020	γ-proteobacteria
Trichodesmium-associated isolate	M63690	γ-proteobacteria
Ruppia maritima root isolates	M63691	γ-proteobacteria
Vibrio diazotrophicus	U23650	γ-proteobacteria
Desulfovibrio gigas	Reference 15	δ-proteobacteria
Chromatium buderi ATCC 25583	U23647	γ-proteobacteria
Bacillus azotofixans	U23649	Low-G+C firmicute
Clostridium pasteurianum sequence 1	X07472	Low-G+C firmicute
Clostridium pasteurianum sequence 2	X07473	Low-G+C firmicute
Clostridium pasteurianum sequence 4	X07475	Low-G+C firmicute
Clostridium pasteurianum sequence 5	X07476	Low-G+C firmicute
Clostridium pasteurianum sequence 6	X07477	Low-G+C firmicute
Rhodobacter capsulatus (*anfH*)	X70033	α-proteobacteria
Azotobacter vinelandii alternative (*anfH*)	M23528	γ-proteobacteria
Clostridium pasteurianum sequence 3	X07474	Low-G+C firmicute
Halodule root DNA	M63688	?
Halodule root DNA	M63689	?
Methanococcus thermolithotrophicus	X13830	Archaea
Methanobrevibacter arboriphilicus DC	U23648	Archaea
Upper mat sequence 1	U23642	
Lower mat sequence 51	U23639	
Lower mat sequence 53	U23638	
Upper mat sequence 413	U23644	
Upper mat sequence 1513	U23646	
Lower mat sequence 47	U23641	
Upper mat sequence 1113	U23643	
Upper mat sequence 913	U23645	
Lower mat sequence J7	U23634	
Lower mat sequence 29	U23636	
Lower mat sequence 55	U23637	
Lower mat sequence 57	U23640	
Lower mat sequence J5	U23633	
Lower mat sequence 25	U23635	
Marine snow	U26186	

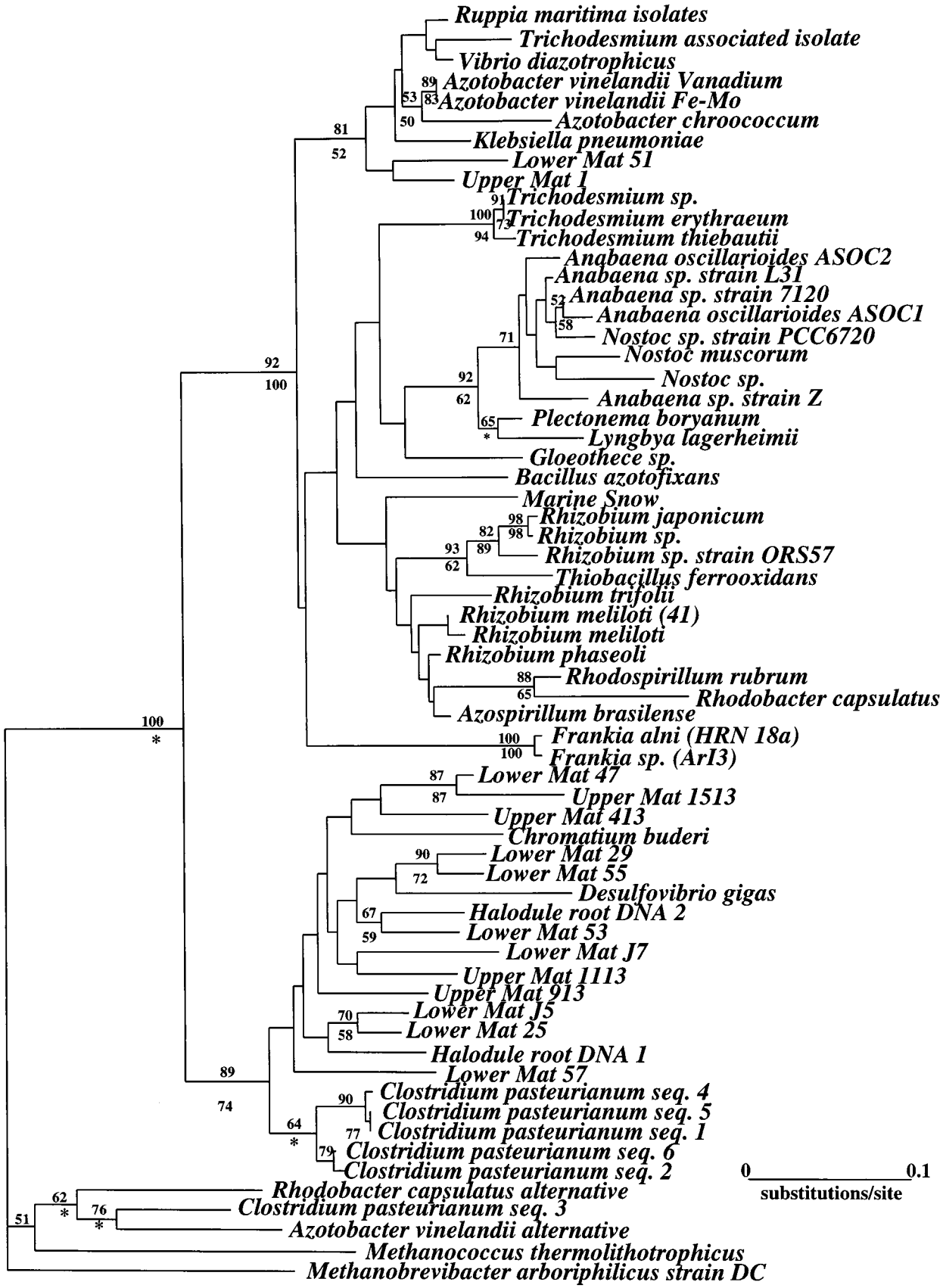

FIG. 1. Phenogram of nitrogenase amino acid sequences (sequences deduced from a 325-bp internal fragment of *nifH*) using 50 sequences from GenBank and 14 *nifH* sequences obtained from North Carolina cyanobacterial mats. The location of the *nifH* fragments used for the analysis corresponds to the *Anabaena* (*Nostoc*) sp. strain 7120 sequence that encodes amino acid residues 48 to 155 (GenBank A00534). *Methanobrevibacter arboriphilicus* DC *nifH* was used as an outgroup. Bootstrap values (percentages) for neighbor joining (above line) and parsimony (below line) are shown for clusters supported by both analyses, and only values greater than 50% are shown. ∗, cluster supported by parsimony but with a bootstrap value of less than 50%. GenBank accession numbers and species description are given in Table 1.

TABLE 2. Similarity of DNA and deduced amino acid *nifH* sequences derived from anaerobic organisms and cyanobacterial mats compared with other bacterial and archaeal *nifH* sequences[a]

	Lower Mat 29	Lower Mat 53	Lower Mat J5	Upper Mat 413	Upper Mat 913	C. pasteurianum 1	C. pasteurianum 4	D. gigas	C. buderi	Anabaena sp. L31	L. lagerheimii	Nostoc sp. 6720	T. erythraeum	Frankia sp. ArI3	R. meliloti	R. phaseoli	K. pneumoniae	V. diazotrophicus	Ruppia maritima	M. thermolitho.
Lower Mat 29		84	86	82	82	86	85	88	85	64	64	63	68	63	66	65	66	68	69	63
Lower Mat 53	74		84	80	81	84	85	84	80	68	66	66	69	62	69	67	66	69	68	65
Lower Mat J5	71	75		81	86	83	84	86	83	69	66	67	73	65	70	68	67	69	70	66
Upper Mat 413	71	70	69		88	85	84	75	84	66	65	64	66	65	65	66	66	68	68	62
Upper Mat 913	69	68	70	73		83	84	84	82	67	65	65	69	67	67	68	65	67	70	65
C. pasteurianum 1	70	66	68	69	68		99	82	84	69	68	69	76	71	71	68	70	72	61	65
C. pasteurianum 4	71	66	69	70	70	94		82	83	70	70	67	77	71	70	67	69	71	70	66
D. gigas	71	75	74	75	68	60	62		82	73	71	73	71	71	73	73	76	76	77	57
C. buderi	72	72	70	75	70	66	67	74		64	64	63	66	62	65	64	65	66	64	65
Anabaena sp. 31	62	65	63	63	59	65	63	64	66		90	97	86	79	82	73	76	78	77	72
L. lagerheimii	63	66	65	67	63	61	58	67	67	77		87	82	76	77	78	80	81	80	58
Nostoc sp. 6720	60	64	62	62	60	66	64	64	64	93	73		84	77	77	75	75	75	75	60
T. erythraeum	58	59	64	61	62	66	66	61	61	80	70	80		77	83	77	80	81	80	63
Frankia sp. ArI3	59	65	65	66	61	59	61	64	66	70	73	68	65		77	76	75	75	75	60
R. meliloti	61	68	68	65	64	62	63	65	67	69	76	67	70	76		88	79	79	80	61
R. phaseoli	62	65	66	66	62	60	61	64	67	67	75	67	67	75	80		82	83	84	61
K. pneumoniae	62	69	68	67	63	61	61	67	69	70	78	68	68	72	76	75		94	94	64
V. diazotrophicus	60	64	65	64	61	65	66	62	65	74	71	73	75	67	72	71	79		98	65
Ruppia maritima	63	64	65	66	62	65	66	64	64	73	71	74	73	66	69	72	78	81		63
M. thermolitho.	61	61	62	59	62	67	67	56	61	63	57	61	51	55	59	58	58	60	63	

% Amino Acid Identical

% DNA Identical

[a] For full species names and GenBank accession numbers, see Table 1.

unknown organisms if they are relatively closely related to known *nifH* sequences.

All of the lower mat sequences are related to sequences from anaerobes, which is consistent with the more reduced conditions in the lower mat regions. In contrast, upper mat sequences include anaerobe *nifH* sequences as well as sequences closely related to facultative anaerobes such as *K. pneumoniae*. The lower mat *nifH* sequences that cluster with the anaerobe sequences are not very closely related to the *Chromatium buderi*, *C. pasteurianum*, or *D. gigas* sequences or to each other (Table 2). The large number of distantly related sequences suggests a high diversity of anaerobic N_2-fixing organisms in the cyanobacterial mat. N_2 fixation in marine systems has been shown to be associated with vibrios (11) or sulfate-reducing bacteria (14, 17). The *nifH* sequence obtained from the upper layer of the North Carolina mat could be derived from a vibrio or another γ-proteobacterium, but most of the *nifH* sequences are most closely related to the anaerobes. Since the *nifH* sequences from the anaerobe and mat *nifH* sequences are not very similar (Table 2), positive identification is difficult.

However, it appears that there are *nifH* sequences that are related to sulfate reducers and purple sulfur bacteria (Fig. 1). Of 19 additional clones that were partially sequenced, 13 are most similar to the *Chromatium buderi* group of sequences, indicating that the purple sulfur *nifH* genes are particularly abundant in the mat. It is noteworthy that two upper mat sequences are in the purple sulfur cluster (Fig. 1). Purple sulfur bacteria are often seen within the upper portion of the mat mingled with the cyanobacteria. Some of the mat sequences are closely related only to *nifH* sequences derived from sea grass (*Halodule*) roots and form a separate cluster with no known organisms (Fig. 1).

The mat *nifH* sequences are more distantly related to each other than would be expected from species within the same genus (e.g., *Anabaena* and *Nostoc*; Fig. 1) or multiple copies within the same organism (e.g., *Clostridium* or *Azotobacter* spp.; Fig. 1). Even if some of the sequences are alternative forms of nitrogenase (*nifH* of the vanadium-containing nitrogenase) within the same organism, there are still numerous unique N_2-fixing organisms represented by the diverse sequences obtained from the mat. A recent report on *nifH* genes amplified from rice roots came to the conclusion that there are very diverse *nifH* sequences in the environment (28). The results of that study are strikingly similar to those of this study, with a large number of sequences clustering near the *Clostridium* sequences (28).

It is interesting to note that the sequences from the second alternative nitrogenase from *Azotobacter* cluster closely with the alternative *nifH* gene from *Rhodobacter capsulatus* (Fig. 1) and the *Clostridium nifH3* sequence (Fig. 1). This is consistent with the suggestion that at least some of the alternative nitrogenase genes evolved independently from the more ubiquitous genes for the Mo-containing form of nitrogenase (18, 30). This family of *nifH* can easily be identified on the basis of the sequence of the amplified fragment of *nifH*.

The clustering of microorganisms based on *nifH* sequences has important implications for the use of the *nifH* gene sequence in ecological studies. The amplified *nifH* fragment provides taxonomic information on organisms from the environ-

ment with the genetic potential for N_2 fixation and can also be used to identify isolates obtained from the environment.

This is the first report of the diversity of genes that encode a protein which is critical for nitrogen cycling in the marine environment. Studies have previously demonstrated the diversity of species in oceanic environments on the basis of 16S rRNA sequences (8), but N_2 fixation capabilities are seemingly randomly distributed throughout prokaryotic taxa such that taxonomic information alone does not provide information on N_2 fixation capabilities. The results of this study demonstrate that we have little knowledge of the organisms responsible for N_2 fixation in the marine environment and that the genetic potential for N_2 fixation is extremely diverse.

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REFERENCES

1. **Bebout, B. M., M. W. Fitzpatrick, and H. W. Paerl.** 1993. Identification of the sources of energy for nitrogen fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. Appl. Environ. Microbiol. **59:**1495–1503.
2. **Bebout, B. M., H. W. Paerl, K. M. Crocker, and L. E. Prufert.** 1987. Diel interactions of oxygenic photosynthesis and N_2 fixation (acetylene reduction) in a marine microbial mat community. Appl. Environ. Microbiol. **53:**2353–2362.
3. **Ben-Porath, J., and J. P. Zehr.** 1994. Detection and characterization of cyanobacterial *nifH* genes. Appl. Environ. Microbiol. **60:**880–887.
4. **Capone, D. G., and E. J. Carpenter.** 1982. Nitrogen fixation in the marine environment. Science **217:**1140–1142.
5. **Cohen, Y., R. W. Castenholz, and H. O. Halvorson (ed.).** 1984. Microbial mats: stromatolites. A. R. Liss, New York.
6. **Cohen, Y., and E. Rosenberg (ed.).** 1989. Microbial mats: physiological ecology of benthic microbial communities. American Society for Microbiology, Washington, D.C.
7. **Felsenstein, J.** 1993. PHYLIP—phylogeny inference package (version 3.5c). Department of Genetics, University of Washington, Seattle.
8. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:** 69–63.
9. **Giovannoni, S. J., E. J. DeLong, T. M. Schmidt, and N. R. Pace.** 1990. Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ. Microbiol. **56:**2572–2575.
10. **Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane, and N. R. Pace.** 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170:**3584–3592.
11. **Guerinot, M. L., and R. R. Colwell.** 1985. Enumeration, isolation, and characterization of N_2-fixing bacteria from seawater. Appl. Environ. Microbiol. **50:**350–355.
12. **Hennecke, H., K. Kaluza, B. Thöny, M. Fuhrman, W. Ludwig, and E. Stackebrandt.** 1985. Concurrent evolution of nitrogenase genes and 16S rRNA in Rhizobium species and other nitrogen-fixing bacteria. Arch. Microbiol. **142:** 342–348.
13. **Hirsch, A. M., H. I. McKhann, A. Reddy, J. Liao, Y. Fang, and C. R. Marshall.** 1995. Assessing horizontal transfer of *nifHDK* genes in eubacteria: nucleotide sequence of *nifK* from *Frankia* strain HFPCcI3. Mol. Biol. Evol. **12:**16–27.
14. **Jones, K.** 1982. Nitrogen fixation in the temperate estuarine intertidal sediments of the River Lune. Limnol. Oceanogr. **27:**455–460.
15. **Kent, H. M., M. Buck, and D. J. Evans.** 1989. Cloning and sequencing of the *nifH* gene of *Desulfovibrio gigas*. FEMS Microbiol. Lett. **61:**73–78.
16. **Kirshtein, J. D., H. W. Paerl, and J. P. Zehr.** 1991. Amplification, cloning, and sequencing of a *nifH* segment from aquatic microorganisms and natural communities. Appl. Environ. Microbiol. **57:**2645–2650.
17. **Nedwell, D. B., and S. A. Aziz.** 1980. Heterotrophic nitrogen fixation in an intertidal saltmarsh sediment. Estuarine Coastal Mar. Sci. **10:**699–702.
18. **Normand, P., and J. Bousquet.** 1989. Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms. J. Mol. Evol. **29:**436–447.
19. **Olsen, G. J., C. R. Woese, and R. Overbeek.** 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176:**1–6.
20. **Paerl, H. W.** 1990. Physiological ecology and regulation of N_2 fixation in natural waters. Adv. Microb. Ecol. **8:**305–344.

20a. **Paerl, H. W., et al.** Unpublished data.

21. **Paerl, H. W., L. E. Prufert, and W. W. Ambrose.** 1991. Contemporaneous N_2 fixation and oxygenic photosynthesis in the nonheterocystous mat-forming cyanobacterium *Lyngbya aestuarii*. Appl. Environ. Microbiol. **57:**3086–3092.
22. **Postgate, J. R. (ed.).** 1982. The fundamentals of nitrogen fixation. Cambridge University Press, Cambridge.
23. **Postgate, J. R., and R. R. Eady.** 1988. The evolution of biological nitrogen fixation, p. 31–40. *In* H. Bothe, F. J. deBruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fischer, New York.
24. **Rippka, R., and J. B. Waterbury.** 1977. The synthesis of nitrogenase by non-heterocystous cyanobacteria. FEMS Microbiol. Lett. **2:**83–86.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Stal, L. J., and B. Bergman.** 1990. Immunological characterization of nitrogenase in the filamentous non-heterocystous cyanobacterium *Oscillatoria limosa*. Planta **182:**287–291.
27. **Stewart, W. D. P. (ed.).** 1975. Nitrogen fixation by free-living microorganisms. Cambridge University Press, London.
28. **Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi.** 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol. **177:**1414–1417.
29. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
30. **Young, J. P. W.** 1992. Phylogenetic classification of nitrogen-fixing organisms, p. 43–86. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
31. **Zehr, J. P., and L. A. McReynolds.** 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **55:**2522–2526.