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Use of Degenerate Oligonucleotides for Amplification of the *nifH* Gene from the Marine Cyanobacterium *Trichodesmium thiebautii*

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***Trichodesmium* spp. are marine filamentous, nonheterocystous, nitrogen-fixing cyanobacteria which are an important component of marine ecosystems. This organism has never been maintained in axenic culture, and there has remained some doubt as to the identity of the organism responsible for nitrogen fixation in *Trichodesmium* aggregates. By using degenerate oligonucleotide primers, it has been possible to amplify, clone, and sequence a segment of the *nifH* gene from a natural assemblage of *Trichodesmium thiebautii*. Examination of the DNA and presumed amino acid sequence shows that the gene is most closely related to that of *Anabaena* spp. and therefore is most likely a cyanobacterial *nifH* gene. The use of degenerate oligonucleotides, in concert with the polymerase chain reaction, can be a powerful tool for the cloning and sequencing of a variety of genes from microorganisms in the environment.**

Trichodesmium spp. are nonheterocystous cyanobacteria which are found in tropical and subtropical oceanic waters (3). Since the discovery of nitrogen fixation in association with this organism (7), many studies have attempted to characterize the nitrogen fixation activity and physiology of *Trichodesmium* spp. (2, 4, 16, 22, 23, 27). *Trichodesmium* spp. form aggregates or bundles of trichomes with which numerous microorganisms are associated. Since *Trichodesmium* spp. have never been maintained in axenic culture, little is known about the nitrogen fixation apparatus, and there has remained some doubt as to the identity of the organism responsible for nitrogen fixation in *Trichodesmium* bundles.

Nitrogenase, which catalyzes the reduction of dinitrogen to ammonium, is rapidly inactivated by oxygen. Oxygenic photoautotrophs which fix nitrogen must protect nitrogenase from the oxygen evolved during photosynthesis. Heterocystous filamentous forms, such as *Anabaena* spp., synthesize nitrogenase only in the low oxygen environment of heterocysts (11). A few species of cyanobacteria have been isolated which fix nitrogen in the same cell in which oxygen is evolved. For example, the marine single-celled cyanobacterium *Synechococcus* spp. appears to limit nitrogen fixation activity to specific times during the growth cycle (19). *Gloeothece* spp., *Oscillatoria* spp., and *Synechococcus* sp. strain RF-1, although capable of fixing nitrogen under constant illumination (8, 9, 15, 34), fix nitrogen mainly during the period of darkness when grown under alternating light and dark conditions, regardless of the growth phase (8, 9, 35). In contrast, the highest rates of nitrogen fixation in *Trichodesmium thiebautii* occur during daylight hours, when photosynthetic activity is maximal (30). This phenomenon has been explained in two ways: (i) that cells along the trichome of *Trichodesmium* spp. are differentiated with respect to oxygen evolution and nitrogen fixation activity (2, 4) or (ii) that the structure of individual cells is such that oxygen production and nitrogen fixation are spatially separated within the cell (22). Alternative explanations are that the nitrogen fixation is due to an associated heterotrophic microorganism or that the *Trichodesmium nif* gene products are less sensitive to inactivation by oxygen than in other nitrogen-fixing microorganisms. Determination of the sequence of the *nif* genes in natural assemblages of *Trichodesmium* spp. can yield information on the type of organism responsible for nitrogen fixation, as well as indicate potential structural differences in the nitrogenase protein. Furthermore, the only complete sequence of a cyanobacterial *nifH* gene (which encodes the Fe protein of nitrogenase) that has been published so far is from *Anabaena* sp. strain PCC 7120, a heterocystous cyanobacterium.

The polymerase chain reaction (20, 28, 29) has been used to clone and sequence low-copy-number genes from small DNA samples (10, 17, 29). This technique uses two primers on opposite DNA strands to amplify the intervening DNA through repeated cycles of denaturation, annealing, and DNA synthesis, by using a DNA polymerase (10). The polymerase chain reaction usually requires prior knowledge of the DNA sequence of interest in order to construct the primers for initiation of DNA synthesis with DNA polymerase. However, even genes that are highly conserved on the amino acid level may not be homologous on the DNA level because of the degeneracy of the translational code. We have found that, in regions of conserved amino acid sequence, degenerate oligonucleotide primers (96- and 128-fold degenerate) can be constructed that will amplify a target DNA sequence with high specificity. The specificity results from amplification between the oligonucleotides, for the degeneracy prohibits their use separately as specific probes. This method makes it possible to clone and sequence a gene of interest without the construction of a DNA library, production of an antibody, or precise knowledge of the DNA sequence. We present here a partial sequence of the *nifH* gene which was amplified from a natural population of *T. thiebautii* by using degenerate primers and the polymerase chain reaction. The DNA sequence was 66 to 79% similar to those of previously sequenced *nifH* genes but appears to be most closely related to the *nifH* gene of an *Anabaena* sp.

MATERIALS AND METHODS

T. thiebautii was collected in the western Caribbean Sea from the research vessel R/V Iselin with a plankton net fitted with Nitex netting (mesh size, 200 μm). Plankton tows were collected from the surface and at a 20-m depth at a towing speed of 1 to 2 knots (1 knot ≃ 1,852 m/h). *Trichodesmium*

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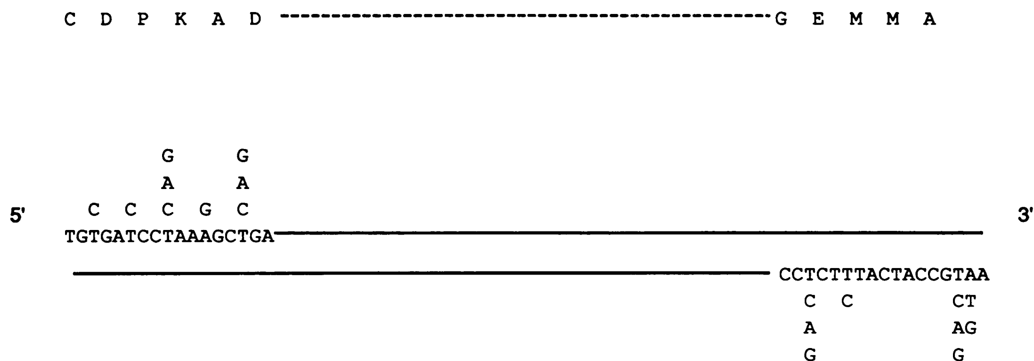

FIG. 1. Structure of degenerate oligonucleotide primers used for amplification of a 359-bp segment of the *nifH* gene. Each primer consists of a mixture of all possible combinations of the base sequences.

bundles were collected from the net tow samples with a Pasteur pipette and placed in buffer (10 mM Tris [pH 8.0], 100 mM EDTA [pH 8.0], 0.3 M NaCl). The *Trichodesmium* bundles were then concentrated with Nitex netting and frozen in microcentrifuge tubes. *T. thiebautii* was the predominant photosynthetic organism in the bundles, and there were no heterocystous cyanobacteria present (E. J. Carpenter, personal communication). The *Trichodesmium* sample (100 μl) was extracted with hot (55°C) buffer-saturated phenol (80 μl) and sodium dodecyl sulfate (1%) for 15 min. Chloroform (80 μl) was added, and the sample was vortexed briefly and spun in a microcentrifuge for 2 min. The phenol-chloroform was back-extracted, and supernatants were combined and extracted with an equal volume of phenol-chloroform. The phenol-chloroform was again back-extracted, and the sample was extracted with phenol-chloroform until the interface was clear. Two volumes of ice-cold propanol and 0.1 volume of 3 M sodium acetate were added, and the DNA was precipitated overnight (−20°C). The DNA was suspended in 100 μl of TE buffer and dialyzed overnight in 1× TE. This procedure resulted in a preparation of high-molecular-weight DNA.

Degenerate oligonucleotide primers (17-mers) were constructed for amplification of a 359-base-pair (bp) fragment of the *nifH* gene on the basis of published DNA sequences. The oligonucleotide sites were chosen from highly conserved amino acid sequences that required less than 200-fold degeneracy of the DNA coding sequences (Fig. 1). The primers were synthesized with every possible combination of the base sequences, resulting in a mixture of 128 and 96 oligonucleotides for the upstream and downstream primers, respectively (Fig. 1). The amplification was performed essentially as described by Saiki et al. (28). The DNA (25 ng) was added to polymerase buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM Tris hydrochloride (pH 8.0), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 200 μM each of dATP, dCTP, dGTP, and dTTP, 100 μg of bovine serum albumin per ml]. Taq polymerase (2.5 U; New England BioLabs, Inc., Beverly, Mass.) was added, and a programmable heating block was used to repeat cycles of denaturation (93°C, 1.2 min), annealing (50°C, 1.0 min), and extension (70°C, 1.5 min). Following 30 rounds of amplification, a single 359-bp band was visible on an ethidium bromide-stained 4% NuSieve (FMC Corp., Rockland, Maine) agarose gel. This band was isolated with DEAE paper (38) (NA45, Schleicher & Schuell, Inc., Keene, N.H.). The fragment was eluted from the DEAE paper in 300 μl of buffer (20 mM Tris [pH 8.0], 0.1 M EDTA [pH 8.0], 1 M NaCl) at 65°C for 1 h. The DNA was then extracted with phenol (100 μl) and precipitated with 750 μl of propanol. Following suspension in TE, the fragment was blunt ended with Klenow fragment (5 U; New England BioLabs) in ligation buffer (50 mM Tris, 10 mM $MgCl_2$, 10 mM dithiothreitol, 100 μM each deoxynucleoside triphosphate [pH 7.6]) at room temperature for 30 min. The enzyme was heat inactivated (65°C for 10 min), ATP (1 mM) and T4 DNA ligase (400 U; New England BioLabs) were added, and the fragment was blunt end ligated into the sequencing vector M13mp19 overnight (39). Cloning procedures were as described by Maniatis et al. (14). Following transformation of the host strain JM101 (New England BioLabs), single-stranded DNA was isolated and sequenced with the dideoxynucleotide termination method (with Klenow fragment or T7 DNA polymerase) and $[^{35}S]$dATP (31). Three clones were sequenced, one of which was in opposite orientation. Both strands were sequenced from one clone. The DNA sequence data were compared with those for previously sequenced *nifH* genes by using the University of Wisconsin Genetics Computer Group data analysis programs (6).

RESULTS

The DNA sequence in two of the three clones sequenced had identical DNA sequences, whereas the third had four nucleotide base changes. The DNA and deduced amino acid sequences of the 359-bp product of amplification (on the basis of the two identical clones) are shown in Fig. 2. Comparisons were made to the corresponding sequences of the other *nifH* genes and gene products, excluding the primer sequences which may have included artifacts due to annealing of a slightly heterologous primer. The DNA sequence was 66 to 79% similar to the corresponding nucleotide sequence of the *nifH* gene from an *Anabaena* sp. (18), *Rhizobium meliloti* (37), *Clostridium pasteurianum* (5), *Azotobacter vinelandii* (1), *Klebsiella pneumoniae* (33, 36), and *Rhodobacter* (*Rhodopseudomonas*) *capsulatus* (32), whereas the deduced amino acid sequence was 76 to 84% similar (exact matches) (Table 1).

Although the amino acid sequence of the *nifH* gene is highly conserved, there are numerous positions where amino acid residues vary between species (Fig. 3). There are 42 variable amino acid positions in the 359-bp segment, of which 8 are unique to *C. pasteurianum*. In the 34 remaining positions, there are 41 amino acids which occur in a specific position in only one species (other than *T. thiebautii*). The amino acids in variable regions of the *T. thiebautii* sequence match the unique amino acid residues used by the *Anabaena*

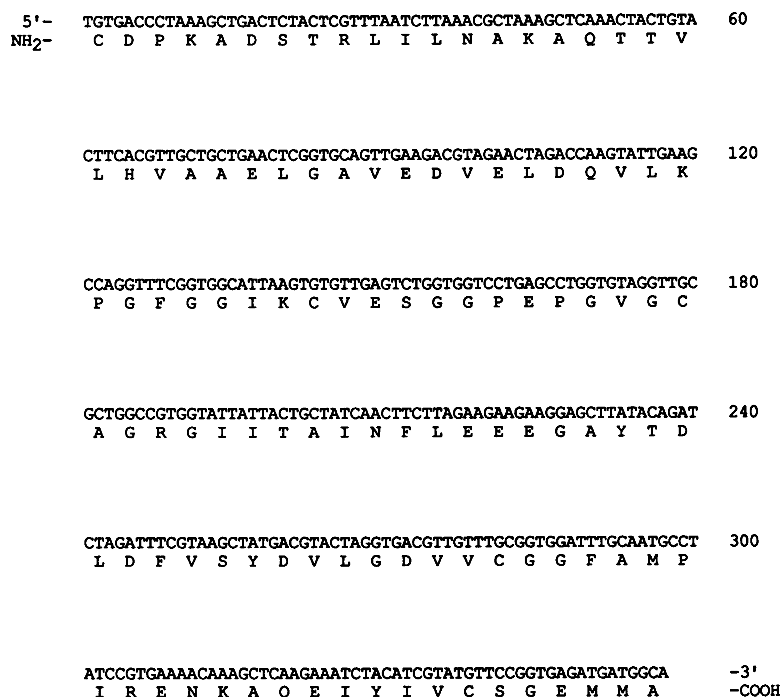

FIG. 2. Nucleotide and deduced amino acid sequence of the *nifH* amplification product from *T. thiebautii*.

sp. the most often (Table 1; shaded boxes in Fig. 3). This includes the sequence, gap-leucine, at position 81 to 82 in the sequence. No exact matches were found with *K. pneumoniae* or *A. vinelandii* at unique amino acid residues (Table 1). Furthermore, the isoleucine at position 65 in the sequence of a ribosylation site (24) is also found only in the *Anabaena* sp. and *C. pasteurianum*.

DISCUSSION

Trichodesmium spp. are believed to be important in nitrogen fixation in aerobic tropical and subtropical oceanic waters (3). However, since it has rarely been maintained in culture (22) and has never been grown in axenic culture, it has never been possible to determine the nature of the nitrogen fixation apparatus in *Trichodesmium* assemblages. With the construction of degenerate oligonucleotide primers and use of the polymerase chain reaction, it has been possible to clone and sequence part of the *nifH* gene from *T. thiebautii*. From examination of the DNA sequence and the deduced amino acid sequence, it is evident that the *nifH* gene in *T. thiebautii* is similar to the *nifH* gene of other nitrogen-fixing microorganisms. The *T. thiebautii* deduced amino acid sequence of the *nifH* gene is similar to that of the other *nifH* genes which have been sequenced. The DNA sequence of the *T. thiebautii nifH* gene has more similarity with the *Anabaena* sp. *nifH* gene than with the other *nifH* sequences (Table 1). GC content of this segment is similar to the GC content of the *Anabaena* sp. segment but quite different from those of the other species, with the exception of *C. pasteurianum* (Table 1). Usage of amino acids in nonconserved positions resembles amino acid usage by the *Anabaena* sp. more than it resembles those of the other species (Fig. 3). Although *C. pasteurianum* has three unique matches at variable amino acid positions, it also has the greatest number of total differences in amino acid residues (Table 1).

TABLE 1. Comparison of the composition of the 359-bp segment of the *T. thiebautii nifH* gene and gene product with the corresponding segments of other *nifH* genes

Species	% Similarity[a]		% GC	No. of unique matches[b]	No. of differences
	Nucleic	Protein			
T. thiebautii			43		
Anabaena sp.	79	84	50	4	17
A. vinelandii	72	83	59	0	19
K. pneumoniae	68	79	61	0	22
R. capsulatus	68	82	60	0	19
C. pasteurianum	66	76	40	3	27
R. meliloti	70	83	58	0	18

[a] Exact matches.
[b] Matches at nonconserved positions between *T. thiebautii* and only one other species.

The iron protein can be inactivated by ribosylation in *Rhodospirillum* spp. (12). The sequence for this site is GRGIIT (24), which is present in most of the *nifH* genes sequenced thus far, with the exception of an *Anabaena* sp. and *C. pasteurianum*. In the *Anabaena* sp., this sequence is GRGVIT, which is also found in the *T. thiebautii* sequence. Considering the GC content, the matches at variable amino acid positions, and the similarity in the ribosylation site, the *T. thiebautii* sequence appears to be most similar to that of the *Anabaena* sp. Thus, the cloned fragment appears to be cyanobacterial in origin and not derived from an associated heterotrophic bacterium. Further confirmation of the similarity of the *T. thiebautii nifH* gene to that of the *Anabaena* sp. may be obtained when the less conserved carboxy-terminal region is sequenced.

Even though the *T. thiebautii* sequence is most closely related to the *Anabaena* sp. sequence (79 and 84% similar at the DNA and protein levels, respectively), it has unique

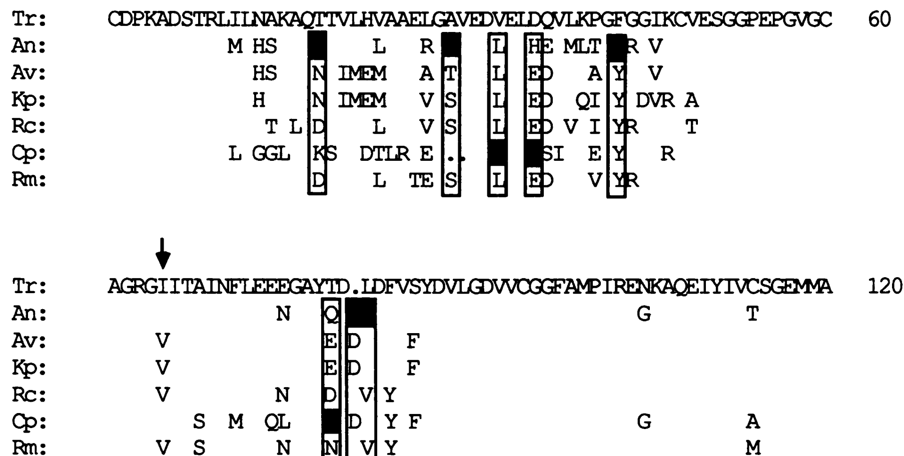

FIG. 3. Comparison of the deduced amino acid sequence of *T. thiebautii* to *Anabaena* sp. strain PCC 7120 (An [18]), *A. vinelandii* (Av [1]), *K. pneumoniae* (Kp [33, 36]), *Rhodobacter capsulatus* (Rc [32]), *C. pasteurianum* (Cp [5]) and *R. meliloti* (Rm [37]). Identical matches between *T. thiebautii* and other sequences are indicated by blank spaces. The positions where the *Trichodesmium* sequence matches an amino acid used by only one other species are boxed, with the shaded portion indicating the location of the exact match. ↓, Change in base at ribosylation site.

characteristics. At a number of variable (nonconserved) amino acid positions, *T. thiebautii* uses an amino acid used by one of the heterotrophic bacterial species or an amino acid not yet found at this position. Third base usage, determined from the 359-bp fragment in *T. thiebautii*, appears to be different from that of the other species (data not presented). These preliminary data suggest that codon usage in *T. thiebautii* may be different from that of the other organisms studied so far, including the *Anabaena* sp.

Two of the three clones (in opposite orientation) sequenced had identical DNA sequences, whereas a third had four nucleotide changes that resulted in amino acid changes. The amino acid sequence of the third clone was even more similar to the *Anabaena* sp. *nifH* protein sequence. The difference in sequence between the three clones could have been a result of (i) artifacts created during amplification, (ii) amplification of *nifH* genes from more than one organism, or (iii) amplification of more than one copy of the *nifH* gene from the same organism. Sequences determined from DNA amplified with Taq polymerase can contain errors due to misincorporation by Taq polymerase (28). However, these errors are relatively infrequent (error frequency of 0.25% [28]) and cannot explain the number of differences found between the *T. thiebautii nifH* sequence and the other reported *nifH* sequences. The variation in sequence between the two clones may also be due to the amplification of *nifH* genes from more than one organism, but it is unlikely that both sequences would be so closely related to that of the *Anabaena* sp. The most plausible explanation is that the two clones are derived from more than one *nifH* gene in *T. thiebautii*, as multiple *nifH* or *nifH*-like sequences have been found in other microorganisms (5, 21, 25, 26). This issue will be resolved when downstream sequence data are obtained.

Degenerate oligonucleotide primers can be constructed for conserved regions in amino acid sequences, even if the precise sequence of the DNA coding for these sequences is not known. Recently, this technique has been used for amplification of a family of related viral genes by using highly conserved octapeptide regions (13). The primers constructed in this study can be used for amplification of *nifH* from other microorganisms as well; they were used successfully to amplify a 359-bp fragment of *nifH* from several marine heterotrophic bacterial isolates (J. Kirshtein and J. P. Zehr, unpublished data). Even though degenerate oligonucleotides (100- to 200-fold degenerate) cannot be used effectively for hybridization experiments because of the relatively high degree of degeneracy, they can be used to amplify low-copy-number genes from genomic DNA with high specificity. This technique makes it possible to amplify, clone, and sequence a variety of genes which it has not been possible to study because of the difficulties of obtaining large amounts of DNA and of screening with polyclonal antibodies or heterologous DNA probes. The use of degenerate oligonucleotide primers in concert with the polymerase chain reaction is a powerful tool for studying the genes of microorganisms in the environment.

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