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PCR Analysis of the Distribution of Unicellular Cyanobacterial Diazotrophs in the Arabian Sea

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An oligonucleotide primer, NITRO821R, targeting the 16S rRNA gene of unicellular cyanobacterial N_2 fixers was developed based on newly derived sequences from *Crocosphaera* sp. strain WH 8501 and *Cyanothece* sp. strains WH 8902 and WH 8904 as well as several previously described sequences of *Cyanothece* sp. and sequences of intracellular cyanobacterial symbionts of the marine diatom *Climacodium frauenfeldianum*. This oligonucleotide is specific for the targeted organisms, which represent a well-defined phylogenetic lineage, and can detect as few as 50 cells in a standard PCR when it is used as a reverse primer together with the cyanobacterium- and plastid-specific forward primer CYA359F (U. Nübel, F. Garcia-Pichel, and G. Muyzer, Appl. Environ. Microbiol. 63:3327–3332, 1997). Use of this primer pair in the PCR allowed analysis of the distribution of marine unicellular cyanobacterial diazotrophs along a transect following the 67°E meridian from Victoria, Seychelles, to Muscat, Oman (0.5°S to 26°N) in the Arabian Sea. These organisms were found to be preferentially located in warm (>29°C) oligotrophic subsurface waters between 0 and 7°N, but they were also found at a station north of Oman at 26°N, 56°35′E, where similar water column conditions prevailed. Slightly cooler oligotrophic waters (<29°C) did not contain these organisms or the numbers were considerably reduced, suggesting that temperature is a key factor in dictating the abundance of this unicellular cyanobacterial diazotroph lineage in marine environments.

Microbial N_2 fixation has traditionally been considered to be of minor importance as a source of fixed nitrogen in oceanic environments (5). Contemporary mass balance estimates, however, have suggested that there is excess removal of combined forms of nitrogen, particularly from areas of the tropical ocean, and it has been proposed that biological N_2 fixation may account for the imbalances (6, 12). This has led to a radically revised view of the quantitative importance of this process in the nitrogen cycle. Current estimates of global N_2 fixation are ~240 Tg of N $year^{-1}$ and a marine contribution of ~80 Tg of N $year^{-1}$ (4). *Trichodesmium*, a filamentous nonheterocystous cyanobacterium broadly distributed throughout tropical and subtropical oceans, has long been considered to be responsible for most of this marine N_2 fixation (see references 3 and 17 for recent reviews). However, recently, Zehr et al. (34) showed that unicellular cyanobacteria that are 3 to 10 μm in diameter may make a significant contribution to oceanic N_2 fixation. Indeed, based on N_2 fixation data for the 0.2- to 10-μm bacterioplankton size fraction and the concentrations of phycoerythrin-containing unicellular cyanobacteria, they estimated that this contribution might equal or exceed that of *Trichodesmium*.

Several unicellular marine cyanobacterial diazotrophs belonging to the genera *Synechococcus* (15, 16) *Cyanothece* (21), and *Crocosphaera* (28) have previously been described. These organisms all have temporal separation of N_2 fixation and photosynthesis, which is likely controlled by an endogenous circadian rhythm (22). In addition to these free-living isolates, 16S rRNA gene (rDNA) sequences derived from cyanobacterial symbionts of the marine diatom *Climacodium fraeuenfeldianum* show close phylogenetic relatedness, suggesting that unicellular cyanobacterial symbionts also have the potential to contribute to N_2 fixation (7, 9, 10). Although several studies have now reported the presence of unicellular phycoerythrin-containing cyanobacteria that are 3 to 10 μm in diameter in geographically separated marine environments (2, 18, 27), suggesting that there is widespread distribution, the exact contribution of these unicellular cyanobacterial strains to global N_2 fixation rates remains to be determined. Indeed, a means to specifically detect or analyze the diversity of these organisms is not yet available. To address this problem, we developed a 16S rDNA oligonucleotide primer that specifically recognizes the discrete marine unicellular cyanobacterial diazotroph lineage within which nearly all previously documented isolates lie. We also describe the utility of this oligonucleotide as a PCR primer for assessing the distribution of these organisms along a transect in the Arabian Sea situated in the northwest Indian Ocean.

MATERIALS AND METHODS

PCR amplification. 16S rDNA sequences were amplified from *Cyanothece* sp. strains WH 8902 and WH 8904 and *Crocosphaera* sp. strain WH 8501 (29) by using the PCR primers OXY107F and OXY1313R (Table 1, 31) and conditions described previously (30) and cloned into the TA vector pCR2.1-TOPO (Invitrogen). Cyanobacterial 16S rDNA clones were identified from heterotrophic contaminants of the culture by PCR screening by using PCR primers CYA359F and OXY1313R (Table 1) prior to sequencing.

DNA sequencing. PCR products and double-stranded plasmid DNAs were sequenced bidirectionally by using an ABI 373A automated sequencer (Applied Biosystems, Foster City, Calif.) at the Warwick University sequencing facility.

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TABLE 1. PCR primers used in this study

Primer	Target	Sequence (5′ to 3′)	Reference
NITRO821R	16S rDNA of unicellular cyanobacterial diazotrophs	CAA GCC ACA CCT AGT TTC	This study
CYA359F	16S rDNA of cyanobacteria	GGG GAA TYT TCC GCA ATG GG	19
OXY107F	16S rDNA of oxygenic phototrophs biased	GGA CGG GTG AGT AAC GCG GTG	31
OXY1313R	16S rDNA of oxygenic phototrophs biased	CTT CAY GYA GGC GAG TTG CAG C	31
CNF	*nifH* of cyanobacteria biased	CGT AGG TTG CGA CCC TAA GGC TGA	20
CNR	*nifH* of cyanobacteria biased	GCA TAC ATC GCC ATC ATT TCA CC	20

Phylogenetic analysis and development of the NITRO821R primer. 16S rDNA sequence alignment and phylogenetic analysis were performed by using the ARB software (13). The NITRO821R oligonucleotide primer (Table 1) was designed to specifically recognize the phylogenetic lineage in which the unicellular cyanobacterial diazotrophs *Cyanothece* sp. strains WH 8902, WH 8904, and PCC8801 (also known as *Synechococcus* sp. strain RF-1 [25]) and *Crocosphaera* sp. strain WH 8501 lie (Fig. 1) by using the probe design and probe match tools from the ARB program.

Genomic DNA isolation. Genomic DNA was isolated from *Trichodesmium* sp. strain WH 9601, *Synechococcus* sp. strain CC9605, *Prochlorococcus* sp. strains EQPAC1 (HLI lineage), TAK9803-2 (HLII lineage), and MIT9313 (LL lineage), *Planktothrix rubescens* strain 97112, *Anabaena variabilis*, *Anabaena* sp. strain PCC7118, *Nostoc* sp. strain PCC7120, *Synechocystis* sp. strain PCC6803, *Microcystis* sp. strain PCC7806, and *Gloeotrichia* sp. for use in the PCR to determine the specificity of the NITRO821R primer described above by using a previously described protocol (11).

Sampling. Water samples for DNA extraction were collected during the NERC-funded AMBITION cruise in the Arabian Sea from 1 to 27 September 2001 aboard the RRS *Charles Darwin*. Samples were obtained from discrete depths at 11 stations along a 5,500-km transect (Table 2 and Fig. 2) between Victoria, Seychelles, and Muscat, Oman, by using 20-liter Niskin bottles on a hydrographic cable. Conductivity, temperature, and barometric pressure were measured simultaneously with a CTD (model Sea-Bird 9/11). Seawater (5 to 10 liters) from each depth was filtered onto 47-mm-diameter, 0.45-μm-pore-size polysulfone filters (Supor-450; Gelman Sciences Inc., Ann Arbor, Mich.) after prefiltration through a 47-mm-diameter, 3-μm-pore-size filter (MCE MF-Millipore filters [Fisher]) with a gentle vacuum (10 mm of Hg). This allowed collection of >3- and 3- to 0.45-μm fractions. The filters were placed in a 5-ml cryovial with 3 ml of DNA lysis buffer (0.75 M sucrose, 400 mM NaCl, 20 mM EDTA, 50 mM Tris HCl [pH 9.0]) and stored at −80°C until extraction.

Environmental DNA isolation. DNA was extracted from the filters in lysis buffer as described by Fuller et al. (11).

Development of the dual analytical PCR. For specific amplification of the 16S rDNA of marine unicellular cyanobacterial diazotrophs, the specific NITRO821R primer was used in conjunction with the cyanobacterium- and plastid-specific forward primer CYA359F (19) (Table 1). For amplification of environmental *nifH* sequences we used the cyanobacterium-biased primer pair described by Olson et al. (20), which we designated CNF-CNR (Table 1). PCRs were carried out in 25-μl mixtures containing 0.5 μl of template (cells or environmental DNA sample), each deoxynucleoside triphosphate at a concentration of 200 μM, 2 mM $MgCl_2$, each primer at a concentration of 0.2 μM (for 16S rDNA amplification) or 1 μM (for *nifH* amplification), and 0.625 U of *Taq* polymerase in 1× enzyme buffer (GIBCO BRL, Life Technologies Ltd., Paisley, Scotland). For amplification of environmental DNA we also included 1 mg of bovine serum albumin (Sigma) ml^{-1}. The amplification conditions comprised a denaturation step of 95°C for 5 min and then 80°C for 1 min, at which time *Taq* polymerase was added, followed by 30 cycles of 95°C for 1 min, 57°C for 30 s, and 72°C for 40 s and a final extension of 6 min at 72°C. Reaction mixtures were stored at 4°C prior to analysis. Products (10 of 25 μl) were resolved by gel electrophoresis on a 1.5% (wt/vol) agarose gel at 100 V. DNA was stained with ethidium bromide (0.5 μg ml^{-1}), visualized under short-wavelength UV, and photographed with a gel documentation system (UVP Inc., Upland, Calif.).

Relative quantification of PCR products. Ethidium bromide-stained agarose gels were quantified by using the array analysis tool in the *Total*Lab software (Nonlinear Dynamics).

Clone libraries. Eight clone libraries were constructed, four each for *nifH* and 16S rDNA, by using the CNF-CNR and CYA359F-NITRO821R primers, respectively. The libraries were constructed by using environmental DNA from the AMBITION cruise transect obtained at station 2 (depth, 10 and 25 m), station 4 (depth, 25 m), and station 11 (depth, 10 m). PCR products from each depth were ligated into the pCR2.1-TOPO cloning vector before transformation into *Escherichia coli* strain TOP10F (Invitrogen Corporation, San Diego, Calif.). Plasmid DNA was isolated from transformants with a QIAprep miniprep kit (QIAGEN Ltd., Crawley, West Sussex, United Kingdom). Ten clones were subsequently sequenced from each library prior to phylogenetic analysis by using the ARB software as described above.

Nucleotide sequence accession numbers. 16S rDNA and *nifH* sequences reported in this paper have been deposited in the GenBank database under accession numbers AY620237 to AY620241 (cultured strains) and AY621666 to AY621747 (AMBITION cruise environmental sequences).

RESULTS

NITRO821R primer design, specificity, and sensitivity. The 16S rRNA sequences obtained for *Cyanothece* sp. strains WH 8904 and WH 8902 and *Crocosphaera* sp. strain WH 8501 were compared with other sequences from the cyanobacterial radiation, and the results are shown as a phylogenetic tree in Fig. 1. *Cyanothece* sp. strain WH 8902 is more closely related to *Crocosphaera* sp. strain WH 8501 (97.1% identity) than to *Cyanothece* sp. strain WH 8904 (94.8% identity), but all three strains form a coherent lineage that is well supported phylogenetically and also contains other marine unicellular cyanobacterial diazotrophs, including 16S rDNA sequences from cyanobacterial symbionts of the diatom *C. frauenfeldianum*. Within this lineage *Cyanothece* sp. strains ATCC 51142 and PCC8801 (= *Synechococcus* sp. strain RF-1 [25]) show the greatest diversity (93.2% identity). Alignment of the 16S rDNA sequences of all members of this unicellular cyanobacterial diazotroph lineage (designated the $UCYN_2$-fix lineage) and comparison with all other cyanobacterial and environmental DNA sequences in the databases allowed the design of an oligonucleotide primer, NITRO821R (Table 1), that showed complete identity to sequences of all members of this $UCYN_2$-fix lineage. In silico alignments showed that members of the genus *Planktothrix* have a 1-bp mismatch with this oligonucleotide. Otherwise, the most closely related 16S rDNA sequences had at least two mismatches with the NITRO821R primer (Table 3), while BLAST searches (1) did not reveal any significant homology with other eubacterial or archaeal 16S rDNA sequences.

The NITRO821R primer was subsequently used as a reverse primer in PCRs together with the cyanobacterium- and plastid-specific forward primer CYA359F (19). Our searches with the sequences in the currently available bacterial 16S rDNA databases, including the new sequences for *Cyanothece* sp. and *Crocosphaera* sp. reported here, confirm the specificity of CYA359F for recognizing all cyanobacterial 16S rDNA sequences, as previously reported (19). By using genomic DNAs extracted from a range of cyanobacterial genera, including the commonly found marine genera *Synechococcus*, *Prochlorococcus*, and *Trichodes-*

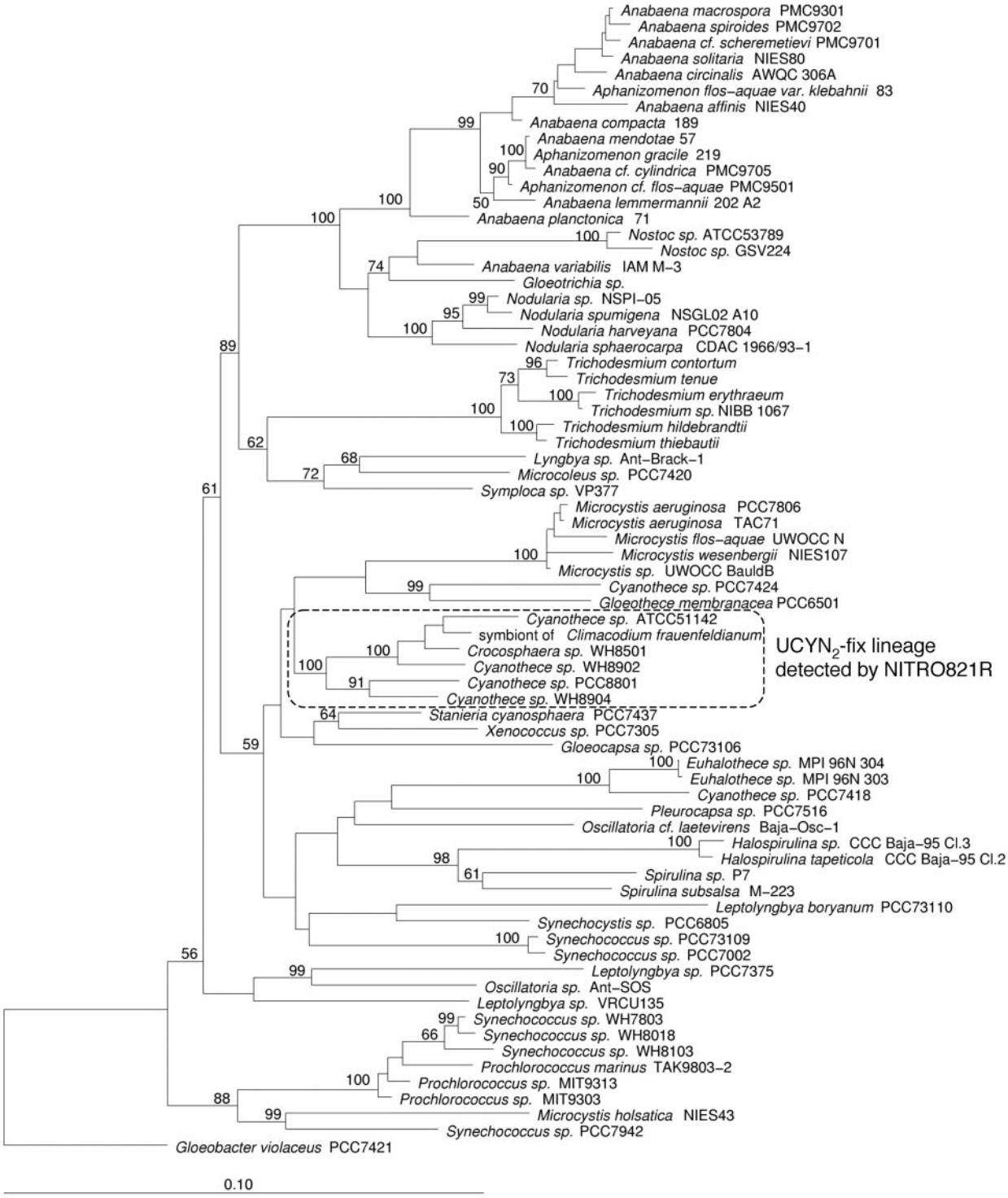

FIG. 1. Phylogenetic tree showing the relationships of marine and freshwater cyanobacteria, including diazotrophs and nondiazotrophs, inferred from 16S rDNA sequences. The tree was constructed by the neighbor-joining method with Jukes-Cantor correction. The percentages of bootstrap replicates supporting the branching order are indicated at the nodes. Partial sequences (<1,190 nucleotides) were added to the tree by using a maximum-parsimony option within ARB. The cyanobacterium *Gloeobacter violaceus* PCC7421 was used as a root. The scale bar represents the equivalent of 0.1 substitution per nucleotide.

TABLE 2. Positions of the principal stations along the AMBITION cruise transect

Station	Latitude	Longitude
1	00°55′S	64°08′E
2	00°00′N	67°00′E
3	03°48′N	67°00′E
4	07°36′N	67°00′E
5	11°24′N	67°00′E
6	15°12′N	67°00′E
7	19°00′N	67°00′E
8	20°55′N	64°00′E
9	22°40′N	60°41′E
10	23°55′N	59°15′E
11	26°00′N	56°35′E

mium, as templates, the specificity of the primer pair in the PCR for the $UCYN_2$-fix lineage was determined (Fig. 3A). As expected, sequences of all members of the $UCYN_2$-fix lineage tested were amplified. In addition, however, the sequence of *P. rubescens*, a representative of the genus *Planktothrix*, was also amplified with this primer pair. This filamentous cyanobacterial genus is, however, restricted to freshwater environments. The sequences of all other marine and freshwater cyanobacteria with two or more mismatches in the 16S rDNA sequence compared with the NITRO821R primer sequence were not amplified. A similar use of the CNF-CNR primer pair for detecting only cyanobacterial diazotrophs was also confirmed (Fig. 3B). Subsequently, by using a dilution series of *Cyanothece* sp. strain WH 8902 cells, the detection limit of the analytical PCR with the CYA359F-NITRO821R primer pair was determined to be as low as 50 cells per PCR under the conditions used (Fig. 4). This combination of specificity and sensitivity suggested that the CYA359F-NITRO821R primer combination should be an extremely useful tool for specific, semiquantitative detection of unicellular cyanobacterial diazotrophs in natural marine samples.

Distribution and abundance of members of the $UCYN_2$-fix lineage in the Arabian Sea. Environmental DNAs extracted from the >3-μm fraction in depth profiles along the AMBITION cruise transect (Table 2 and Fig. 2) were used as the templates in PCRs with the CYA359F-NITRO821R primer pair. The results showed that the $UCYN_2$-fix lineage was present only in subsurface waters at stations 2, 3, 4, and 11; the peak abundance occurred at a depth of around 25 m, and the lineage was undetectable at depths greater than 50 m (Fig. 2). Semiquantitative analysis of these data by using the *Cyanothece* sp. strain WH 8902 standard curve allowed relative cell abundance estimates to be made (Fig. 5). This analysis showed that the peak estimated levels were $3.8 \times 10^5 \pm 8 \times 10^4$ cells per liter (mean ± standard error) at a depth of 25 m at station 2, and lower numbers (around $2.4 \times 10^4 \pm 1.6 \times 10^3$ to $4 \times 10^4 \pm 4.9 \times 10^3$ cells per liter) were obtained at stations 3, 4, and 11. The $UCYN_2$-fix lineage was either absent or undetectable at all other stations along the transect.

The distribution of cyanobacterial diazotrophs in the >3-μm fraction along the AMBITION cruise transect was also investigated by using the *nifH*-specific primers CNF and CNR. PCR products were obtained from stations 1 and 4 and at station 11; however, whereas at stations 1 to 4 products were confined to the upper 50 m, at station 11 products were detected throughout the water column, down to a depth of 95 m. At each of these stations the brightest signals were at a depth of 10 m (data not shown). No cyanobacterial *nifH* products were detectable at the other stations along the transect when this primer pair was used.

Genetic diversity of cyanobacterial diazotrophs along the AMBITION cruise transect. Clone libraries of 16S rDNA and *nifH* were constructed by using the CYA359F-NITRO821R and CNF-CNR primer pairs, respectively, for station 2 at depths of 10 and 25 m, for station 4 at a depth of 25 m depth, and for station 11 at a depth of 10 m. All sequenced 16S rDNA

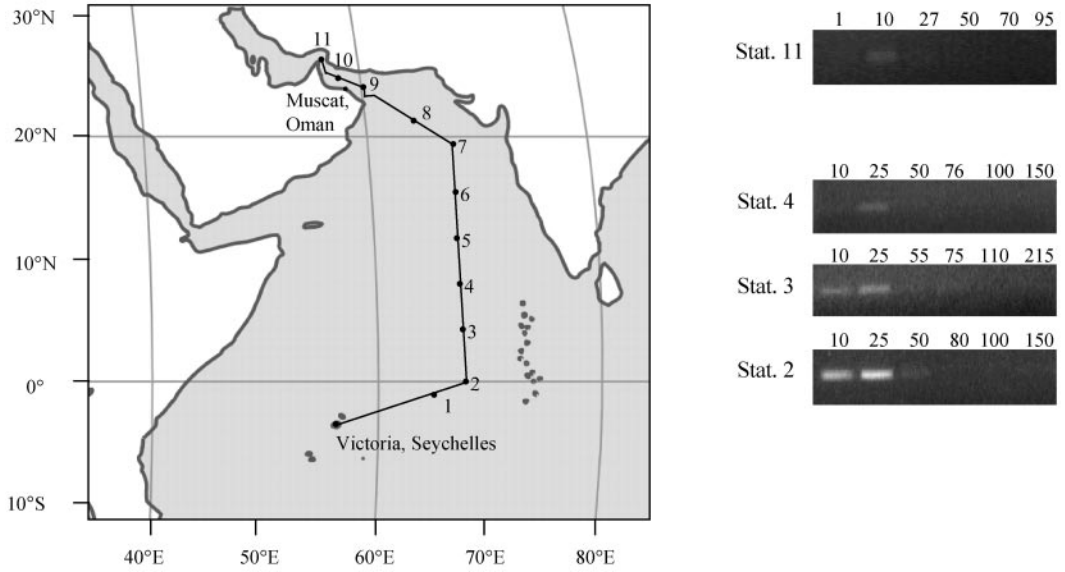

FIG. 2. Distribution of the $UCYN_2$-fix lineage along the AMBITION cruise transect in the Arabian Sea. The sampling stations are indicated by dots and are numbered in the order of sampling. Representative agarose gels for stations giving detectable PCR products with the CYA359F-NITRO821R primer pair are shown on the right. The numbers above the lanes indicate the depth of sampling (in meters). Sta., station.

TABLE 3. Specificity of the NITRO821R oligonucleotide illustrated, by alignment of the primer and target sequences with the 16S rDNA sequences of different marine unicellular diazotrophs and other cyanobacteria

Probe, target, or organism	Sequence[a]
Probe NITRO821R	3′-CTT TGA TCC ACA CCG AAC-5′
Target	5′-GAA ACT AGG TGT GGC TTG-3′
Crocosphaera sp. strain WH 8501	... ...
Cyanothece sp. strain WH 8902	... ...
Cyanothece sp. strain WH 8904	... ...
Planktothrix rubescens 97112	... ... C..
Anabaena variabilis	..T ... ... C.. ... ...
Anabaena sp. strain PCC7118	..T ... ... C.. ... ...
Gloeotrichia sp.	..T ... ... C.. ... ...
Nostoc sp. strain PCC7120	..T ... ... C.. ... ...
Microcystis sp. strain PCC7806	..T ... ... C.. ... ...
Trichodesmium erythraeum/*T. thiebautii*	..T ... ... T.. C..
Synechocystis sp. strain PCC6803	..T ... ... T.T C..
Synechococcus sp. strain CC9605	A.C ... ... C.G GG.
Prochlorococcus sp. strain EQPAC1 (- HLI)	A.C ... ... C.G GG.
Prochlorococcus sp. strain TAK9803-2 (- HLII)	A.C ... ... C.G GG.
Prochlorococcus sp. strain MIT9313 (- LL)	A.C ... ... C.G GG.

[a] Dots indicate bases identical to those of the target sequence.

clones from these environmental libraries fell in the $UCYN_2$-fix lineage (Fig. 6). The majority of the sequences (27 of 40 sequences), all derived from station 2 or 11, were phylogenetically most closely related to *Crocosphaera* sp. strain WH 8501. Within this group of sequences, those obtained from station 11 formed a distinct clade. 16S rDNA sequences from station 4 formed two separate clusters within the lineage and were generally not closely related to any known cultured unicellular diazotroph. Of the 40 environmental sequences obtained along the transect, clone 8 obtained from station 4 at a depth of 25 m and clone 5 obtained from station 11 at a depth of 10 m showed the greatest diversity (93.6% identity for the 462-bp product), suggesting that there is wide genetic diversity of these organisms in the Arabian Sea region.

The overwhelming majority of *nifH* sequences obtained from the four clone libraries (38 of 42 sequences) were closely related to the *Trichodesmium-Katagnymene* group (Fig. 7), which is consistent with the observed presence of filaments of these organisms as determined from surface plankton tows (K. Orcutt, unpublished data). Interestingly, though, two sequences were obtained from station 2 at a depth of 10 m which were 99.7% identical to *nifH* from *Crocosphaera* sp. strain WH 8501, while two other sequences, one from station 11 at a depth of 10 m and one from station 2 at a depth of 25 m, were phylogenetically more similar to *Anabaena* spp. (Fig. 7).

Role of sea temperature in defining the distribution of the $UCYN_2$-fix lineage along the AMBITION cruise transect. The distribution of the $UCYN_2$-fix lineage along the AMBITION cruise transect was compared to environmental parameters, including water column temperature and inorganic nutrient concentrations (specifically, the concentrations of nitrate and soluble reactive phosphate [SRP]). The nitrate concentrations varied between <0.01 and 0.15 μM and between <0.02 and 20 μM in the top 50 m at stations 1 to 7 and 8 to 11, respectively, and the SRP concentrations varied between 0.02 and 2.5 μM at all stations (M. Woodward unpublished data). The water column temperature showed a strong trend with the distribution of the unicellular diazotroph lineage (Fig. 8), and the organisms appeared generally to be preferentially located in warm (>29°C) oligotrophic subsurface waters. In cooler oligotrophic waters (<29°C) these organisms were absent or the numbers were considerably reduced.

DISCUSSION

We describe here the design and utilization of a 16S rDNA oligonucleotide, NITRO821R, which we used in the PCR to detect marine unicellular cyanobacterial diazotrophs. This primer recognizes all known cultured isolates and environmental sequences in a well-defined lineage (Fig. 1), which suggests

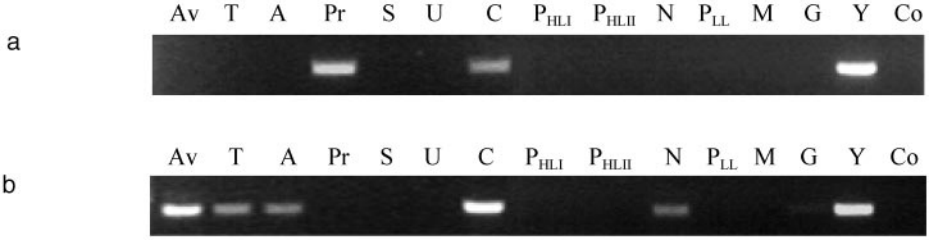

FIG. 3. Specificity of amplification with 16S rDNA primers CYA359F and NITRO821R (a) or *nifH* primers CNF and CNR (20) (b). Lane Av, *Anabaena variabilis*; lane T, *Trichodesmium* sp. strain WH 9601; lane A, *Anabaena* sp. strain PCC7118; lane Pr, *Planktothrix rubescens*; lane S, *Synechocystis* sp. strain PCC6803; lane U, *Synechococcus* sp. strain CC9605; lane Y, *Cyanothece* sp. strain WH 8902; lane C, *Cyanothece* sp. strain WH 8904; lane P_{HLI}, *Prochlorococcus* sp. strain EQPAC1; lane P_{HLII}, *Prochlorococcus* sp. strain TAK9803-2; lane P_{LL}, *Prochlorococcus* sp. strain MIT9313; lane N, *Nostoc* sp. strain PCC7120; lane M, *Microcystis* sp. strain PCC7806; lane G, *Gloeotrichia* sp.; lane Co, control (no DNA).

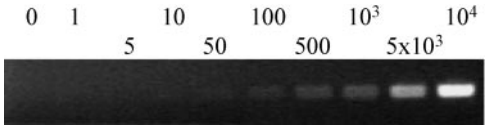

FIG. 4. Limit of detection of the CYA359F-NITRO821R PCR. The numbers of *Cyanothece* sp. strain WH 8902 cells in the 25-μl reaction mixtures are indicated at the top.

that it should have broad utility for detection of these organisms in the natural environment. Confirmation that the cyanobacterial symbionts of the marine diatom *C. frauenfeldianum* can fix atmospheric dinitrogen is still required, however. This would require isolation of these cyanobacterial symbionts in axenic culture. Assuming that such organisms do fix nitrogen, then the NITRO821R primer shows excellent specificity for marine unicellular cyanobacterial diazotrophs, particularly in conjunction with the cyanobacterium- and plastid-specific primer CYA359F (19), in PCRs (Fig. 3), as shown in this study.

Several 16S rDNA sequences of freshwater unicellular cyanobacterial diazotrophs which represent the genera *Gloeocapsa*, *Gloeothece*, and *Aphanothece* (accession numbers AB067575 to AB067581 and AB119259) and which are in the $UCYN_2$-fix lineage described here (data not shown) have recently been deposited in the GenBank database. These sequences are also targeted with complete identity by the NITRO821R oligonucleotide, which potentially broadens its utility to freshwater systems. However, care is needed when this primer is used to enumerate this lineage in freshwater environments, given that we have shown that sequences of members of the genus *Planktothrix*, with a 1-bp mismatch with the NITRO821R primer, are amplified in PCRs with the CYA359F-NITRO821R primer pair. Specific size fractionation of freshwater environmental samples to remove the filamentous *Planktothrix* spp. would be one route to circumvent such a problem.

The utility of this molecular approach for revealing the distribution of unicellular cyanobacterial diazotrophs in natural marine systems appears to be well demonstrated, however, by the data obtained along the AMBITION cruise transect. Although the relative abundance compared to the nondiazotrophic picocyanobacterial genera *Prochlorococcus* and *Synechococcus*, which were the dominant organisms at the southern and northern stations, respectively (G. Tarran unpublished data), was low, significant numbers of the $UCYN_2$-fix lineage were detectable, as determined by the semiquantitative analytical PCR developed in this study. Interestingly, the range of cell abundance observed along this single transect ($7 \times 10^3 \pm 1.6 \times 10^2$ to $3.8 \times 10^5 \pm 8 \times 10^4$ cells liter^{-1}) is very similar to the range of average cell counts of unicellular cyanobacteria in the 2.5- to 7-μm size class reported by Falcon et al. (10) for the tropical North Atlantic and subtropical North Pacific. In that study the cell numbers in the two ocean systems were considerably different, and it was proposed that the higher Fe flux to the North Atlantic was one of the factors that were important in determining the abundance of these organisms. Although we have no data on Fe flux at the different stations along the AMBITION cruise transect, this ocean basin is known to have high and variable deposition of dust (24), which results in pulsed iron addition in this region (14). Certainly, the fact that markedly different relative abundance values for the $UCYN_2$-fix lineage were observed along this transect shows that the abundance of these organisms can vary considerably over relatively small spatial scales. Indeed, a comparison of a suite of environmental parameters obtained from the AMBITION cruise suggests that seawater temperature (Fig. 8) accompanied by low surface SRP and nitrogen levels (generally <0.25 μM) is significant in shaping the conditions in which these organisms can proliferate. Temperature has recently been shown to be important in dictating the distribution of heterocystous and nonheterocystous cyanobacteria in oceanic systems (23). In that study, differences in the temperature dependence of oxygen flux, respiration, and N_2 fixation activity were shown to be critical in explaining how *Trichodesmium* performs better than heterocystous species at higher temper-

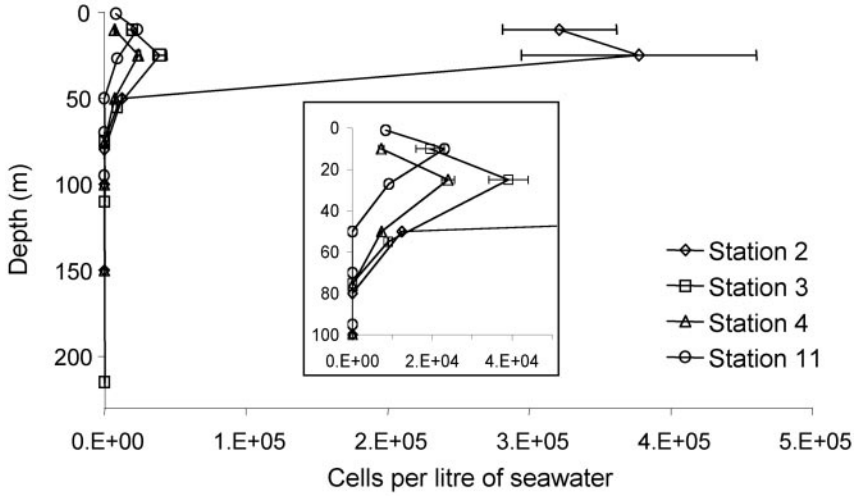

FIG. 5. Relative abundance of the $UCYN_2$-fix lineage along the AMBITION cruise transect in the Arabian Sea. The error bars indicate standard errors. The inset shows the data for stations 3, 4, and 11 in more detail.

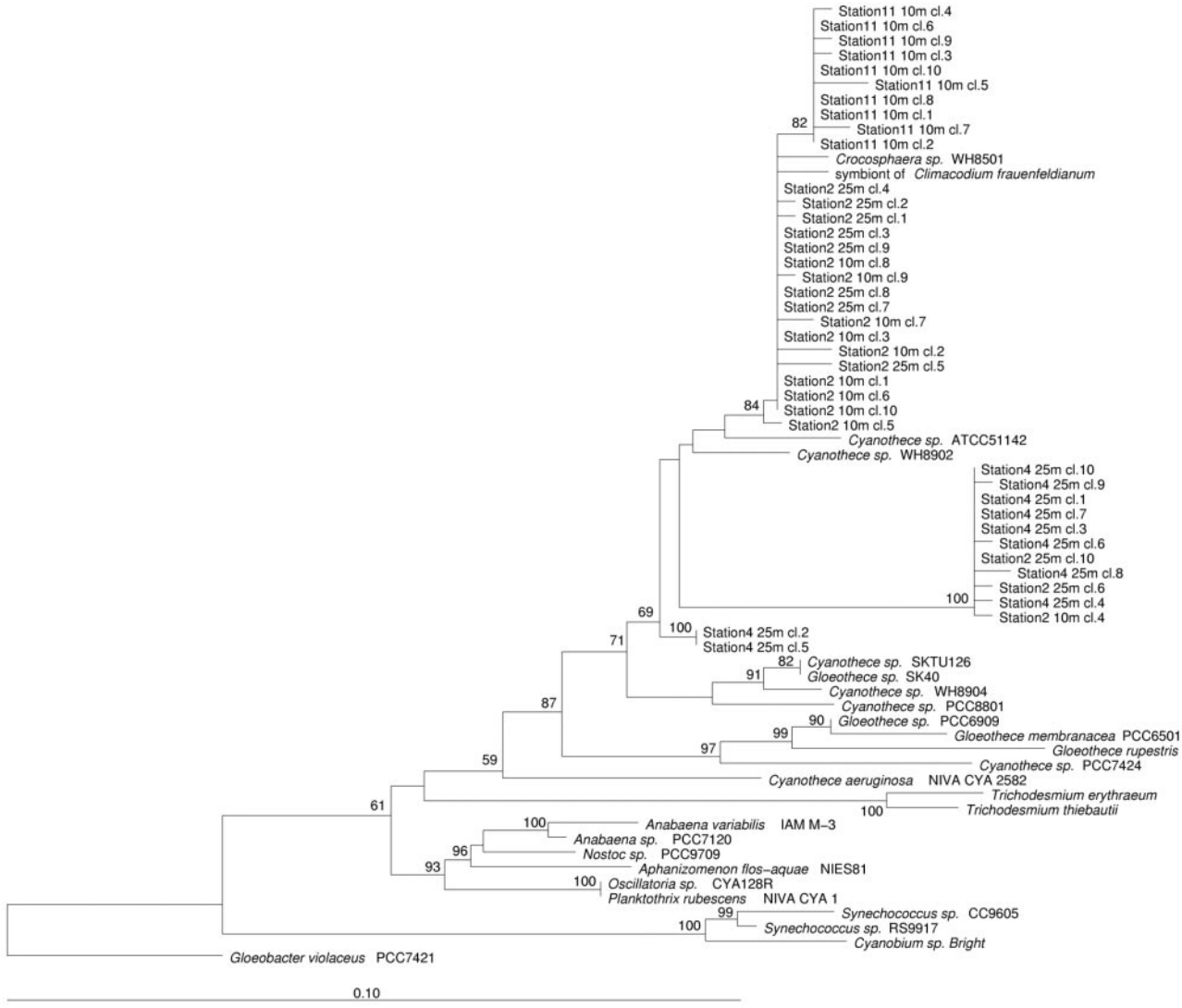

FIG. 6. Phylogenetic tree showing the relationships of the AMBITION cruise CYA359F-NITRO821R environmental 16S rDNA sequences. Each AMBITION cruise environmental sequence is referred to by station number, depth of sampling, and clone (cl.) number. The tree was constructed by the neighbor-joining method with Jukes-Cantor correction based on nearly full-length 16S rDNA sequences from marine and freshwater cyanobacteria. The percentages of bootstrap replicates supporting the branching order are indicated at the nodes. Partial sequences (e.g., sequences of the 462-bp product amplified by the CYA359F-NITRO821R primer set and other sequences <1,190 nucleotides long) were added to the tree by using a maximum-parsimony option within ARB. The cyanobacterium *Gloeobacter violaceus* PCC7421 was used as a root. The scale bar represents the equivalent of 0.1 substitution per nucleotide.

atures, and it may be that some of these factors are important for unicellular nitrogen-fixing species too.

The construction of environmental clone libraries based on the CYA359F-NITRO821R primer pair allowed us to obtain direct insight into the diversity of the $UCYN_2$-fix lineage along the AMBITION cruise transect. The wide genetic diversity among the derived 16S rDNA sequences for the three stations analyzed was noticeable (the lowest level of identity was 93.6% for the 462-bp product), and this diversity was much greater than the diversity that has been found for the picocyanobacterial genera *Prochlorococcus* and *Synechococcus* (11, 26), for which the lowest levels of identity between strains and environmental sequences were 96 to 97% (for sequences usually >1,000 bp long) in their respective lineages. How this translates to physiological diversity requires further culture isolation since several of the sequences, particularly those from station 4, had no closely related cultured counterparts. Within a station, particularly stations 2 and 11, the genetic diversity observed was generally lower, and interestingly, sequences from the same station formed discrete clusters or clades (Fig. 6), suggesting the potential for ecotype adaptation of members of the $UCYN_2$-fix lineage in specific water columns. The utility of the CYA359F-NITRO821R primer pair for focusing directly on the $UCYN_2$-fix lineage is mirrored by data for the *nifH* clone libraries, where the primers used are not targeted to a particular cyanobacterial lineage. Here, sequences closely related to the *Trichodesmium-Katagnymene* group dominated the libraries. Two sequences from station 2, however, were closely

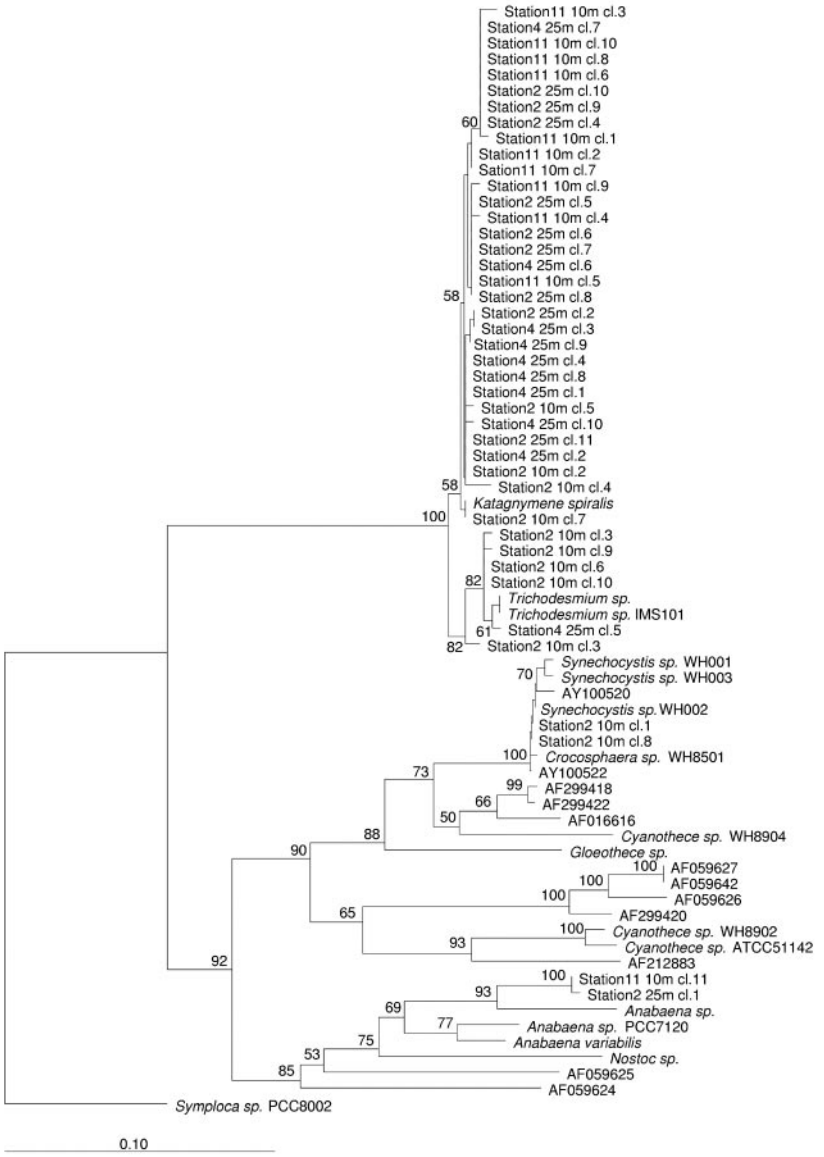

FIG. 7. Phylogenetic tree showing the relationships of the AMBITION cruise environmental *nifH* sequences. Each AMBITION cruise environmental sequence is referred to by station number, depth of sampling, and clone (cl.) number. The tree was constructed by the neighbor-joining method with Jukes-Cantor correction based on partial *nifH* sequences (e.g., a 359-bp fragment amplified with the CNF-CNR PCR primer set used in this study). The percentages of bootstrap replicates supporting the branching order are indicated at the nodes. The cyanobacterium *Symploca* sp. strain PCC8002 was used as the root. The scale bar represents the equivalent of 0.1 substitution per nucleotide.

related to the *nifH* sequence of *Crocosphaera* sp. strain WH 8501, which was in good agreement with the 16S rDNA environmental sequence data from that station, where sequences closely related to this strain dominated. These sequences thus correspond to the group B *nifH* sequences designated by Zehr et al. (34), while the group A *nifH* sequences reported in that study may be related to the deeply branching 16S rDNA clusters that include the station 4 clones.

Given the wide utility of the *nifH* gene for investigating the diversity of marine nitrogen fixers (32, 33, 35), it is noteworthy that we also obtained two environmental sequences (from stations 2 and 11) whose closest known relatives are *nifH* sequences from *Anabaena* sp. We found no previous reports of *nifH* sequences from members of this genus for oligotrophic open-ocean marine environments. However, these sequences may well be equivalent to the sequence of the novel *Anabaena* sp. described by Carpenter and Janson (8), who reported the presence of a heterocystous cyanobacterium in the southwest Pacific Ocean and Arabian Sea at a low level (1 to 4 trichomes liter^{-1}).

The NITRO821R oligonucleotide and the analytical PCR developed here thus provide an excellent tool for rapid and sensitive screening of environmental samples to establish the presence and relative abundance of unicellular cyanobacterial diazotrophs, organisms that play important roles in oceanic new production and biogeochemistry. Use of the oligonucleo-

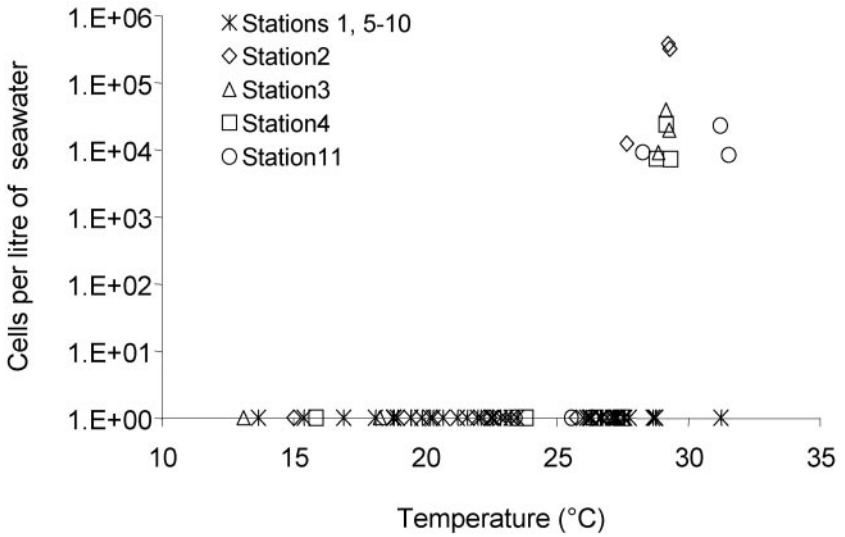

FIG. 8. Plot of the relative abundance of the $UCYN_2$-fix lineage along the AMBITION cruise transect as a function of seawater temperature.

tide in fluorescent in situ hybridization or quantitative PCR experiments, in which absolute quantitation of cell numbers can be obtained, or use in reverse transcription-PCR to determine the members of the community that are active in situ would further enhance its value.

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