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Detection and Characterization of Cyanobacterial *nifH* Genes

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The DNA sequence of a 359-bp fragment of *nifH* was determined for the heterocystous strains *Anabaena* sp. strain CA (ATCC 33047), *Nostoc muscorum* UTEX 1933, a *Nostoc* sp., *Gloeothece* sp. strain ATCC 27152, *Lyngbya lagerheimii* UTEX 1930, and *Plectonema boryanum* IU 594. Results confirmed that the DNA sequence of the 359-bp segment is sufficiently variable to distinguish cyanobacterial *nifH* genes from other eubacterial and archaeobacterial *nifH* genes, as well as to distinguish heterocystous from nonheterocystous *nifH* genes. Nonheterocystous cyanobacterial *nifH* sequences were greater than 70 and 82% identical on the DNA and amino acid levels, respectively, whereas corresponding values for heterocystous cyanobacterial *nifH* sequences were 84 and 91%. The amplified *nifH* fragments can be used as DNA probes to differentiate between species, although there was substantial cross-reactivity between the *nifH* amplification products of some strains. However, an oligonucleotide designed from a sequence conserved within the heterocystous cyanobacteria hybridized primarily with the amplification product from heterocystous strains. The use of oligonucleotides designed from amplified *nifH* sequences shows great promise for characterizing assemblages of diazotrophs.

Cyanobacteria are oxygen-evolving phototrophs which are often conspicuous components of aquatic ecosystems. Some species of unicellular, heterocystous, and nonheterocystous cyanobacteria also have the ability to fix atmospheric nitrogen (17, 19, 23). Substantial progress has been made in understanding the ecology, biochemistry, and molecular biology of nitrogen fixation in cyanobacteria (6, 9, 15). Cyanobacterial nitrogen fixation is potentially important in regulating primary productivity in nitrogen-deficient aquatic environments, yet relatively little is known about the ecology of natural populations of nitrogen-fixing cyanobacteria. One of the difficulties in studying natural populations is that nitrogen-fixing microbial communities can be complex mixtures of many species of bacteria and cyanobacteria. Species which develop heterocysts are clearly capable of fixing nitrogen, but other species are less easily identified since nitrogen fixation genes are present in many prokaryotic taxa. Problems with identification of nitrogen-fixing microorganisms in nature are exacerbated by difficulties in culturing microorganisms from the environment (7, 21). The objective of this study was to obtain information on cyanobacterial nitrogen fixation genes, such that a molecular approach could be developed for studying diazotrophic cyanobacterial assemblages in the environment.

Nitrogenase, the enzyme that reduces N_2 to ammonium, is composed of two multisubunit proteins (the MoFe protein, composed of subunits encoded by *nifD* and *nifK*, and the Fe protein, composed of identical subunits encoded by *nifH*), both of which are conserved among nitrogen-fixing organisms. The amino acid sequence of the Fe protein is very similar among organisms, even those of very different taxonomic groups. Even the alternative nitrogenase Fe protein amino acid sequences are greater than 63% identical to the conventional nitrogenase Fe protein sequence (91% between *nifH* and *vnfH* and 63% between *nifH* and *anfH* [3]). This conserved feature of the Fe protein gene, *nifH*, provides a convenient way to develop probes for studying nitrogen fixation genes in diverse organisms, as well as for characterizing nitrogen fixation in natural microbial communities (24, 25).

Data from the limited number of *nif* genes that have been sequenced suggest that broad taxonomic groups as well as closely related species can potentially be distinguished by comparison of *nifH* DNA and deduced amino acid sequences (2, 11, 12, 14, 24). However, prior to this study the *nifH* gene has been sequenced from very few cyanobacterial species (11, 13, 25). We investigated the possibility of distinguishing different types of cyanobacteria on the basis of the DNA sequence of an amplified fragment of *nifH*. Following comparison of *nifH* DNA and amino acid sequences from representatives of different groups of cyanobacteria, we tested the use of the amplified fragments as DNA probes to distinguish individual species or groups of species. We also used the DNA sequence data to design oligonucleotides that may be useful for characterizing natural populations of diazotrophs. Finally, we demonstrated the utility of this approach in identifying diazotrophs in a marine cyanobacterial mat.

MATERIALS AND METHODS

Source of DNA. Six pure cultures representing both heterocystous and nonheterocystous cyanobacteria were chosen. *Anabaena* sp. strain CA (ATCC 33047), a heterocystous cyanobacterium, was obtained from the American Type Culture Collection. *Nostoc muscorum* UTEX LB 1933, a heterocystous cyanobacterium, and *Lyngbya lagerheimii* UTEX 1930, a nonheterocystous cyanobacterium, were obtained from the University of Texas Culture Collection. A *Nostoc* sp. strain, a heterocystous cyanobacterium, and *Gloeothece* sp. strain ATCC 27152, a unicellular cyanobacterium, were provided by J. Gallon. *Plectonema boryanum* IU 594, a nonheterocystous cyanobacterium, was obtained from R. S. Safferman. DNA from *Fischerella ambigua* UTEX 1903, a heterocystous branching cyanobacterial strain, was obtained from the laboratory of S. Nierzwicki-Bauer and was used only for the oligonucleotide hybridization tests.

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Samples of a marine cyanobacterial mat were collected from Bird Shoal Island, N.C. (provided by B. Bebout and H. Paerl).

DNA extraction. DNA was extracted from cells and filaments with a microcentrifuge tube minihomogenizer (Kontes Co.) and incubated with 1 mg of lysozyme per ml, 160 μg of proteinase K per ml, and 3 mg of RNase per ml at 37°C for 2 h. The samples were then extracted with phenol at 56°C for 20 min, and the aqueous phase was extracted twice with phenol-chloroform. Two volumes of cold propanol and 0.1 volume of 3 M sodium acetate were added to the samples, and the DNA was left to precipitate overnight. The samples were then centrifuged, and the DNA pellet was resuspended in 1× TE (10 mM Tris [pH 7.4], 0.1 mM EDTA [pH 8.0]).

DNA extraction from mats followed essentially the same procedure as that for cultures, except that the DNA was purified by using Elutip-D columns (20) or by agarose gel electrophoresis.

Amplification of *nifH*. Two different sets of degenerate oligonucleotide primers were used for the PCR amplification of the *nifH* gene fragment. One set, described by Zehr and McReynolds (25), was used to amplify DNA from *N. muscorum* and from marine cyanobacterial mats. The other DNA samples were amplified with a set of oligonucleotides that had restriction sites for the enzymes *Eco*RI and *Bam*HI, which facilitated cloning and sequencing.

Primer 1: 5′-G**GAATTC**CTGYGAYCCNAARGCNGA-3′
Primer 2: 5′-C**GGATCC**GDNGCCATCATYTCNCC-3′

where Y is T or C; N is A, C, G or T; R is A or G; and D is A, G or T. Restriction sites are shown in boldface.

Primer 1, located at positions 318 to 335 in reference to the *Anabaena* sp. strain PCC7120 *nifH* sequence (13), has the *Eco*RI site. Primer 2, located in positions 659 to 674 of the opposite strand, has the *Bam*HI site.

A 359-bp fragment of the *nifH* was amplified from all the samples by using 2.5 to 5 U of *Taq* DNA polymerase (Promega) and 35 cycles of denaturation (93°C, 1.2 min), annealing (50°C, 1.0 min), and extension (70°C, 1.5 min). The resulting 359-bp fragment was visualized on a 4% NuSieve agarose gel, stained with ethidium bromide, and isolated with DEAE paper (22).

Cloning and sequencing. The *nifH* gene fragments with the restriction sites were ligated into M13 phage DNA that was digested with the restriction enzymes *Eco*RI and *Bam*HI. The *nifH* gene segments without the restriction site were blunt-ended with Klenow fragment and ligated into the *Hinc*II site of M13mp18 or M13mp19. Overnight ligation performed at 25°C was followed by transformation into *E. coli* JM101 (25). Single-stranded DNA was extracted from recombinant clones. The opposite strand was sequenced by recloning the insert into the M13 vector with the reversed polylinker cloning site.

The recombinant DNA clones were sequenced by the dideoxynucleotide chain termination method (18) with Sequenase version 2.0 (U.S. Biochemical Corp.) and ^{35}S-dATP (New England Nuclear, DuPont de Nemours). For each species, both strands of the cloned amplification product were sequenced. The DNA sequence data were analyzed by using the University of Wisconsin Genetic Computer Group (5) or the HIBIO DNAsis and PROsis (Hitachi Software Engineering America, Ltd., Brisbane, Calif.) data analysis programs. Each DNA sequence was obtained from one clone derived from one PCR. Previous results of replicated amplifications (2) demonstrated that PCR errors occur at a frequency of about 0.3%, which corresponds to about 6 bases per 2,000 bases of sequence. This magnitude of error is significant only when comparing very closely related sequences (2). A sequence for a cloned *nifH* fragment from *P. boryanum* M101 has recently been submitted to GenBank (accession number D00666). The two *P. boryanum* sequences differ by only 2 bp.

DNA hybridization. DNA hybridizations were performed with digoxigenin-labelled DNA probes (Genius system; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The full-length probes (359 bp) were labelled with digoxigenin-dUTP during PCR amplification, by using 5 μl of the dUTP labelling mixture (Boehringer Mannheim) in place of the normal deoxynucleoside triphosphate mixture. Synthetic oligonucleotides (cyanobacterial, 5′-TCTGGTGGTCCAGAACCCGGT GTA-3′; heterotrophic eubacterial, 5′-TCCGGTGGCCCGG AGCCG/AGGCGTC-3′) were designed from the *nifH* sequences (Fig. 1) and were synthesized by National Biosciences. The oligonucleotides were labelled by tailing with digoxigenin-dUTP (Boehringer Mannheim). Amplified *nifH* DNA fragments from recombinant clones containing the 359-bp insert from individual species were used as the target DNA on slot blots. The target DNA (25 ng) was denatured and filtered through a nylon membrane (Hybond-N; Amersham) by using a slot blot manifold according to the manufacturer's protocol (Bio-Rad Laboratories, Richmond, Calif.). The DNA was fixed to the membrane by UV irradiation (Bios Corp., New Haven, Conn.).

The slot blots were hybridized to the digoxigenin-labelled 359-bp *nifH* probes overnight at 55°C in 50% formamide by using the hybridization solution recommended by the kit manufacturer (Boehringer Mannheim). The oligonucleotide probes were hybridized at 45°C in 50% formamide. The blots were washed, and the hybridized probes were detected by chemiluminescence according to the manufacturer's protocols.

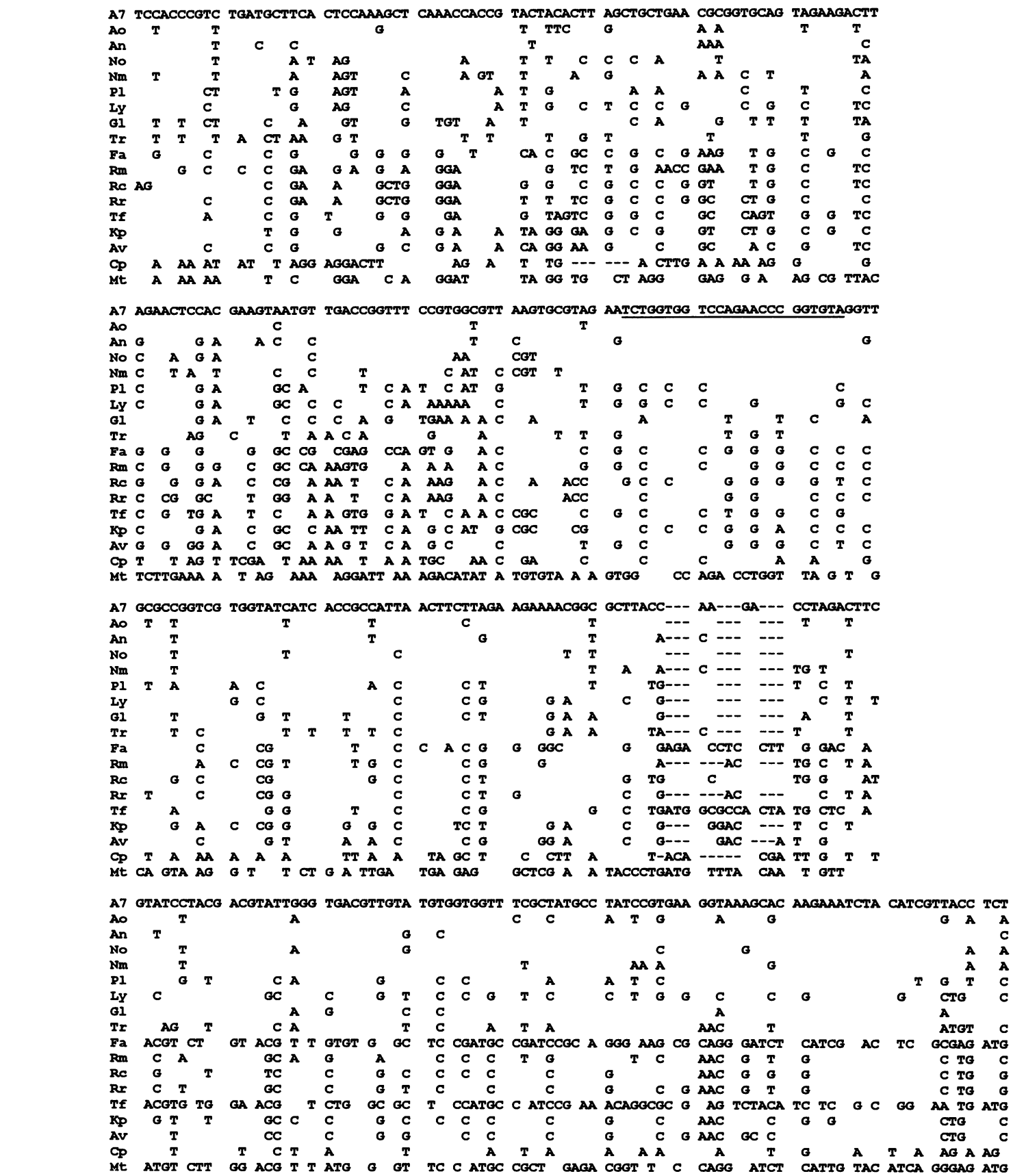
Nucleotide sequence accession numbers. Sequences have been submitted to GenBank under accession numbers L15553 (*Anabaena* sp. strain CA), L15551 (*Nostoc* sp.), U04054 (*N. muscorum*), L15554 (*Gloeothece* sp. strain 1430/3), L15552 (*P. boryanum*), and L15550 (*L. lagerheimii*).

RESULTS

The DNA and deduced amino acid sequences of the amplified *nifH* fragment from the cyanobacterial species are shown in Fig. 1 and 2, together with the corresponding sequence of *nifH* from nine previously reported eubacterial and archaeobacterial sequences. The similarities among DNA and amino acid sequences are presented in Tables 1 and 2. All of the cyanobacterial sequences were composed of the same number of nucleotides within the amplified region, although some of the other species have insertions and deletions in this region (Fig. 1 and 2).

The DNA sequences of heterocystous cyanobacterial species are 84 to 92% identical to each other and more similar to the sequences of other cyanobacterial species (72 to 81% identical) than to those of the other eubacterial or archaeobacterial species (60 to 73% identical) (Table 1). The nonheterocystous cyanobacterial sequences are generally as similar to heterocystous sequences (72 to 81% identical) as to other nonheterocystous sequences (70 to 81% identical) (Table 1). The highest degree of similarity within the nonheterocystous cyanobacteria is between *L. lagerheimii* and *P. boryanum* (81% identical). The other eubacterial sequences (not including the archaeobacterium *Methanococcus thermolithotrophicus*) are 61 to 87% identical to each other, generally having lowest similarity values with the *Clostridium* and *M. thermolithotrophicus* sequences (Table 1).

The sequenced fragments were used as probes to determine whether they could be used to distinguish *nifH* genes from



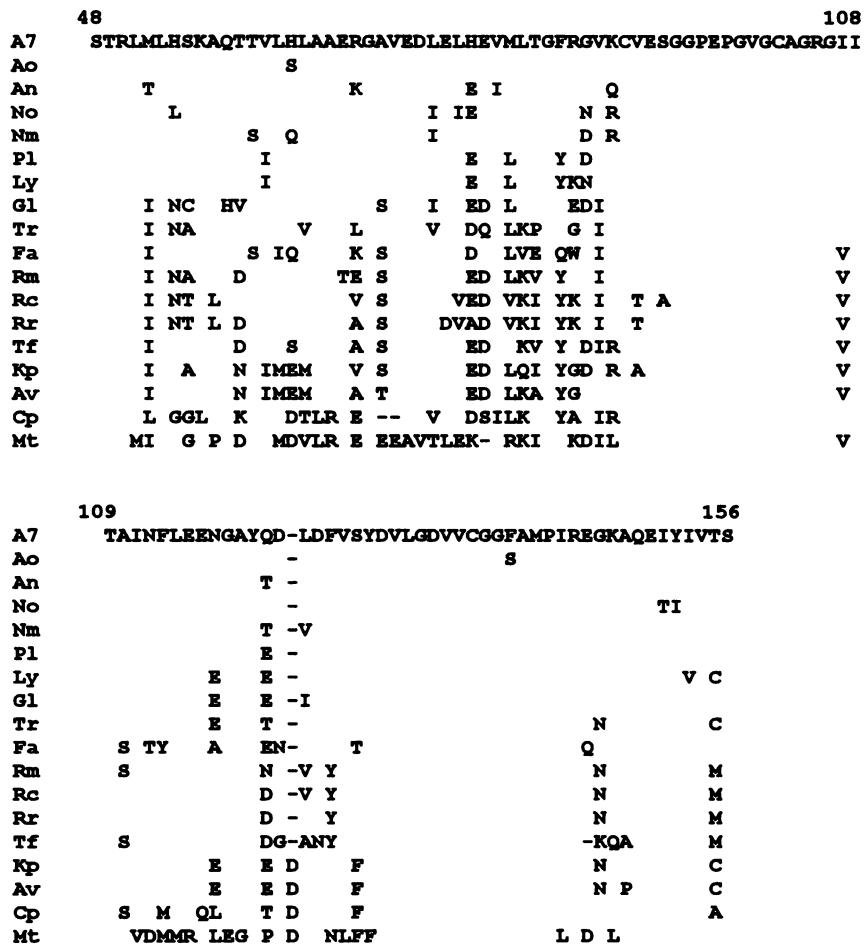


FIG. 2. Comparison of the deduced amino acid sequences of the *nifH* gene from cyanobacteria and the *nifH* sequences of other diazotrophs. Sequences are compared with residues 48 to 155 of the *Anabaena* (*Nostoc*) sp. strain 7120 sequence (13) (GenBank accession no. A00534). Abbreviations are defined in the legend to Fig. 1.

different groups of cyanobacteria. The labelled *Anabaena* sp. fragment hybridized most strongly to itself and the two *Nostoc nifH* fragments (Fig. 3). The *Gloeothece* probe hybridized most strongly to itself but cross-hybridized with the other sequences to a larger extent than the *Anabaena* probe (Fig. 3). The *Lyngbya* probe was fairly specific but did cross-hybridize to the *Anabaena* sp. strain CA and *Nostoc* sp. *nifH* fragments (Fig. 3). Both *Nostoc* probes hybridized strongly to each other but also cross-hybridized with *Anabaena* sp. CA, *Gloeothece* sp., and *Trichodesmium thiebautii* (Fig. 3). The *Plectonema* probe cross-hybridized with *Anabaena* sp. strain CA, *Nostoc* sp., and *T. thiebautii* (Fig. 3). The *T. thiebautii* probe was the most specific, with no detectable cross-hybridization with the other amplified *nifH* fragments (Fig. 3).

The *nifH* amplification product obtained from a *Lyngbya*-dominated cyanobacterial mat was probed with three cyanobacterial *nifH* probes: *Anabaena* sp. strain CA, *L. lagerheimii*, and *N. muscorum*. Hybridization was detected with the *Lyngbya* probe, but not with the *Anabaena* or *Nostoc* probe (Fig. 4).

An oligonucleotide probe (designed from heterocystous cyanobacterial *nifH* DNA sequences [Fig. 1]) hybridized to various degrees with the amplified *nifH* fragments (Fig. 5). The strongest signal was obtained with *Anabaena* sp. strain CA, *N. muscorum*, and *Nostoc* sp., with slightly less signal being obtained with *P. boryanum*, *L. lagerheimii*, and *F. ambigua nifH* DNA. Weak signals were obtained with the *Gloeothece* sp. and *T. thiebautii nifH* fragments.

FIG. 1. DNA sequence of a *nifH* fragment amplified from several species of heterocystous and nonheterocystous cyanobacteria, compared with corresponding sequences from other cyanobacterial, eubacterial, and archaeobacterial sequences. Sequences correspond to bases 335 to 659 of the sequence from *Anabaena* (*Nostoc*) sp. strain PCC7120 (13). Identical matches are indicated by blank spaces. *Anabaena* sp. strain CA (An), *Nostoc* sp. (No), *N. muscorum* (Nm), *Gloeothece* sp. (Gl), *P. boryanum* (Pl), and *L. lagerheimii* (Ly) sequences were obtained from this work. The following sequences were obtained from GenBank (accession numbers are given in parentheses): *Anabaena* sp. strain PCC7120 (V00001) (A7), *Anabaena oscillarioides* (M63686) (Ao), *Trichodesmium thiebautii* (M29709) (Tr), *Rhizobium meliloti* (J01781) (Rm), *Rhodobacter capsulata* (X07866) (Rc), *Rhodospirillum rubrum* (M33774) (Rr), *Frankia* sp. strain Ar13 (X12866) (Fa), *Klebsiella pneumoniae* (J01740) (Kp), *Azotobacter vinelandi* (M11579) (Av), *Clostridium pasteurianum* (M21537) (Cp), *M. thermolithotrophicus* (X13830) (Mt), and *Thiobacillus ferrooxidans* (M15238) (Tf). The region used for design of oligonucleotide probes is underlined. Dashes indicate gaps in the sequence.

TABLE 1. DNA sequence similarities (percent identical match) between cyanobacterial and other bacterial *nifH* sequences[a]

Species	Heterocystous cyanobacteria					Nonheterocystous cyanobacteria				Other eubacteria and archaeobacteria								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 (A7)	100																	
2 (Ao)	90	100																
3 (An)	92	86	100															
4 (No)	89	86	86	100														
5 (Nm)	85	84	85	86	100													
6 (Gl)	81	78	78	81	76	100												
7 (Tr)	80	78	78	77	75	77	100											
8 (Pl)	80	80	79	80	77	77	74	100										
9 (Ly)	76	74	74	77	73	75	70	81	100									
10 (Rm)	69	68	69	68	65	72	70	72	76	100								
11 (Rc)	71	68	70	68	66	71	69	70	75	79	100							
12 (Rr)	72	70	69	71	66	71	69	72	78	81	87	100						
13 (Fa)	72	68	71	68	64	70	65	69	74	77	74	77	100					
14 (Kp)	69	67	69	69	67	70	68	75	78	76	79	78	73	100				
15 (Av)	73	72	72	71	68	71	72	73	78	77	78	78	78	82	100			
16 (Cp)	66	64	66	63	64	65	66	65	60	64	63	63	61	66	63	100		
17 (Mt)	62	60	62	61	62	63	59	60	57	59	58	56	56	58	58	65	100	
18 (Tf)	69	68	69	69	68	69	65	69	72	76	77	78	72	73	76	61	57	100

[a] Abbreviations as in the legend to Fig. 1.

An oligonucleotide probe designed from the same region as the heterocystous cyanobacterial probe but from the other eubacterial sequences did not hybridize significantly to any of the amplified cyanobacterial *nifH* fragments. The blot, although hybridized under the same conditions, had to be exposed to the film for a longer period in order to show the faint nonspecific background observed in Fig. 5.

DISCUSSION

Although the *nifH* gene is highly conserved in the amino acid sequence, there is sufficient variation in amino acid and DNA sequences to distinguish broad taxonomic groups. Analysis of *nif* and 16S rRNA genes from a limited number of bacterial species indicated that *nif* and 16S rRNA genes have evolved in a similar fashion (10). If this is the case for all diazotrophs, heterocystous cyanobacterial *nifH* sequences should be most closely related to each other, since phylogenetic analysis of 16S rRNA sequences indicates that heterocystous species are most closely related (8). The *nifH* DNA and deduced amino acid sequence data presented here are consistent with the results of the 16S rRNA analysis. The significance of this for studies of natural populations is that it should ultimately be possible to determine taxonomic identities of nitrogen-fixing organisms from the sequence of the *nifH* gene.

TABLE 2. Amino acid sequence similarities (percent identical match) between cyanobacterial and other bacterial *nifH* sequences[a]

Species	Heterocystous cyanobacteria					Nonheterocystous cyanobacteria				Other eubacteria and archaeobacteria								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 (A7)	100																	
2 (Ao)	98	100																
3 (An)	94	93	100															
4 (No)	94	93	92	100														
5 (Nm)	94	93	91	93	100													
6 (Gl)	85	83	83	85	84	100												
7 (Tr)	85	83	84	82	81	85	100											
8 (Pl)	94	93	92	92	91	87	83	100										
9 (Ly)	90	88	87	88	85	84	82	94	100									
10 (Rm)	80	78	79	77	76	82	81	81	77	100								
11 (Rc)	78	76	77	76	73	80	80	78	76	88	100							
12 (Rr)	79	77	77	76	73	79	81	78	76	86	95	100						
13 (Fa)	80	79	79	75	77	77	77	80	76	77	72	73	100					
14 (Kp)	77	76	77	76	76	79	80	82	80	79	79	79	75	100				
15 (Av)	79	79	79	76	74	79	82	83	82	79	79	80	77	92	100			
16 (Cp)	72	71	74	71	71	71	77	72	71	71	68	69	70	71	72	100		
17 (Mt)	61	60	61	60	59	62	63	61	58	60	62	62	60	64	63	66	100	
18 (Tf)	78	78	78	77	77	77	75	79	74	86	82	82	75	77	77	69	62	100

[a] Abbreviations as in the legend to Fig. 1.

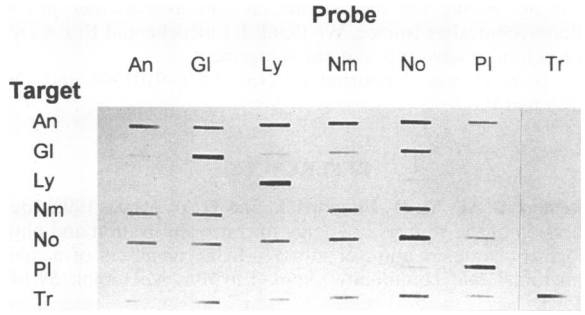


FIG. 3. Slot blot showing cross-hybridization among 359-bp *nifH* fragments of cyanobacterial strains. Each column has been probed with the individual cyanobacterial *nifH* fragment indicated. The slots on the diagonal from the upper left to the lower right are 100% identical. An, *Anabaena* sp. strain CA; Gl, *Gloeothece* sp.; Ly, *L. lagerheimii*; Nm, *N. muscorum*; No, *Nostoc* sp.; Pl, *P. boryanum*; Tr, *T. thiebautii.*

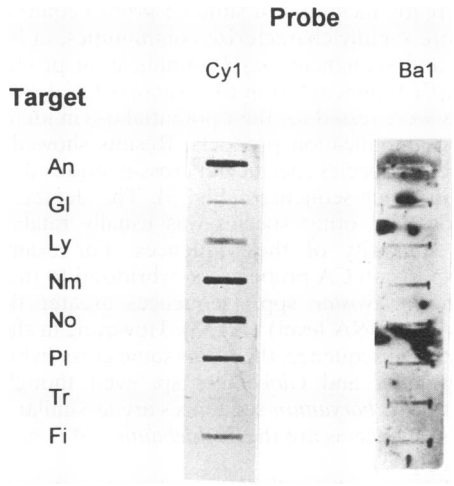


FIG. 5. Hybridization of oligonucleotides designed from heterocystous cyanobacterial (Cy1) and other eubacterial (Ba1) *nifH* sequences (corresponding to bases 468 to 492 of the *Anabaena* sp. strain 7120 sequence. An, *Anabaena* sp. strain CA; Gl, *Gloeothece* sp.; Ly, *L. lagerheimii*; Nm, *N. muscorum*; No, *Nostoc* sp.; Pl, *P. boryanum*; Tr, *T. thiebautii*; Fi, *F. ambigua.*

The DNA sequence similarity values were generally higher between representatives of the same group (e.g., heterocystous or nonheterocystous), indicating that DNA sequence similarity potentially can be used for characterizing an unknown sequence obtained from a natural assemblage (Table 1). Similarities among the cyanobacterial *nifH* sequences (≥70% for DNA and ≥81% for amino acids) are generally higher than between cyanobacteria and other bacteria (≤75% for DNA and ≤83% for amino acids), with the exception of the *L. lagerheimii* sequence, which has similarities with other eubacterial sequences ranging up to 78 and 82% identical on the DNA and amino acid levels, respectively. Strikingly similar sequences are found among heterocystous species (≥84% for DNA and ≥91% for amino acids). The two filamentous, heterocystous, nonbranching genera studied here, *Anabaena* and *Nostoc*, are probably more closely related to each other than to some other heterocystous cyanobacterial genera. Liquid axenic cultures of *Anabaena* and *Nostoc* species are morphologically very similar and are distinguished only on the basis of the presence of hormogonia (*Nostoc* spp.) and motility of the filaments (*Anabaena* spp.) or motility of the hormogonia (*Nostoc* spp.) (4). The lower degree of hybridization of the heterocystous *nifH* oligonucleotide probe to the *nifH* fragment from the filamentous, heterocystous, branching strain *F. ambigua* (Fig. 5) is consistent with the greater divergence of *nifH* sequences between the *Anabaena-Nostoc* group and other heterocystous genera, although the *nifH* sequence from *F. ambigua* has not yet been determined.

The degree of similarity of the *nifH* DNA sequences among representatives within the nonheterocystous cyanobacteria or the other eubacterial species groups was lower than that within the heterocystous cyanobacteria, except between *L. lagerheimii* and *P. boryanum* (81%). Although this is not as high a degree of similarity as that among the *Anabaena* spp. and *Nostoc* spp. (84 to 92% but only 70 to 81% among nonheterocystous sequences), it is consistent with the fact that *L. lagerheimii* and *P. boryanum* are taxonomically included within the *Lyngbya-Plectonema-Phormidium* group (16, 17). The amino acid similarity between *L. lagerheimii* and *P. boryanum* (94%) is much more striking (compared with 82 to 87% between other nonheterocystous sequences). Interestingly, the *L. lagerheimii* DNA sequence is more similar to the other bacterial sequences than is any of the other cyanobacterial sequences.

The results indicate that heterocystous *nifH* sequences (specifically the *Anabaena-Nostoc* group) can be identified with some certainty, whereas nonheterocystous or other eubacterial identifications may be dependent upon how closely related the uncharacterized *nifH* sequence is to one of the sequenced genes. The lower degrees of similarity within the nonheterocystous cyanobacteria and other bacterial sequences reflect the greater diversity of these groups, compared with the similarity within the *Anabaena-Nostoc* group. Sequences of the amplified 359-bp *nifH* fragment from species within the same genus can differ by only a few base pairs (*T. thiebautii* and *Trichodesmium erythraeum* [2]) or a few percent (*Frankia* strains [14]). The sequences of the *nifH* fragment from species that are taxonomically related, such as between the sequences from *Rhodobacter capsulata* and *Rhodospirillum rubrum* (87% for DNA and 95% for amino acids), have higher degrees of similarity than those from pairs of less closely related nonheterocystous cyanobacterial or other eubacterial genera. The results indicate that it will be useful to obtain a more diverse data base of sequences of the *nifH* fragment for characterizing unknown sequences. Fortunately, the use of PCR can facilitate the process of obtaining a data base of *nifH* sequences from pure cultures of representative organisms.

Although DNA sequences provide the most-definitive information on the characteristics of diazotrophs, ecological studies

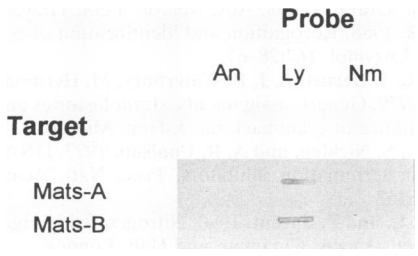


FIG. 4. Hybridization of *Lyngbya nifH* probe to amplified *nifH* fragment from North Carolina; cyanobacterial mats collected in March (A) and May (B) 1992. An, *Anabaena* sp. strain CA; Ly, *L. lagerheimii*; Nm, *N. muscorum.*

would be greatly facilitated if suitable probes could be developed to more rapidly characterize communities, at least prior to cloning and sequencing *nifH* amplification products. The amplified *nifH* fragments from the cyanobacterial strains used in this study were tested for their potential use in identification of unknown amplification products. Results showed that the probes were not species specific but cross-hybridized to various degrees with other sequences (Fig. 3). The degree of cross-hybridization with other species was usually related to the degree of similarity of the sequences. For example, the *Anabaena* sp. strain CA probe cross-hybridized to the greatest extent with the *Nostoc* spp. (sequences greater than 84% identical on the DNA level) (Fig. 3). However, in the case of the *N. muscorum* sequence, there was some cross-hybridization with *T. thiebautii* and *Gloeothece* sp. even though the *L. lagerheimii* and *P. boryanum* sequences are as similar to the *N. muscorum* sequence as are the *T. thiebautii* and *Gloeothece* sp. sequences (Fig. 3).

The probes cross-hybridize to some extent between *nifH* sequences, yet the probes are still potentially useful for general characterization of unknown assemblages. The *Anabaena* sp. strain CA, *L. lagerheimii*, and *N. muscorum* probes were used to probe the amplification product from a cyanobacterial mat from North Carolina (Fig. 4). Microscopic examination of the mat indicates that *Lyngbya* is an important component, although experimental evidence indicated that other diazotrophs may also be involved in nitrogen fixation (1). The results of the probe hybridization indicate that *Lyngbya* species (or perhaps those of another nonheterocystous genus) are the dominant nitrogen-fixing microorganisms, at least among the cyanobacterial species in the mat community. Full characterization of the mat diazotrophic community is currently in progress, and preliminary results indicate that although heterocystous cyanobacteria are probably not important, other eubacterial nitrogen-fixing microorganisms are present.

The use of oligonucleotides, designed from the amplified *nifH* sequence, holds great promise for rapidly characterizing unknown or mixed *nifH* amplification products. The oligonucleotide designed from a conserved region within the heterocystous cyanobacteria hybridized with the cyanobacterial *nifH* amplification products to various degrees, primarily reflecting the number of mismatches between the probe and the target sequences (Fig. 5). In contrast, a probe designed to reflect the other eubacterial sequences did not hybridize to any of the cyanobacterial *nifH* fragments (Fig. 5). These two probes, and other similar oligonucleotides designed for other taxonomic groups will facilitate the characterization of diazotrophs in mixed assemblages, without the necessity for cloning and sequencing.

One of the interesting aspects of nitrogen fixation is that it is so widely, but seemingly randomly, distributed throughout microbial taxa. However, as proposed by Young (23), the distribution of N_2 fixation capability may simply have been overlooked to a great extent because of our preconceived notion that N_2 fixation is a special capability. The information presented here provides a foundation for analysis of the ecology of N_2-fixing communities in nature and also provides a tool for investigating the diversity and distribution of nitrogen fixation genes in the microbial world.

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