APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2645–2650 Vol. 57, No. 9
0099-2240/91/092645-06$02.00/0
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Amplification, Cloning, and Sequencing of a *nifH* Segment from Aquatic Microorganisms and Natural Communities

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Received 25 March 1991/Accepted 1 July 1991

By use of the polymerase chain reaction and degenerate oligonucleotide primers for highly conserved regions of *nifH*, a segment of *nifH* DNA was amplified from several aquatic microorganisms, including an N_2-fixing bacterium closely associated with the marine filamentous cyanobacterium *Trichodesmium* sp., a heterotrophic isolate from the root/rhizome of the seagrass *Ruppia maritima*, and the heterocystous freshwater cyanobacterium *Anabaena oscillarioides*. *nifH* segments were amplified directly from DNA extracted from the rhizosphere of roots of the seagrass *Halodule wrightii*. The *nifH* fragments were then cloned and sequenced. The DNA and deduced amino acid sequences were compared with known sequences, revealing distinct differences between taxonomic groups. This technique was shown to be useful for (i) the detection of N_2-fixing microorganisms and (ii) rapidly obtaining the DNA sequence of the *nifH* gene, which provides information about general taxonomic groups of N_2-fixing microorganisms.

Primary production in coastal and marine systems is often limited by biologically available nitrogen (9, 10, 34). It has remained enigmatic that biological nitrogen fixation, which could alleviate N depletion in coastal and marine waters by bringing in new nitrogen, does not appear to do so to a greater extent (4). Planktonic N_2 fixation in oceanic waters is thought to contribute <1% of total nitrogen requirements to the system (5, 16). Most of the nitrogen fixation in the euphotic zone is attributed to *Trichodesmium* blooms (4, 20). However, numerous studies have demonstrated the presence of N_2-fixing microheterotrophs in marine waters (14, 18, 23, 50). Further, addition of organic substrates to coastal North Carolina waters resulted in stimulation of N_2 fixation (as measured by the acetylene reduction technique) on a year-round basis (34). Other N_2-fixing microorganisms, such as unicellular cyanobacteria and endosymbionts in diatoms (e.g., *Richelia* spp.), could also be major sources of fixed N in oceanic waters (22, 26, 46, 47). It is therefore possible that both the numbers of nitrogen fixers and their nutritional impact on primary productivity have been greatly underestimated (14, 19).

Previous studies have been limited in two ways. Measurement of nitrogenase activity is dependent on active N_2 fixation at the time of sampling and may not detect the presence of inactive N_2-fixing microorganisms. Nitrogenase activity can occur at a time different from that of sampling and incubation (e.g., at night), or specific environmental conditions favorable to nitrogenase activity may be disrupted during sampling and incubation. Also, while culturing diazotrophs from marine waters is the most direct way to demonstrate their presence, a small percentage (<1 to 10%) of marine bacteria may actually be able to be cultured (3, 11, 17).

Studies of the genetics of N_2-fixing microorganisms have been hampered by related problems. Until recently, in order to obtain genetic information, a DNA library was constructed from an organism in culture (or at least a dominant organism in a sample), the genes of interest were identified and cloned, and the DNA was sequenced. It is now possible to obtain DNA sequence information directly from genomic DNA by using the polymerase chain reaction (PCR) (27, 39). This technique is particularly useful for amplifying sequences of interest from environmental samples having an unknown diversity.

PCR has recently been used to detect and study *Trichodesmium* spp. and *Frankia* spp. (28, 30, 52). In the *Trichodesmium* study, it was shown that degenerate oligonucleotides could be used to amplify a segment of the *Trichodesmium nifH* gene (52). Using these oligonucleotides, we amplified a segment of the *nifH* gene from (i) *Anabaena oscillarioides*, a freshwater heterocystous cyanobacterium, (ii) a bacterium designated RM1-2 (and RM2-2, both believed to be the same species), a salt-requiring *Klebsiella* sp. isolated from *Ruppia maritima* roots, (iii) a flagellated bacterium designated M1, which was isolated from a natural *Trichodesmium* aggregate, and (iv) DNA extracted from *Halodule wrightii* root rhizospheres. The amplified fragments were then cloned, and the DNA was sequenced. Here we show that this technique makes it possible not only to detect the presence of *nif* genes but also to differentiate between *nif* genes from cyanobacteria and those from other eubacteria.

MATERIALS AND METHODS

Two *R. maritima* isolates (designated RM1-2 and RM2-2), both belonging to the same salt-requiring *Klebsiella* sp., were isolated from the roots/rhizomes as previously described (7). *A. oscillarioides* was isolated (K. Lam) from the Waikato River, New Zealand. A microaerophilic bacterium (unidentified, designated M1) was isolated from *Trichodesmium* aggregates as previously described (33, 35).

DNA was extracted from the cultures of heterotrophs (RM1-2, RM2-2, and M1) with phenol-chloroform following cell lysis (21). *A. oscillarioides* DNA was extracted according to the protocol of Mazur et al. (24). *H. wrightii* plants were collected from Middle Marsh, near Beaufort, N.C. The roots were dipped briefly in filtered seawater to remove sediment, placed in TNE buffer (10 mM Tris-Cl [pH 8.0], 150

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mM NaCl, 100 mM EDTA), and sonicated to release the bacteria into the buffer. The DNA was extracted by standard methods except that the DNA was treated twice with polyvinylpolypyrrolidone to remove humic compounds contaminating the sample (15, 48).

Primers were those used by Zehr and McReynolds (52). Primer 1 is located at positions 123 to 139 in reference to the *Anabaena* strain 7120 *nifH* sequence (which is identical to the *nifH* mRNA), and primer 2 is located on the opposite strand, corresponding to positions 466 to 482 (25).

Primer 1: 5′-TGXGAXCCYAAZGCYGA-3′ X = T or C, Y = A,C,G, or T
Primer 2: 5′-AWYGCCATCATXTCYCC-3′ Z = A or G, W = A,T, or G

Amplification was performed as follows. The DNA (1 to 10 ng) was amplified in polymerase buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl (pH 8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 200 μM each dATP, dCTP, dGTP, and dTTP, 100 μM each primer, and 100 μg of bovine serum albumin per ml, with 2.5 U of *Taq*1 polymerase added to the 100-μl reaction mixture]. Thirty-five cycles of denaturation (93.5°C for 1.2 min), annealing of primers (57°C for 1 min), and extension (70°C for 1.5 min) were performed. Amplification products were visualized on 4% NuSieve (FMC Bioproducts) agarose gels stained with ethidium bromide. The fragment was isolated with DEAE paper as previously described (49). DNA fragments were blunt ended with Klenow fragment and ligated into the *Sma*I site of the phage M13mp18 or M13mp19 vector (51). The fragment was ligated to the vector at 14 or 25°C with 6 to 7 U of T4 DNA ligase. *Escherichia coli* 7118 or JM101 was transformed with the ligated DNA, and single-stranded DNA was prepared from positive plaques. The cloned inserts were sequenced with Sequenase (United States Biochemical Corp.) and [^{35}S]dATP (40). In order to sequence the opposite strand, *Eco*RI-*Hin*dIII fragments which included the *nifH* inserts were isolated from the phage and ligated into the M13 vector with reversed polylinker cloning sites. Single-stranded DNA was prepared and sequenced. Sequence data were analyzed with the University of Wisconsin Genetics Computer Group data analysis programs (8).

RESULTS

Figures 1 to 4 show the DNA and deduced amino acid sequence data for the *nifH* amplification products from *A. oscillarioides* (two clones), M1, RM1-2 (and RM2-2), and *H. wrightii* root DNA (HRD) (two clones). Table 1 shows DNA and deduced amino acid sequence similarity between organisms; sequences were determined from PCR products or are from a representative group of previously published sequences (percent similarity is based on exact matches).

The two *A. oscillarioides nifH* segments are not identical; there are 12 different bases resulting in three different amino acids between the primer regions (Fig. 1). The number of differences cannot be attributed to the error rate of *Taq*1 polymerase during amplification (error rate = 0.25% [39]). The sequences are most similar to other cyanobacterial *nifH* sequences (Table 1). Two sequences were obtained from the HRD (Fig. 4). There are 103 different bases resulting in 17 different amino acids between the two fragments. The HRD amplification products are most similar in DNA sequence and amino acid usage to the *Clostridium pasteurianum* sequence (Table 1). Amino acid usages unique to *C. pasteurianum* and HRD in these comparisons include GL (amino acids 8 and 9), DTLR (amino acids 16 to 19), and the two consecutive gaps (amino acids 23 and 24) in variable regions of *nifH* (Fig. 5). HRD also has two extra amino acids (amino acids 76 and 77) that do not occur in *C. pasteurianum*. The site of ribosylation in a *Rhodospirillum* sp. (which inactivates the iron protein) has the sequence GRGVIT (37). The cyanobacteria sequenced and *C. pasteurianum* have the sequence GRGIIT, as does HRD.

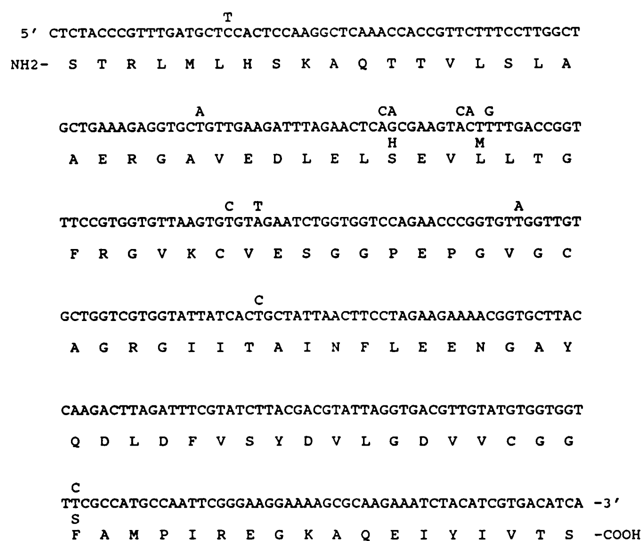

FIG. 1. Nucleotide and deduced amino acid sequences of two *nifH* amplification products from *A. oscillarioides*.

The sequences of the *R. maritima* isolates, RM1-2 and RM2-2, are identical, and these isolates are believed to be the same species (Fig. 2). RM1-2 and RM2-2, belonging to a *Klebsiella* sp., are most similar in sequence to *Klebsiella pneumoniae*, as expected (Table 1). The *Trichodesmium*-associated bacterium M1, whose genus is not known, is most similar to *K. pneumoniae*, RM1-2 (or RM2-2), and *Azotobacter vinelandii* (Table 1). These four species also share amino acid usage, with NTIMEM in positions 12 to 17, a variable region in the *nifH* gene (Fig. 5).

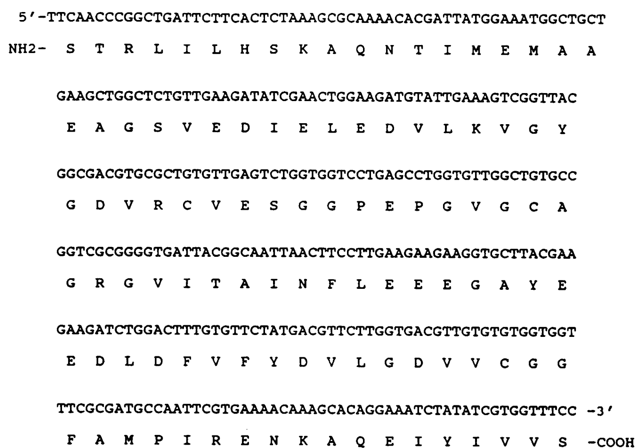

FIG. 2. Nucleotide and deduced amino acid sequences of the *nifH* amplification product of RM1-2 and RM2-2.

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5'-TTCTACCCGTTTGATTCTGCATTCAAAAGCTCAAAATACCATCATGGAGATGGCAGCA
NH2- S  T  R  L  I  L  H  S  K  A  Q  N  T  I  M  E  M  A  A
   GAAGCCGGTACCGTTGAAGACATCGAACTGGAAGATGTATTGAAGATTGGTTTT
    E  A  G  T  V  E  D  I  E  L  E  D  V  L  K  I  G  F
   GGTGATGTTCGCTGTGTTGAATCCGGTGGTCCTGAGCCAGGTGTCGGCTGTGCA
    G  D  V  R  C  V  E  S  G  G  P  E  P  G  V  G  C  A
   GGACGTGGTGTCATTACCGCGATTAACTTCCTTGAAGAGGAAGGTGCATATGAA
    G  R  G  V  I  T  A  I  N  F  L  E  E  E  G  A  Y  E
   GATGACATCGATTTTGTATTTTACGATGTGCTCGGGGATGTGGTCTGTGGCGGA
    D  D  I  D  F  V  F  Y  D  V  L  G  D  V  V  C  G  G
   TTTGCCATGCCGATTCGTGAATACAAAGCTGAAGAAATCTATATCGTCGTATCC -3'
    F  A  M  P  I  R  E  Y  K  A  E  E  I  Y  I  V  V  S  -COOH
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *nifH* amplification product from M1, a bacterium associated with a *Trichodesmium* sp.

DISCUSSION

The two *nifH* sequences from *A. oscillarioides* suggest that *A. oscillarioides* has multiple *nifH* genes. Although the possibility of both PCR primers annealing to and amplifying a 359-bp segment of a similar but non-*nif* gene is remote, a more similar *nifH*-like gene from an N_2-fixing microorganism could be detected. A *nifH*-like gene has been found in *Anabaena* strain 7120, although it is not known if this is a functional gene and perhaps an alternative *nifH* (38). Similarly, one of the sequences found here may not be functional, although the similarity between the two is quite high (96% on the DNA level and 97% on the amino acid level). It is possible that an alternative nitrogenase exists in *A. oscillarioides*, such as that found in *A. vinelandii* and other organisms (1). The possibility also exists that the *A. oscillarioides* culture contains two *Anabaena* strains or that the error rate for *Taq*1 polymerase in this experiment was higher than published estimates, leading to artifacts when the cloned product was sequenced (32). The two *nifH* sequences obtained from the HRD have 69% similarity on the DNA level and 84% similarity on the amino acid level and are too

```
      C TC T  G  G  CGGC  CC CT C  AC     TG        T   T   G
5'-CTCAACAAGGCTCCTCCTGAATGGATTGGCACAGAAAACCATATTGGACACCCTGCGT

                     G        S     Q  V
NH2- S  T  R  L  L  L  N  G  L  A  Q  K  T  I  L  D  T  L  R

     A    C  G    GGAA  A  G   A T  C   GCA  T TTATG  A
   GATGAAGGAGAAGATGTCCTGTTGGAAGATGTCCGAAAAATCGGATACGGTGGC

    E              E           I        A     F  M
    D  E  G  E  D  V  L  L  E  D  V  R  K  I  G  Y  G  G

    CA T   GTA  G  G  C     G  G     T  T  C        A  C
   ACTTTGTGTACGGAATCAGGAGGACCCGAACCAGGCGTCGGGTGCGCCGGCCGA

    I     V
    T  L  C  T  E  S  G  G  P  E  P  G  V  G  C  A  G  R

    A     T  G  G     TA  T G  G   CCT     G  C CA     C
   GGTATTATCACCTCTATTAACCTGCTTGAACAGTTAGGCGCTTATGGCGAAGAT

                        M        P           A
    G  I  I  T  S  I  N  L  L  E  Q  L  G  A  Y  G  E  D

    AC TT G     C CA  C  T  C   C C  C              C  A
   GAGAAACTCGATTATGTGTTTTACGATGTATTGGGTGATGTTGTCTGCGGTGGT

    H        A
    E  K  L  D  Y  V  F  Y  D  V  L  G  D  V  V  C  G  G

       T        T  C     C  G        G  T        G  C  C
   TTTGCCATGCCGATCCGGGAAGGAAAAGCCCAGGAAATCTATATTGTTGTTTCT -3'

    F  A  M  P  I  R  E  G  K  A  Q  E  I  Y  I  V  V  S  -COOH
```

FIG. 4. Nucleotide and deduced amino acid sequences of *nifH* amplification products from DNA extracted from *H. wrightii* roots.

TABLE 1. Comparison of nucleotide and deduced amino acid percent similarities of *nifH* amplification products between organisms

Organism[a]	% Similarity[b]																											
	T.th.		A.os1		A.os2		An7120		RM1-2		M1		HRD 1		HRD 2		K.pneu.		R.phas.		R.mel.		Rh.ca.		C.pas.		A.vin.	
	DNA	AA[c]	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA	DNA	AA
T.th.			77	82	77	84	79	84	74	81	73	79	63	72	64	72	68	79	65	77	70	83	68	82	66	76	72	83
A.os1	77	82			96	97	89	98	73	77	73	76	64	68	60	69	67	76	64	77	68	79	68	76	64	70	72	80
A.os2	77	84	96	97			87	97	73	79	72	78	64	69	61	70	67	78	63	77	68	81	67	77	64	72	71	82
An7120	79	84	89	98	87	97			74	78	70	77	65	69	62	70	69	77	66	77	69	81	70	78	63	71	74	81
RM1-2	74	81	73	77	73	79	74	78			80	94	66	74	65	71	78	93	72	84	70	81	71	77	65	72	78	93
M1	73	79	73	76	72	78	70	77	80	94			66	71	63	69	77	90	70	81	70	76	69	75	64	70	77	92
HRD 1	63	72	64	68	64	69	65	69	66	74	66	71			69	84	66	72	65	73	68	74	65	71	69	84	66	73
HRD 2	64	72	60	69	61	70	62	70	65	71	63	69	69	84			66	67	64	70	67	72	63	68	66	85	66	64
K.pneu.	68	79	67	76	67	78	69	77	78	93	77	90	66	72	66	67			75	82	76	80	79	80	61	71	83	93
R.phas.	65	77	64	77	63	77	66	77	72	84	70	81	65	73	64	70	75	82			80	89	77	83	59	71	75	81
R.mel.	70	83	68	79	68	81	69	81	70	81	70	76	68	74	67	72	76	80	80	89			78	89	61	75	79	81
Rh.ca.	68	82	68	76	67	77	70	78	71	77	69	75	65	71	63	68	79	80	77	83	78	89			58	70	79	80
C.pas.	66	76	64	70	64	72	63	71	65	72	64	70	69	84	66	85	61	71	59	71	61	75	58	70			61	73
A.vin.	72	83	72	80	71	82	74	81	78	93	77	92	66	73	66	64	83	93	75	81	79	81	79	80	61	73		

[a] T.th., *Trichodesmium thiebautii*; A.os, *A. oscillarioides*; An7120, *Anabaena* strain 7120; K.pneu., *K. pneumoniae*; R.phas., *Rhizobium phaseoli*; R.mel., *Rhizobium meliloti*; Rh.ca., *Rhodobacter capsulatus*; C.pas., *C. pasteurianum*; A.vin., *A. vinelandii*.

[b] Percent similarity is based on exact matches. Sequence data obtained from references 2, 6, 25, 29, 41, 42, 44, 45, and 52.

[c] AA, amino acid.

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Tr.t:STRLILNAKAQTTVLHVAAELGAVEDVELDQVLKPGFGGIKCVESGGPEPGVGCAGRGIITAINFLEEEGAYTD...LDFVSYDVLGDVVCGGFAMPIRENKAQEIYIVCS

Ana :STRLMLHSKAQTTVLHLAAERGAVEDLELHEVMLTGFRGVKCVESGGPEPGVGCAGRGIITAINFLEENGAYQD...LDFVSYDVLGDVVCGGFAMPIREGKAQEIYIVTS

Aos1:STRLMLHSKAQTTVLSLAAERGAVEDLELHEVMLTGFRGVKCVESGGPEPGVGCAGRGIITAINFLEENGAYQD...LDFVSYDVLGDVVCGGSAMPIREGKAQEIYIVTS

Aos2:STRLMLHSKAQTTVLSLAAERGAVEDLELSEVLLTGFRGVKCVESGGPEPGVGCAGRGIITAINFLEENGAYQD...LDFVSYDVLGDVVCGGFAMPIREGKAQEIYIVTS


C.pa:STRLLLGGLAQKSVLDTLREEG..EDVELDSILKEGYGGIRCVESGGPEPGVGCAGRGIITSINMLEQLGAYTDD..LDYVFYDVLGDVVCGGFAMPIREGKAQEIYIVAS

HRD1:STRLLLNGLAQKTILDTLRDEG..EDVLLEDVRKIGYGGTLCTESGGPEPGVGCAGRGIITSINLLEQLGAYGEDEKLDYVFYDVLGDVVCGGFAMPIREGKAQEIYIVVS

HRD2:STRLLLGGLSQQTVLDTLREEG..EDVELEDIRKAGFMGTICVESGGPEPGVGCAGRGIITSINMLEQPGAYAEDEHLDYAFYDVLGDVVCGGFAMPIREGKAQEIYIVVS


M1  :STRLILHSKAQNTIMEMAAEAGTVEDIELEDVLKIGFGDVRCVESGGPEPGCGCAGRGVITAINFLEEEGAYEDD..IDFVFYDVLGDVVCGGFAMPIREYKAEEIYIVVS

R1-2:STRLILHSKAQNTIMEMAAEAGSVEDIELEDVLKVGYGDVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYEED..LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVVS

K.pn:STRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGDVRCAESGGPEPGVGCAGRGVITAINFLEEEGAYEDD..LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCS

A.vi:STRLILHSKAQNTIMEMAAEAGTVEDLELEDVLKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDD..LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCS

Rz p:STRLILHAKAQDTILSLAASAGSVEDLELEDVMKVGYKDIRCVESGGPEPGVGCAGRGVITSINFLEENGAYEN...IDYVSYDVLGDVVCGGFAMPIRENKAQEIYIVMS

Rz m:STRLILNAKAQDTVLHLAATEGSVEDLELEDVLKVGYRGIKCVESGGPEPGVGCAGRGVITSINFLEENGAYND...VDYVSYDVLGDVVCGGFAMPIRENKAQEIYIVTS

R.ca:STRLILNTKLQDTVLHLAAEVGSVEDLEVEDVVKIGYKGIKCTEAGGPEPGVGCAGRGVITAINFLEENGAYDD...VDYVSYDVLGDVVCGGFAMPIRENKAQEIYIVMS
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FIG. 5. Comparison of deduced amino acid sequences of *nifH* segments, showing distinct taxonomic groupings. Tr.t, *Trichodesmium thiebautii*; Ana, *Anabaena* strain 7120; Aos, *A. oscillarioides*; C.pa, *C. pasteurianum*; R1-2, RM1-2; K.pn, *K. pneumoniae*; A.vi, *A. vinelandii*; Rz p, *Rhizobium phaseoli*; Rz m, *Rhizobium meliloti*; R.ca, *Rhodobacter capsulatus*. Amino acids which differ between taxonomic groups are underlined.

different to be caused by *Taq*1 polymerase errors. The fragments may be from the same organism, containing multiple *nifH* genes, or they may be from different but closely related microorganisms. Because the HRD fragments are most similar in DNA sequence and amino acid usage to the *C. pasteurianum* sequence, they may actually be from a sediment organism rather than being closely associated with *H. wrightii* roots.

The inability to culture most marine bacteria has greatly hampered the study of function, diversity, and community structure of pelagic and benthic microorganisms. The advent of PCR has allowed genes from the environment to be amplified to track genetically engineered organisms in the environment (43), to detect phototrophs by amplifying the *rbcL* gene (36), to conduct phylogenetic and genetic diversity analysis of marine picoplankton (12, 13), and now to detect nitrogen-fixing microorganisms in the environment.

The overall rates of biological N_2 fixation and the degree to which N_2 fixers supplement (satisfy N requirements for) primary production in marine systems are still not known. Although most N_2 fixation measurements made in coastal and open-ocean planktonic environments yield nitrogenase activity at ecologically insignificant rates (unless they are taken during *Trichodesmium* or *Rhizosolenia-Richelia* blooms), it is possible that past measurements do not adequately assess N_2 fixation by heterotrophic bacteria, microorganisms in consortia, unicellular cyanobacteria, and endosymbionts in the *Rhizosolenia* sp. diatoms. Problems with most rate measurement (i.e., acetylene reduction or $^{15}N_2$ incorporation) techniques include (i) detection only if active fixation occurs at the time of sampling, (ii) disruption of natural conditions necessary for nitrogenase activity (i.e., bottle effects), (iii) changes in community structure during incubations, and (iv) inhibition of normal metabolism in certain microorganisms by acetylene (e.g., methane-oxidizing bacteria) (11, 31).

Clearly, conclusions on the degree and extent of N_2 fixation in pelagic waters cannot be obtained until accurate measurements can be made which are not limited by current experimental constraints. PCR allows for the detection of N_2 fixers by detection of the *nifH* gene and thus reflects genetic potential for nitrogen fixation in a particular environment. PCR using degenerate primers enables diverse groups of N_2-fixing microorganisms to be detected. We have successfully amplified *nifH* fragments from every diazotroph tested thus far. If the amplification products are then cloned and sequenced, the genetic diversity of diazotrophs in that system can also be assessed in addition to the dominant taxonomic group. Many questions can now be addressed such as the relative importance of free-living versus particle-associated nitrogen fixers, general quantitation of *nif* genes in different regimes and at different depths, the possible presence of alternative or multiple *nif* genes, and the relative importance of autotrophic versus heterotrophic and cyanobacterial versus other eubacterial diazotrophs. Lastly, application of PCR as a shipboard screening technique will

greatly facilitate locating and ultimately quantifying sources of marine and freshwater N_2 fixation.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation (NSF) grants OCE 8820036 and BSR 8918482 and NC Biotechnology Center grant ARIG 88013 to H.W.P. and NSF grant OCE 8916356 to J.Z.

We thank Paul Bishop for graciously providing laboratory space for the DNA sequencing work and for helpful discussions. We also thank Elizabeth Wolfinger for sequencing advice and assistance with the computer analysis and Carolyn Currin for the RM isolates.

REFERENCES

1. **Bishop, P. E., D. M. L. Jarlenski, and D. R. Hetherington.** 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **77**:7342–7346.
2. **Brigle, K. E., W. E. Newton, and D. R. Dean.** 1985. Complete nucleotide sequence of the *Azotobacter vinelandii* nitrogenase gene cluster. Gene **37**:37–44.
3. **Brock, T. D.** 1987. The study of microorganisms *in situ* progress and problems. Symp. Soc. Gen. Microbiol. **41**:1–17.
4. **Carpenter, E. J.** 1983. Nitrogen fixation by marine *Oscillatoria* (*Trichodesmium*) in the world's oceans, p. 65–104. *In* E. J. Carpenter and D. G. Capone (ed.), Nitrogen in the marine environment. Academic Press, Inc., New York.
5. **Carpenter, E. J., and D. G. Capone.** 1982. Nitrogen fixation in the marine environment. Science **217**:1140–1142.
6. **Chen, K. C. K., J. S. Chen, and J. L. Johnson.** 1986. Structural features of multiple *nifH*-like sequences and very biased codon usage in nitrogenase genes of *Clostridium pasteurianum* J. Bacteriol. **166**:162–172.
7. **Currin, C. A., H. W. Paerl, G. K. Suba, and R. S. Alberte.** 1990. Immunofluorescence detection and characterization of N_2-fixing microorganisms from aquatic environments. Limnol. Oceanogr. **35**:59–71.
8. **Devereux, J., P. Haebelli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the Vax. Nucleic Acids Res. **12**:387–395.
9. **Dugdale, R. C., and J. J. Goering.** 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol. Oceanogr. **12**:196–206.
10. **Eppley, R. W., E. H. Renger, E. L. Venrick, and M. M. Mullin.** 1973. A study of plankton dynamics and nutrient cycling in the central gyre of the North Pacific Ocean. Limnol. Oceanogr. **18**:534–551.
11. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**:49–55.
12. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345**:60–63.
13. **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.
14. **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.
15. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
16. **Howarth, R. W., R. Marino, and J. Lane.** 1988. Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 1. Rates and importance. Limnol. Oceanogr. **33**:669–687.
17. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
18. **Kawai, A., and I. Sugahara.** 1971. Microbiological studies on nitrogen in aquatic environments. II. On the nitrogen fixing bacteria in offshore regions. Bull. Jpn. Soc. Sci. Fish. **37**:981–985.
19. **Legendre, L., and M. Gosselin.** 1989. New production and export of organic matter to the deep ocean: consequences of some recent discoveries. Limnol. Oceanogr. **34**:1374–1380.
20. **Mague, T. H., F. C. Mague, and O. Holm-Hansen.** 1977. Physiology and chemical composition of nitrogen-fixing phytoplankton in the Central North Pacific Ocean. Mar. Biol. (New York) **41**:213–227.
21. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
22. **Martinez, L., M. W. Silver, J. M. King, and A. L. Alldredge.** 1983. Nitrogen fixation by floating diatom mats: a source of new nitrogen to oligotrophic ocean waters. Science **221**:152–154.
23. **Maruyama, Y., N. Taga, and O. Matsuda.** 1970. Distribution of nitrogen-fixing bacteria in the Central Pacific Ocean. J. Oceanogr. Soc. Jpn. **26**:360–366.
24. **Mazur, B. J., D. Rice, and R. Haselkorn.** 1980. Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridization probes. Proc. Natl. Acad. Sci. USA **77**:186–190.
25. **Mevarech, M., D. Rice, and R. Haselkorn.** 1980. Nucleotide sequence of a cyanobacterial *nifH* gene coding for nitrogenase reductase. Proc. Natl. Acad. Sci. USA **77**:6476–6480.
26. **Mitsui, A., S. Kumazawa, A. Takahashi, H. Ikemoto, S. Cao, and T. Arai.** 1986. Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. Nature (London) **323**:720–722.
27. **Mullis, K., and F. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. **155**:335–350.
28. **Myrold, D. D., A. B. Hilger, and S. H. Strauss.** 1990. Detecting *Frankia* using PCR. Nitrogen Fixation Congress, Knoxville, Tenn.
29. **Norel, F., and C. Elmerich.** 1987. Nucleotide sequence and functional analysis of the two copies of *nifH* in *Rhizobium* ORS571. J. Gen. Microbiol. **133**:1563–1576.
30. **Normand, P., P. Simonet, B. Cournoyer, and S. Nazaret.** 1990. Use of *rrn* and *nif* genes from *Frankia* as targets for PCR detection. Nitrogen Fixation Congress, Knoxville, Tenn.
31. **Oremland, R. S., and B. F. Taylor.** 1975. Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acetylene reduction assay for anaerobic microcosms. Appl. Microbiol. **30**:707–709.
32. **Paabo, S., D. M. Irwin, and A. C. Wilson.** 1990. DNA damage promotes jumping between templates during enzymatic amplification. J. Biol. Chem. **265**:4718–4721.
33. **Paerl, H. W., B. M. Bebout, and L. E. Prufert.** 1989. Bacterial associations with *Oscillatoria* sp. (*Trichodesmium* sp.) populations: ecophysiological implications. J. Phycol. **25**:773–784.
34. **Paerl, H. W., K. M. Crocker, and L. E. Prufert.** 1987. Limitation of N_2 fixation in coastal marine waters: relative importance of molybdenum, iron, phosphorus and organic matter availability. Limnol. Oceanogr. **32**:525–536.
35. **Paerl, H. W., J. C. Priscu, and D. L. Brawner.** 1989. Immunochemical localization of nitrogenase in marine *Trichodesmium* aggregates: relationship to N_2 fixation potential. Appl. Environ. Microbiol. **55**:2965–2975.
36. **Paul, J. H., L. Cazares, and J. Thurmond.** 1990. Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. Appl. Environ. Microbiol. **56**:1963–1966.
37. **Pope, M. R., S. A. Murrell, and P. W. Ludden.** 1985. Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue. Proc. Natl. Acad. Sci. USA **82**:3173–3177.
38. **Rice, D., B. J. Mazur, and R. Haselkorn.** 1982. Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **257**:13157–13163.
39. **Saiki, R., D. Gelfland, S. Stoffel, S. Scharf, R. Higuchi, G. Horn, K. Mullis, and H. Erlich.** 1988. Primer-directed amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

41. **Schumann, J. P., G. M. Waitches, and P. A. Scolnick.** 1986. A DNA fragment hybridizing to a *nif* probe in *Rhodobacter capsulatus* is homologous to a 16S rRNA gene. Gene **48**:81–92.
42. **Scott, K. F., B. G. Rolfe, and J. Shine.** 1981. Biological nitrogen fixation: primary structure of the *Klebsiella pneumoniae nifH* and *nifD* genes. J. Mol. Appl. Genet. **1**:71–81.
43. **Steffan, R. J., and R. M. Atlas.** 1989. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
44. **Sundaresan, V., and F. M. Ausubel.** 1981. Nucleotide sequence of the gene coding for the nitrogenase iron protein from *Klebsiella pneumoniae*. J. Biol. Chem. **256**:2808–2812.
45. **Torok, I., and A. Kondorosi.** 1981. Nucleotide sequences of the *R. meliloti* nitrogenase reductase (*nifH*) gene. Nucleic Acids Res. **9**:5711–5723.
46. **Waterbury, J. B., S. W. Watson, and F. W. Valois.** 1988. Temporal separation of photosynthesis and dinitrogen fixation in the marine unicellular cyanobacterium *Erythrosphaera marin*. EOS Trans. Am. Geophys. Union (Washington, D.C.) **69**:1089.
47. **Waterbury, J. B., S. W. Watson, F. W. Valois, and D. G. Franks.** 1986. Biological and ecological characterization of the marine unicellular bacterium *Synechococcus*. Can. Bull. Fish. Aquat. Sci. **214**:71–120.
48. **Weller, R., and D. M. Ward.** 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. Appl. Environ. Microbiol. **55**:1818–1822.
49. **Winberg, G., and M. L. Hammanskjold.** 1980. Isolation of DNA from agarose gels using DEAE paper; application to restriction site mapping of adenovirus type 16 DNA. Nucleic Acids Res. **8**:253–264.
50. **Wynn-Williams, D. D., and M. E. Rhodes.** 1974. Nitrogen fixation in seawater. J. Appl. Bacteriol. **37**:203–216.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
52. **Zehr, J., and L. McReynolds.** 1989. Use of degenerate oligonucleotide primers for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **55**:2522–2526.