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NOTE

Determination of N_2 fixation potential in the marine environment: application of the polymerase chain reaction

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ABSTRACT: Since determinations of aquatic N_2 fixation potentials have been hampered by the inability to quantitatively culture diazotrophs, we evaluated the polymerase chain reaction (PCR) for the detection of N_2-fixing microorganisms in marine samples. We used previously described degenerate oligonucleotide primers which are universal for the *nifH* gene to determine the levels of detection for N_2-fixing genes in seawater samples. A marine isolate of *Klebsiella* sp. was used as an internal standard in DNA samples obtained from natural marine microbial assemblages to establish a limit of detection. We obtained a limit of detection equivalent to 10 cells ml^{-1} This approach is useful for determining the potential for nitrogen fixation in the marine environment, regardless of whether or not N_2 fixation is occurring at the time of sampling.

Biological nitrogen fixation, the reduction of atmospheric dinitrogen to ammonia by microorganisms, represents a 'new' nitrogen input in open ocean waters which are chronically nitrogen-depleted (Dugdale & Goering 1967, Eppley et al. 1973). Oceanic N_2-fixing microorganisms might be expected to have a selective advantage over microorganisms which depend on regenerated nitrogen for growth and reproduction. However, the role of N_2-fixing microorganisms in alleviating nitrogen limitation is currently believed to be minor, except in certain eutrophic lakes prone to N_2-fixing cyanobacterial blooms and during large blooms of the marine planktonic cyanobacterium *Trichodesmium* spp. (Fogg et al. 1973, Carpenter & Capone 1983, Howarth et al. 1988). It appears that either current N_2 fixation measurements underestimate the importance of N_2 fixation in aquatic systems or that N_2 fixation potentials and hence N inputs are limited by other factors (Legendre & Gosselin 1989).

Due to the difficulties in culturing and quantifying diazotrophs and determining their activities in natural communities, it is advantageous to detect N_2-fixing microorganisms directly using a method which does not depend on rate measurements of nitrogenase activity or culturing. We have evaluated and present here an alternative approach for determining the presence of N_2-fixing microorganisms in seawater based on amplification of a segment of *nifH*, the structural gene for dinitrogenase reductase, using the polymerase chain reaction (PCR) (Mullis & Faloona 1987, Saiki et al. 1988), a technique which is quickly finding environmental applications (e.g. Steffan & Atlas 1989, Paul et al. 1990). This approach should facilitate determinations of aquatic N_2-fixing potential and community composition.

The use of PCR for detection of *nif* genes in environmental samples is dependent upon the development of a suitable extraction technique, removal of compounds which inhibit the PCR and a reliable method for detection of the amplified product. Although DNA purification techniques often involve CsCl density gradient ultracentrifugation, the broad application of PCR by marine scientists is enhanced by more efficient techniques that do not require expensive specialized equipment such as an ultracentrifuge. We describe here results of experiments to determine the level of detection of *nif* genes in seawater samples using simple procedures that do not require expensive or specialized equipment. These experiments determined (1) the optimal DNA extraction protocol, (2) a satisfactory method for eliminating or reducing the inhibition of PCR by seawater samples, and (3) the level of detection of nitrogen fixing cells in seawater.

Materials and methods. ***Cell cultures:*** *Klebsiella* sp. RM2-2 and RM1-2 (believed to be the same species) were isolated as previously described and obtained from C. Currin (Currin et al. 1990). Liquid 'FL' N-

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sufficient medium (Currin et al. 1990) was inoculated with RM2-2 and incubated at 25°C overnight in the dark. The cell density was determined using acridine orange direct counts (AODC) as described by Hobbie et al. (1977).

DNA extraction: Two ml of culture (3.6×10^8 cells) were added to 1 l of filtered seawater. This amendment of seawater allowed us to check the recovery of a known amount of DNA during processing of the seawater sample. The sample was then filtered onto Gelman GA8 47 mm polysulfone 0.2 µm pore size filters and quickly placed into 0.4 ml TNE Buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 100 mM EDTA) to avoid drying of the membrane and DNA degradation. The sample was frozen at -20°C and thawed. Several protocols were tested with the goal of achieving maximum yield with DNA purity sufficient for enzymatic manipulation (see also Fuhrman et al. 1988, Somerville et al. 1989). The DNA extraction protocol was modified from Marmur (1961). Lysozyme (Sigma grade III) was added at 0.5 mg ml^{-1} and samples incubated 10 min at 37°C. Sodium dodecyl sulfate (Sigma lauryl sulfate, sodium salt 95%) was added to a final concentration of 1% and the sample was incubated 10 min at 37°C. One phenol-chisam (chisam = chloroform : isoamyl alcohol 24:1 v/v) extraction was performed followed by an isopropanol/ammonium acetate DNA precipitation (Marmur 1961). The DNA in TE buffer (10mM Tris-Cl pH 8.0, 1 mM EDTA) was treated with 1 mg ml^{-1} RNaseA (Sigma Type 1-AS) at 37°C for 30 min. One phenol: chisam extraction was performed and the DNA was precipitated as described above.

Enzyme inhibition experiments: One liter of seawater from Bogue Sound (Morehead City, North Carolina, USA) was prefiltered through 20 µm Nitex and a 5 µm porosity, 47 mm diameter polycarbonate filter. The filtrate was filtered onto a 0.2 µm porosity 47 mm diameter polysulfone filter, which was then placed in TNE buffer. The DNA was extracted as described for the RM2-2 seawater samples except that the RNaseA step was omitted. The DNA in Treatment 3 was not purified further. The DNA in Treatment 2 was dialyzed against TE buffer using Spectra/Por 4 dialysis membrane tubing, and the DNA was re-precipitated and resuspended in 20 µl TE. The DNA in Treatment 1 was treated with RNase as described for the RM2-2 amended seawater DNA, phenol:chloroform extracted once more, re-precipitated and resuspended in 20 µl TE.

*Eco*RI restriction digests were performed using RM1-2 DNA and varying concentrations of picoplankton DNA from each treatment in order to determine if *Eco*RI activity (and potentially *Taq* polymerase activity) was inhibited by each DNA sample. One microliter of 1:5 diluted picoplankton DNA was added to digest A for each of Treatments 1 to 3. These are called A1, A2, A3. Different amounts of diluted picoplankton DNA (3, 5 and 10 µl) were added to digests B, C and D (for each treatment).

PCR and sensitivity experiments: The level of detection was determined with serial dilutions of *Klebsiella* sp. cells. In the first experiment, serial dilutions were prepared in TE buffer from 1:10 (DNA from 10^6 cells) to 1:100 000 (DNA from 100 cells). PCR was performed on the dilutions. A second PCR run was performed as described using DNA from 10 to 10^5 cells.

PCR conditions for all experiments were essentially as described previously with slight modification (Saiki et al. 1988, Zehr & McReynolds 1989). A Coy tempcycler was used. Degenerate primers to 2 highly conserved regions of *nifH* were used (Zehr & McReynolds 1989):

Primer 1: 5'-TGYGAYCCNAARGCNGA-3'
Y = T or C, N = A, C, G, or T
Primer 2: 5'-ADNGCCATCATYTCNCC-3'
R = A or G, D=A,T,or G

The final primer concentration was 100 µM for each primer. Buffer consisted of 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl pH 8.0, 6.7 mM MgCl2, 10 mM beta-mercaptoethanol, 200 µM each dATP, dCTP, dGTP, and dTTP, 100 µg BSA ml^{-1}. DNA was denatured at 95°C for 5 min and then cooled to 57°C for 5 min, during which 2.5 U *Taq* polymerase was added to each 100 µl reaction. The samples were heated to 70°C for 1.5 min, followed by 45 cycles of 1.2 min at 93.5°C, 1 min at 57°C, and 1.5 min at 70°C. Ten microliters of each sample were electrophoresed on a 4% NuSieve gel (FMC Bioproducts) and visualized with ethidium bromide using a UV transilluminator.

Results and discussion. DNA yields were not different between RM2-2 extractions directly from culture (where cells were pelleted, resuspended in buffer and the DNA extracted) and RM2-2 cells added to seawater, and filtered onto 0.2 µm filters followed by extraction. This determination was made by comparing DNA yields between 2 serial dilution sets (direct vs added to seawater) on an ethidium-bromide-stained agarose gel. However, impurities that are purified with DNA during extraction of DNA from seawater caused inhibition of *Taq* polymerase activity (as well as selected restriction enzymes). The inhibition could be reduced with RNaseA treatment, and a second phenol:chisam extraction and NH_4OAc/EtOH precipitation. Apparently, an unknown inhibitor of enzymatic activity exists in seawater samples which can be reduced or eliminated with these steps.

Inhibition of *Eco*RI activity was evident in samples which had not been treated with RNase when 3, 5 or 10 µl of diluted picoplankton DNA was added to the RM1-2 digest (Fig. 1). One microliter of diluted pico-

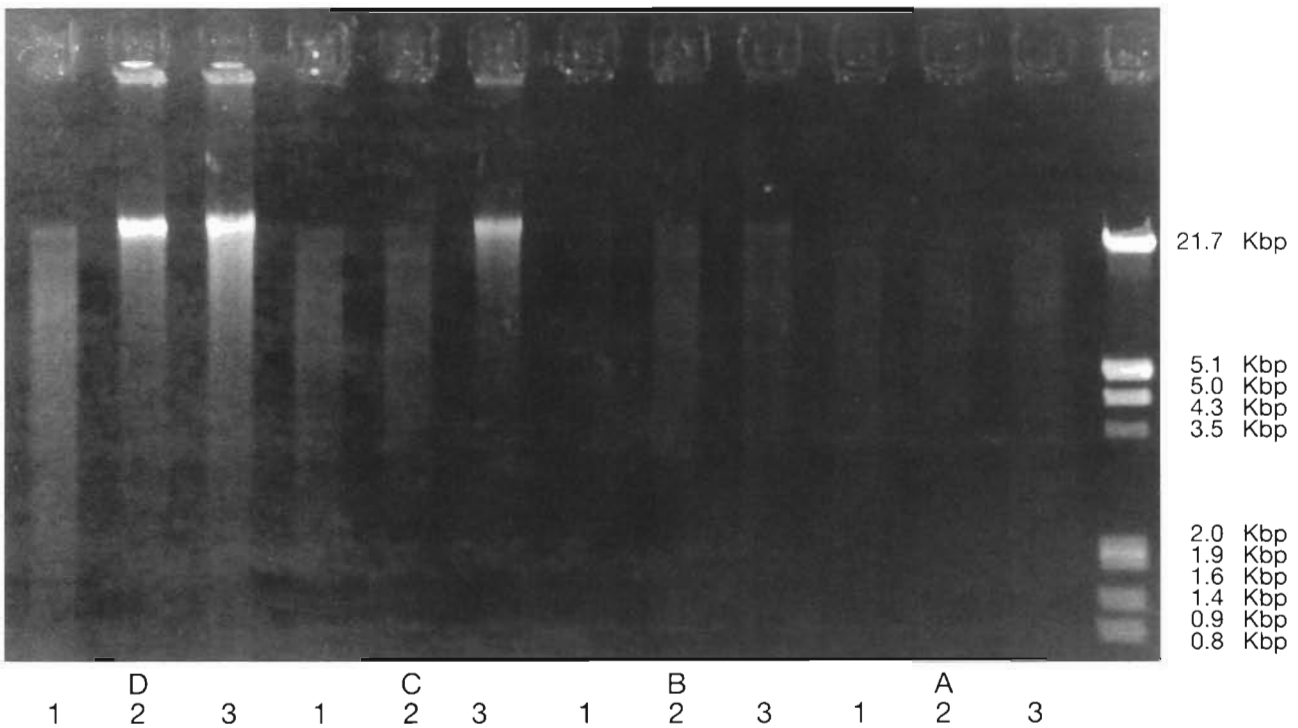

Fig. 1. *Eco*RI digests of RM1-2 and picoplankton DNA comparing purity of DNA preparations. (A) 1 µl picoplankton DNA added to digest; (B) 3 µl pico DNA; (C) 5 µl pico DNA; (D) 10 µl pico DNA. (1) RNase A treated; (2) dialyzed; (3) no RNase A or dialysis

plankton DNA did not appear to inhibit enzyme activity with any treatments, but 5 and 10 µl picoplankton DNA samples from Treatment 3 were not digested. Dialyzing against TE improved digestion marginally (5 µl digested, but 10 µl did not). Treatment 1 DNA which had been treated with RNase digested completely at all levels. Six microliters of undiluted picoplankton DNA from Treatment C was added to *Anabaena oscillarioides* DNA and the DNA digested completely (data not shown).

An inhibitory agent which reduces or eliminates *Taq* polymerase (and some restriction endonuclease) activity apparently exists in seawater samples. A similar problem was found with Sargasso Sea samples during a study of phylogenetic analysis of marine picoplankton (Giovannoni et al. 1990b). In other studies, CsCl density ultracentrifugation was used to purify the DNA for enzymatic manipulations (Fuhrman et al. 1988, Somerville et al. 1989). However, such a procedure requires relatively large amounts of DNA, which necessitates concentration of large volumes of seawater with expensive, specialized equipment such as tangential flow ultrafiltration devices, as well as an ultracentrifuge. A simplified procedure such as described here will make it possible for the polymerase chain reaction to be applied by marine ecologists and biogeochemists of diverse background and interest.

A *nifH* amplification product, ca 360 bp, was visible on an ethidium bromide-stained gel from PCR using as few as 100 cells (Fig. 2). No amplification product was visible with DNA from 10 or 0 cells, or from *Escherichia coli* DNA (amplification no. 2; data not shown), but non-specific products were evident in the *E. coli* sample. The level of detection is 100 cells. Since we were unable to amplify DNA extracted from 10 ml of seawater without apparent inhibition, this makes our level of detection 10 cells ml^{-1} It is important to note however that the level of detection can vary with sample composition. Therefore, the level of detection should be determined on each sample type.

In order to quantify N_2-fixing microorganisms in seawater samples by PCR, rigorous controls must be included. First, a dilution series such as that presented above should be included in every PCR run to establish limits of detection for that experiment. A small amount of DNA should be added to one of the natural samples (i.e. close to the anticipated detection limit) to ensure that the DNA preparations are pure enough for PCR. Because the technique is so sensitive, a negative control and a no-DNA control should be included to ensure that the DNA extraction buffers and PCR reagents have not become contaminated with *nif* DNA.

The *Klebsiella* sp. used in this experiment is a relatively large bacterium in culture (ca 1 µm). Microorganisms in culture are typically 10 to 100 times more

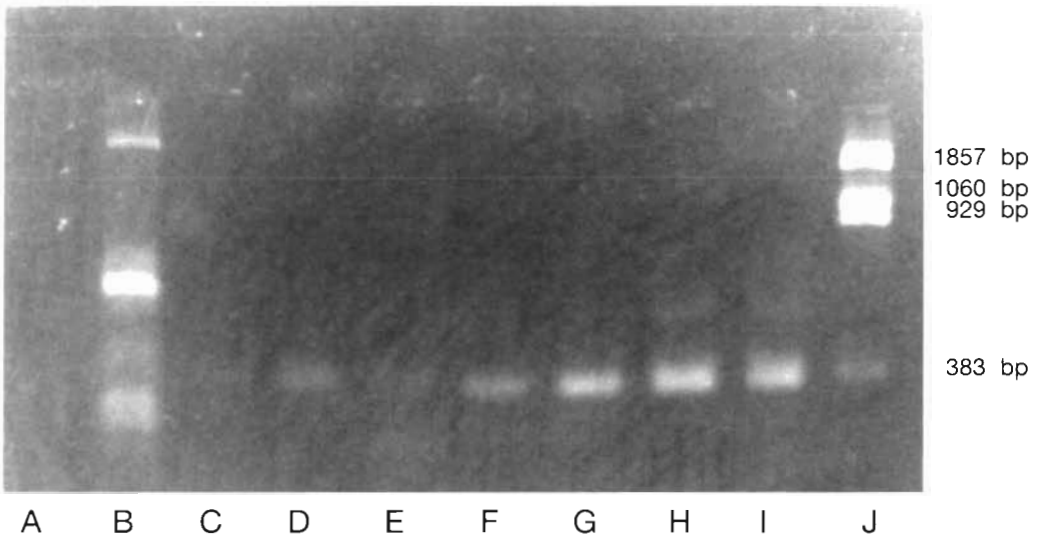

Fig. 2. Amplification #1 of *nifH* from RM2-2 from seawater to assess sensitivity. (A) no DNA. (B) *E. coli* DNA. (C) 102 cells. (D) 3×10^2 cells. (E) 10^3 cells. (F) 3×10^3 cells. (G) 10^4 cells. (H) 10^5 cells. (I) 10^6 cells. (J) pBR322/*Bst*NI

voluminous than organisms from a natural sample (Simon & Azam 1989). Therefore, the limit of detection in seawater may be lower due to less DNA per cell. However, total DNA per unit volume appears to be higher in smaller cells (Simon & Azam 1989). Accordingly, the results of these experiments serve to give general estimates of detection limits rather than absolute numbers. The detection limit of 100 cells observed in this study may not be the lower detection limit. Theoretically, only one copy of the gene is needed in order to amplify the target DNA sequence. Lower detection limits may be dependent upon developing better procedures for extraction of high quality DNA from seawater.

The polymerase chain reaction, due to its high sensitivity, is finding many practical applications in the detection of microorganisms in nature. It has recently been used to detect genetically-engineered microorganisms in the environment (Steffan & Atlas 1988). Photoautotrophs were detected in a Florida (USA) reservoir by amplifying a segment of the *rbcL* gene (Paul et al. 1990). Preliminary studies of genetic diversity and phylogenetic analysis of marine picoplankton used PCR as well (Giovannoni et al. 1990a, b). Recently, PCR with degenerate primers was used to amplify a *nifH* segment from a natural *Trichodesmium thiebautii* aggregate for cloning and sequencing (Zehr & McReynolds 1989). The same primers were used to amplify a *nifH* segment from *Anabaena oscillarioides*, RM1-2 and 2-2 (a *Ruppia maritima* root/rhizome associated *Klebsiella* sp.), M1 (a bacterium associated with *Trichodesmium* spp.), and from a bulk DNA extraction from *Halodule wrightii* roots (Kirshtein et al. 1991).

The adaptation of PCR to amplify a *nifH* segment in seawater samples allows natural populations to be screened for the presence of N_2-fixing microorganisms. This approach should make it possible to determine the occurrence and distribution of N_2-fixing microorganisms in diverse N-deplete coastal and open ocean environments where determinations of abundance and community dynamics have thus far been hampered by the inability to quantitatively culture diazotrophs. The ability to detect N_2-fixing microorganisms will highlight environments where further investigation and characterization of N_2 fixation is warranted.

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LITERATURE CITED

Carpenter, E. J., Capone, D. G. (1982). Nitrogen fixation in the marine environment. Science 217: 1140–1142

Currin, C. A., Paerl, H. W., Suba, G. K., Alberte, R. S. (1990). Immunofluorescence detection and characterization of N_2-fixing microorganisms from aquatic environments. Limnol. Oceanogr. 35: 59–71

Dugdale, R. C., Goering, J. J. (1967). Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol. Oceanogr. 12: 196–206

Eppley, R. W., Renger, E. H., Venrick E. L., Mullin, M. M. (1973). A study of plankton dynamics and nutrient cycling in the central gyre of the North Pacific Ocean. Limnol. Oceanogr. 18: 534–551

Fogg, G. E., Stewart, W. D. P., Fay P., Walsby, A. E. (1973). The blue-green algae. Academic Press, London

Fuhrman, J. A., Comeau, D. E., Hagstrom, A., Chan, A.M. (1988). Extraction from natural planktonic organisms of DNA suitable for molecular biological studies. Appl. environ. Microbiol. 54: 1426–1429

Giovannoni, S. J., Britschgi, T. B., Moyer, C. L., Field, K. G. (1990). Genetic diversity in Sargasso Sea bacterioplankton. Nature 345: 60–63

Giovannoni, S. J., DeLong, E. J., Schmidt, T. M., Pace, N. R. (1990). Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. environ. Microbiol. 56: 2572–2575

Guerinot, M. L., Colwell, R. R. (1985). Enumeration, isolation and characterization of N_2-fixing bacteria from seawater. Appl. environ. Microbiol. 50: 350–355

Hobbie, J. E., Daley, R. J., Jasper, S. (1977). Use of Nuclepore filters for counting bacteria by flourescence microscopy. Appl. environ. Microbiol. 56: 1963–1966

Howarth, R. W., Marino, R., Lane J., Cole, J. (1988). Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 1. Rates and importance. Limnol. Oceanogr. 33: 669–687

Kirshtein, J. D., Paerl H. W., Zehr, J. (1991). Amplification, cloning and sequencing of a *nifH* segment from aquatic microorganisms and natural communities. Appl. environ. Microbiol. 57: 2645–2650

Legendre, L., Gosselin, M. (1989). New production and export of organic matter to the deep ocean:consequences of some recent discoveries. Limnol. Oceanogr. 34: 1374–1380

Marmur, J. (1961). A procedure for the isolation of deoxy-ribonucleic acid from microorganisms. J. molec. Biol. 3: 208–218

Mullis, K., Faloona, F. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed reaction. Meth. Enzymol. 155: 335–350

Paul, J. H., Cazares, L., Thurmond, J. (1990). Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. Appl. environ. Microbiol. 56: 1963–1966

Saiki, R., Gelfland, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., Erlich, H. (1988). Primer-directed amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491

Simon, M., Azam, F. (1989). Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Ecol. Prog. Ser. 51: 201–213

Somerville, C. C., Knight, I. T., Straube, W. L., Colwell, R. R. (1989). Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. environ. Microbiol. 55: 548–554

Steffan, R. J., Atlas, R. M. (1989). DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. environ. Microbiol. 54: 2185–2191

Zehr, J., McReynolds, L. (1989). Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. environ. Microbiol. 55: 2522–2526

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