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Temporal Patterns of Nitrogenase Gene (*nifH*) Expression in the Oligotrophic North Pacific Ocean

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Dinitrogen (N_2)-fixing microorganisms (diazotrophs) play important roles in ocean biogeochemistry and plankton productivity. In this study, we examined the presence and expression of specific planktonic nitrogenase genes (*nifH*) in the upper ocean (0 to 175 m) at Station ALOHA in the oligotrophic North Pacific Ocean. Clone libraries constructed from reverse-transcribed PCR-amplified mRNA revealed six unique phylotypes. Five of the *nifH* phylotypes grouped with sequences from unicellular and filamentous cyanobacteria, and one of the phylotypes clustered with γ-proteobacteria. The cyanobacterial *nifH* phylotypes retrieved included two sequence types that phylogenetically grouped with unicellular cyanobacteria (termed groups A and B), several sequences closely related (97 to 99%) to *Trichodesmium* spp. and *Katagnymene spiralis*, and two previously unreported phylotypes clustering with heterocyst-forming *nifH* cyanobacteria. Temporal patterns of *nifH* expression were evaluated using reverse-transcribed quantitative PCR amplification of *nifH* gene transcripts. The filamentous and presumed unicellular group A cyanobacterial phylotypes exhibited elevated *nifH* transcription during the day, while members of the group B (closely related to *Crocosphaera watsonii*) unicellular phylotype displayed greater *nifH* transcription at night. In situ *nifH* expression by all of the cyanobacterial phylotypes exhibited pronounced diel periodicity. The γ-proteobacterial phylotype had low transcript abundance and did not exhibit a clear diurnal periodicity in *nifH* expression. The temporal separation of *nifH* expression by the various phylotypes suggests that open ocean diazotrophic cyanobacteria have unique in situ physiological responses to daily fluctuations of light in the upper ocean.

The growth of dinitrogen (N_2)-fixing microorganisms partially regulates ocean nutrient cycles and carbon export (3, 4, 15, 24). In the subtropical ocean gyres, N_2 fixation appears to directly influence particulate and dissolved organic matter elemental stoichiometries and ecosystem productivity (12, 23, 29). Geochemical estimates of new production (the fraction of primary production by exogenous sources of nitrogen) in the Sargasso Sea are more than twofold greater than modeled nitrate fluxes (21), and diazotrophic N_2-fixing bacteria may provide an additional source of exogenous nitrogen to this ecosystem (2, 15, 29). Similarly, time series analyses of particulate nitrogen fluxes suggest that N_2 fixation may account for more than one-half of the total N export in the oligotrophic North Pacific Ocean (12, 23).

The biological reduction of N_2 is catalyzed by the metalloprotein nitrogenase. Nitrogenase consists of two multisubunit proteins: dinitrogenase reductase, encoded by the *nifH* gene, and dinitrogenase, encoded by the *nifD* and *nifK* genes. Studies on the diversity of *nifH*-containing plankton in the open ocean have revealed a diverse suite of potential diazotrophs, including cyanobacteria and proteobacteria, as well as *nifH* sequences from anaerobic bacteria (1, 52, 54).

Among the open ocean cyanobacteria, *nifH* sequences with a high degree of similarity to *Trichodesmium* species *nifH* sequences are often prevalent in PCR clone libraries (52). However, in addition to *Trichodesmium* spp., *nifH* sequences clustering with unicellular cyanobacteria have also been retrieved from the Pacific and Atlantic oceans (13, 54). Size-fractionated N_2 fixation rate measurements indicate that unicellular oceanic diazotrophs can contribute 3 to 70% of the total planktonic N_2 fixation in open ocean ecosystems (12, 13, 34). Cultivation efforts in the Pacific Ocean were successful in isolating 5- to 10-μm-diameter phycoerythrin-containing unicellular cyanobacteria (termed group B) whose *nifH* DNA sequences are 93 to 100% similar to *Crocosphaera watsonii* sequences (54). To date, however, the other phylotype of presumed unicellular cyanobacteria (termed group A) has not been maintained in culture.

Various studies have evaluated how environmental cues, such as temperature, light, and nutrients, influence cyanobacterial nitrogenase activity, *nif* gene transcription, and rates of N_2 fixation (7, 10, 18, 32, 37). Nitrogenase activity appears to be tightly regulated on both the transcriptional and posttranslational levels, in part due to the energy demands of N_2 fixation (16 molecules of ATP for each molecule of N_2 fixed) and the sensitivity of nitrogenase to O_2 (11, 16, 25, 28). Cyanobacteria utilize various strategies to decouple the apparently incompatible processes of N_2 fixation and oxygenic phototrophy, including physical separation of N_2 fixation and photosynthesis by intercellular spatial compartmentalization (e.g., formation of nonvegetative heterocysts) and temporal separation of photosynthesis and N_2 fixation (14).

The TaqMan 5′-fluorogenic exonuclease quantitative PCR (QPCR) assay has been used to assess the abundance and distribution of various ecologically relevant marine pro-

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karyotes (41, 45, 46). In this study, we examined the sequence diversity of plankton actively expressing *nifH* genes at Station ALOHA and quantified in situ *nifH* gene transcripts over a diurnal period. Our results indicate that natural populations of open ocean diazotrophs have distinct temporal rhythms of *nifH* transcription and suggest that phylogenetically distinct N_2-fixing bacteria have unique physiological responses to daily environmental fluctuations of light in the upper ocean.

MATERIALS AND METHODS

Sample collection. Samples were collected aboard R/V *Kilo Moana* during a research cruise in December 2002 to Station ALOHA (22°45′N, 158°00′W). Seawater was collected at discrete depths using 10-liter polyvinyl chloride bottles attached to a conductivity-temperature-depth rosette sampler. To evaluate day-to-night changes in *nifH* expression, depth profiles of planktonic RNA were collected at eight discrete depths (5, 25, 45, 75, 100, 125, 150, and 175 m) from midnight and midday hydrocasts. Additional RNA samples were collected from 25 m at approximately 4-h intervals over a 36-h period to evaluate in situ temporal changes in *nifH* gene expression. Seawater subsamples were collected from the conductivity-temperature-depth rosette in 2-liter, acid-rinsed polycarbonate bottles and immediately processed as described below.

To harvest planktonic RNA, 1 liter of seawater was pressure filtered onto 25-mm-diameter 0.2-μm-pore-size Supor filters (Pall Gelman, Inc.) using a peristaltic pump. The filters were removed from the in-line filter holders and placed in 2-ml bead beater tubes (Fisher Scientific) containing 600 μl RLT buffer (QIAGEN RNeasy), 1% β-mercaptoethanol, and 0.2 g of 0.1-mm glass beads (Biospec Products, Inc.). Samples were stored frozen in liquid nitrogen until they were processed for RNA extraction.

RNA extraction, reverse transcription, amplification, cloning, and sequencing. To extract planktonic RNA, bead beater tubes containing filters and glass beads were placed inside a Fast Prep machine (Bio 101, Carlsbad, CA) and agitated for 1.5 min. The tubes were then centrifuged at 8,500 × *g* for 30 s, and the supernatants were transferred to clean 2-ml microcentrifuge tubes with an equal volume of 70% ethanol. Samples were applied to QIAGEN RNeasy minicolumns (QIAGEN), and the RNA was purified and eluted by following the manufacturer's specifications. To eliminate carry-over DNA, samples were treated with DNase I by using the QIAGEN on-column DNase I RNA extraction protocol. RNA was eluted off the RNeasy minicolumn with 30 μl RNase-free water and frozen at −80°C. RNA concentrations were quantified using the RiboGreen RNA quantification protocol (Molecular Probes, Eugene, OR), and concentrations were determined fluorometrically using a spectrofluorometer (Varian, Palo Alto, CA).

nifH gene transcripts were reverse transcribed and PCR amplified (RT-PCR) by using a nested PCR protocol described by Zehr and Turner (53). In the initial reaction, *nifH* gene transcripts were reverse transcribed and amplified using a one-step Access RT-PCR kit (Promega). The following two pairs of degenerate outer primers were used in the initial PCRs: nifH3 (5′-ATRTTRTTNGCNGC RTA-3′) plus nifH4 (5′-TTYTAYGGNAARGGNGG-3′) (50) and nif2F (5′-T GAGACAGATAGCTATYTAYGGHAA-3′) plus nif623R (5′ GATGTTCGC GCGGCACGAADTRNATSA-3′) (44). The RT-PCR mixtures consisted of 2 ng plankton RNA, 1× buffer, 1 mmol liter^{-1} $MgSO_4$, 0.8 mmol liter^{-1} of each deoxynucleoside triphosphate (dNTP), 1 μmol liter^{-1} of each outer primer, 5 U *Tfl* DNA polymerase, and 5 U avian myeloblastosis virus reverse transcriptase (Promega). The total reaction mixture volume was 50 μl. Negative control reactions without reverse transcriptase were run in parallel to ensure that there was no contaminating DNA and that the resulting amplification resulted from cDNA synthesis. Samples were reverse transcribed at 48°C for 45 min followed by a 2-min incubation at 94°C to inactivate the reverse transcriptase. The thermal cycling conditions for the reactions with the nifH3 and nifH4 primers were as follows: 40 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 7 min. The cycling conditions for the reactions with the nif32F and nif623R primers were 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 7 min. For the second round of the nested PCR we used the protocol described by Zehr and Turner (53).

PCR products were run on a 1.2% agarose gel, and the 359-bp amplified *nifH* fragments were excised and purified with a QIAEX II gel purification kit (QIAGEN). Purified PCR products were ligated and transformed with a pGEM T vector kit (Promega). Plasmid DNA from 5 to 25 clones from each depth was purified by using the Montage 96-well miniprep kit protocol (Millipore). Plasmid inserts were sequenced using Big Dye Terminator V.3 sequencing chemistry (ABI) and the T7 and SP6 primers with an automated capillary sequencer (ABI 3100). Sequences were edited using the Seqlab GCG Wisconsin Package software (V. 10.3), and the edited *nifH* sequences were translated and imported into an amino acid sequence database that had been aligned using a hidden Markov model built iteratively with HMMER 2.2 (51). Phylogenetic distances were calculated using the evolutionary distance algorithm in GCG. The aligned nucleic acid sequences were imported into ARB (http://www.arbhome.de/) for construction of neighbor-joining phylogenetic trees. Sequences exhibiting >95% similarity were grouped for clarity. For phylogenetic trees, the *nifH*-like *frxC* gene from *Plectonema boryanum* (accession no. D00665) used as an outgroup, and bootstrap values were based on 1,000 replicate tree reconstructions.

RT-QPCR. For reverse transcription-quantitative PCR (RT-QPCR) assays, total RNA was reverse transcribed using a SuperScript III first strand cDNA synthesis kit (Invitrogen) by following the manufacturer's specifications. The cDNA reaction mixtures consisted of 20 ng RNA, 0.5 μmol liter^{-1} of each antisense gene-specific primer (nifH2 and nifH4), 1 mmol liter^{-1} of each dNTP, 1× RT buffer, 5 mmol liter^{-1} $MgCl_2$, 10 mmol liter^{-1} dithiothreitol, 40 U RNaseOUT (Invitrogen), and 200 U SuperScript III reverse transcriptase. Upon completion of the cDNA synthesis, 1 U RNase H was added to each reaction mixture to eliminate any residual RNA. Forty microliters of nuclease-free water was added to each reaction mixture, and the cDNA was stored at −20°C until it was utilized in QPCR assays.

For this study, we used the TaqMan primers and probes described by Church et al. (9) to evaluate *nifH* expression by the group A (accession no. AF059642), group B (AF299418), and *Trichodesmium* spp. cyanobacterial phylotypes (Table 1). As a result of the high levels of genetic similarity between *Katagnymene spiralis* (accession no. AF395130) and *Trichodesmium* spp. (27, 36), a single set of TaqMan primers and probe was used to amplify the *nifH* phylotypes clustering with these sequences. We also designed TaqMan primers and probes to quantify *nifH* expression by one of the *nifH* phylotypes clustering with heterocystous cyanobacteria (termed heterocyst-1 [accession no. AY706888 and AY706898]) and the *nifH* phylotype grouping with the γ-proteobacteria (termed γ-proteobacteria [AY706889 and AY706890]) (Table 1).

The thermal cycling conditions and reaction mixtures used for the QPCR assays have been described previously by Short et al. (41). Briefly, triplicate 25-μl QPCR mixtures were used for each sample and standard. The reaction mixtures contained the equivalent of 1 ng of RNA (typically 4 μl of diluted cDNA reaction mixture), 1× TaqMan PCR buffer (Applied Biosystems), 2.0 mmol liter^{-1} $MgCl_2$, 200 μmol liter^{-1} (each) dATP, dGTP, and dCTP, 400 μmol liter^{-1} dUTP, 400 nmol liter^{-1} (each) TaqMan forward and reverse primers, 200 nM fluorogenic probe, 0.25 U of AmpErase uracyl *N*-glycosylase, and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems). A GeneAmp 5700 (Applied Biosystems) was used for quantitative detection of amplified PCR products using the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s, followed by 60°C for 1 min.

The TaqMan primers and probes were checked for nonspecific amplification by addition of nontarget controls (consisting of plasmids with the nontarget *nifH* inserts) to QPCR mixtures (41). None of the primers or probes used in this study amplified nontarget *nifH* clones. We also evaluated whether the addition of cDNA influenced the amplification efficiency of positive controls (plasmids containing the target *nifH* sequences); for these experiments, duplicate QPCR mixtures were spiked with the positive control (0.5 pg target plasmid DNA), and the results were compared to the amplification of positive controls alone. The presence of cDNA did not influence the amplification of the positive controls.

nifH cDNA copies were quantified relative to a standard curve for plasmids containing the target *nifH* gene inserts. Standards were made from serial dilutions of plasmids in nuclease-free water, and 2 μl of each dilution was added to the 25-μl QPCR mixtures, providing a range of *nifH* targets containing between 1 and 10^9 *nifH* gene copies. Model I least-squares linear regressions of $\log_{10}$ target gene copies versus the cycle threshold were used to quantify the target gene copies in each sample.

RESULTS

RT-PCR clone library sequences. In December 2002, the average depth of the upper ocean mixed layer, based on the 0.125 potential density criterion (33), at Station ALOHA was 82 m, and the average temperature in the mixed layer was 24.7°C. The average mixed-layer nitrate plus nitrite (NO_3^- plus NO_2^-) concentration was 2.5 nmol liter^{-1}, and this concentration was ~300 nmol liter^{-1} at 100 m. *nifH* transcripts

TABLE 1. Oligonucleotide primers and probe sequences utilized for QPCR analyses[a]

Phylotype	Forward primer (5′-3′)	Probe (5′-3′)	Reverse primer (5′-3′)	Source
Heterocyst-1	CGGTTTCCGTGGTGTACGTT (105–124)	TCCGGTGGTCCTGAGCCTGGTGT (133–155)	AATACCACGACCCGCACAAC (158–177)	This study
Group A	AGCTATAACAACGTTTTATGCGTTGA (106–131)	TCTGGTGGTCCTGAGCCTGGA (133–153)	ACCACGACCAGCACATCCA (156–174)	Church et al.[b]
Group B	TGGTCCTGAGCCTGGAGTTG (138–157)	TGTGCTGGTCGTGGTAT (160–176)	TCTTCTAGGAAGTTGATGGAGGTGT (178–203)	Church et al.[b]
Trichodesmium	GACGAAGTATTGAAGCCAGGTTTC (217–241)	CATTAAGTGTGTTGAATCTGGTGGTCCTGAGC (246–278)	CGGCCAGCGCAACCTA (284–300)	Church et al.[b]
γ-Proteobacteria	TTGGCTTTGGCGACATCAA (104–122)	TGCGTGGAGTCAGGTGGTCCT (124–144)	ACGACCAGCACAGCCAACTC (152–177)	This study

[a] The numbers in parentheses are the target region, which is the region of the aligned nucleotide sequence targeted by the primers and probes relative to *Azotobacter vinelandii* (accession number M11579).
[b] See reference 1.

were amplified by RT-PCR from samples collected in the top 100 m of water during a midnight depth profile analysis and from five different depths in the top 175 m during a noontime profile analysis (Table 2). In total, 135 clones were sequenced, and six distinct (<95% similarity) *nifH* phylotypes were recovered (Fig. 1). Five of the sequence types grouped with the *nifH*-containing cyanobacteria, and one of the sequence types grouped with *nifH*-containing γ-proteobacteria (Fig. 1).

Within the *nifH*-containing cyanobacteria, 25 of the sequences (~19% of the clone libraries) grouped with unicellular cyanobacteria, including 24 sequences that were 97 to 100% identical to group A *nifH* sequences previously obtained from Station ALOHA (54) and 1 *nifH* sequence that was 96% identical to the *nifH* sequence of the marine organism *C. watsonii* (accession no. AF300829) (Table 2 and Fig. 1). In total, 30 *nifH* sequences (22% of the total clone libraries) clustered with filamentous cyanobacteria of the genus *Trichodesmium*; these sequences included 29 sequences that were 98 to 99% identical to *K. spiralis* (accession no. AF395130) and *Trichodesmium thiebautii* (accession number L00688) sequences and 1 sequence 99% that was identical to the *nifH* sequence of *Trichodesmium erythraeum* IMS 101 (accession number AF167538) (Table 2 and Fig. 1). In addition, 58 of the RT-PCR-amplified *nifH* sequences (43% of the total clone libraries) were novel phylotypes that clustered with *nifH* sequences of heterocyst-forming cyanobacteria (Table 2). The *nifH* sequence types clustering with these presumed heterocystous cyanobacteria formed two distinct phylogenetic groups; there were 24 clones in one of the groups (heterocyst-1) and 34 clones in the other (heterocyst-2) (Table 2 and Fig. 1). Finally, 23 of the *nifH* sequences (17% of the clone libraries) were 97 to 99% identical to uncultivated γ-proteobacterial sequences previously retrieved from PCR clone libraries from the Sargasso Sea (accession no. AF059623) and Station ALOHA (accession number AF059629) (52) (Table 2 and Fig. 1).

Depth-dependent patterns of *nifH* gene expression. Depth profiles of reverse-transcribed *nifH* cDNA from the various phylotypes were quantified from the midnight and noon samplings (Fig. 2). At midnight, *nifH* expression by the unicellular cyanobacterial group A phylotype was detected throughout the upper 100 m, and the concentrations ranged from $\sim 2 \times 10^3$ to 1×10^5 *nifH* cDNA copies liter^{-1} (Fig. 2A). At noon, the group A expression in the upper ocean was 4 to 10 times greater than that at midnight, and the concentrations of group A transcripts ranged from 2×10^4 to 4×10^5 *nifH* cDNA copies liter^{-1} (Fig. 2A). In contrast, *nifH* expression by the unicellular cyanobacterial group B phylotype was greater at midnight than at noon, and concentrations of group B transcripts ranged from $\sim 1 \times 10^3$ to 2×10^6 *nifH* cDNA copies liter^{-1} in the top 100 m. Group B expression declined approximately 3 orders of magnitude by noon, and the average *nifH* transcript abundance was $\sim 2.0 \times 10^2$ *nifH* cDNA copies liter^{-1} in the upper 100 m (Fig. 2B).

Trichodesmium spp. *nifH* transcription was detectable in the top 75 m at midnight and detectable at depths down to 125 m at noon. Transcription in the top 75 m of the water was 1 to 2 orders of magnitude lower at midnight than during the noon sampling (Fig. 2C); the *Trichodesmium* spp. *nifH* transcript concentrations ranged from 1×10^3 to 2×10^4 cDNA copies liter^{-1} at midnight and increased to 6×10^3 to 1×10^5 cDNA

TABLE 2. Summary of *nifH* RT-PCR clone library sequences from Station ALOHA in December 2002

Time	Depth (m)	No. of clones[a]					
		Group A	Group B	*Trichodesmium* spp.	Heterocyst-1	Heterocyst-2	γ-Proteobacteria
Midnight	5	0	0	17	1	5	0
	25	6	0	1	8	2	4
	45	5	0	1	10	0	3
	75	4	1	6	0	7	0
	100	0	0	0	4	0	0
Noon	5	1	0	3	1	0	4
	25	7	0	0	0	16	0
	75	0	0	1	0	2	4
	100	0	0	1	0	2	3
	175	0	0	0	0	0	5

[a] Sequences exhibiting >95% similarity were grouped as monophyletic phylotypes.

copies liter^{-1} by noon. Similarly, the *nifH* transcript abundance of the heterocyst-1 phylotype also decreased with depth, and *nifH* expression was approximately 10-fold lower at midnight than at noon. In the midnight depth profile, the heterocyst-1 transcript concentrations varied from 1×10^3 to 7×10^3 cDNA copies liter^{-1}, and the values increased to 3×10^4 to 5×10^5 cDNA copies liter^{-1} in the noon depth profile (Fig. 2D). The γ-proteobacterial *nifH* transcript concentrations were typically

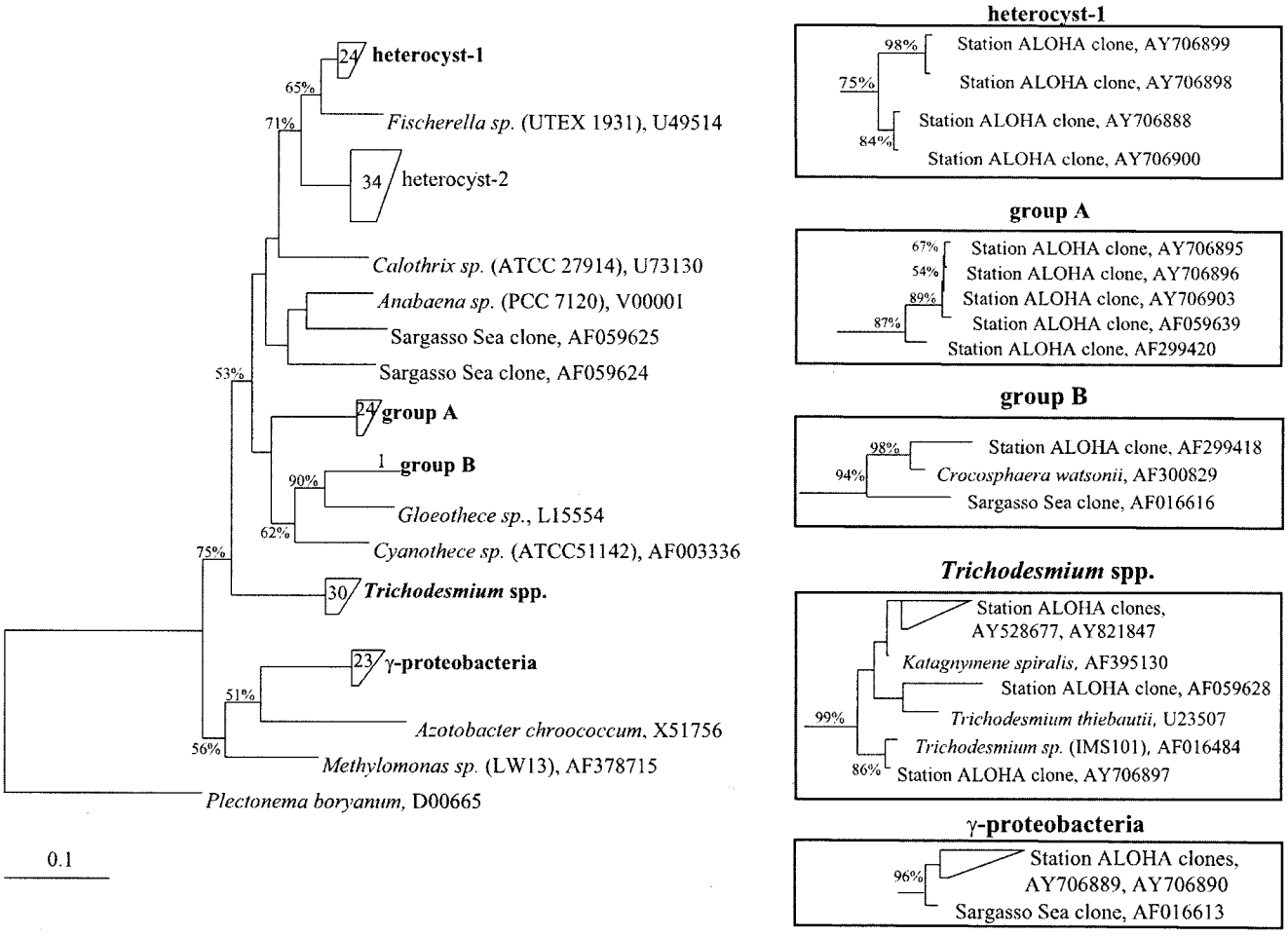


FIG. 1. Neighbor-joining phylogenetic relationships of *nifH* nucleic acid sequences obtained from upper ocean plankton samples at Station ALOHA. The polygons represent *nifH* sequences exhibiting >95% similarity; the numbers of sequences retrieved from RT-PCR clone libraries from this study are indicated inside the polygons. Sequences in boldface type were used to develop phylotype-specific QPCR primers and probes. The accession numbers of representative sequences are indicated next to the phylotypes. The trees in boxes include the sequence types targeted by QPCR. Distances were determined using a Jukes-Cantor correction (22); trees were bootstrapped 1,000 times, and bootstrap values of >50% are indicated at the nodes.

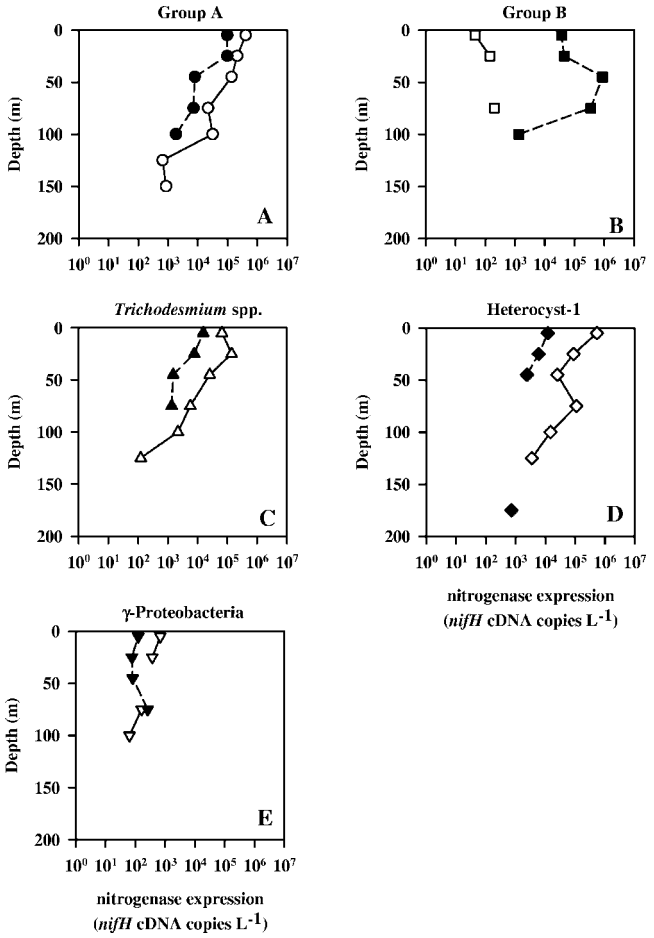


FIG. 2. Depth profiles for *nifH* transcript abundance (*nifH* cDNA copies liter^{-1}) for five different *nifH* phylotypes. Samples were obtained from midnight (solid symbols) and noon (open symbols) depth profiles in December 2002 at Station ALOHA. Transcript abundance was determined by QPCR amplification of reverse-transcribed *nifH* cDNA; the symbols indicate mean *nifH* cDNA concentrations from triplicate QPCR assays. (A) Group A transcripts; (B) group B phylotype; (C) *Trichodesmium* spp.; (D) heterocyst-1; (E) γ-proteobacterial phylotypes.

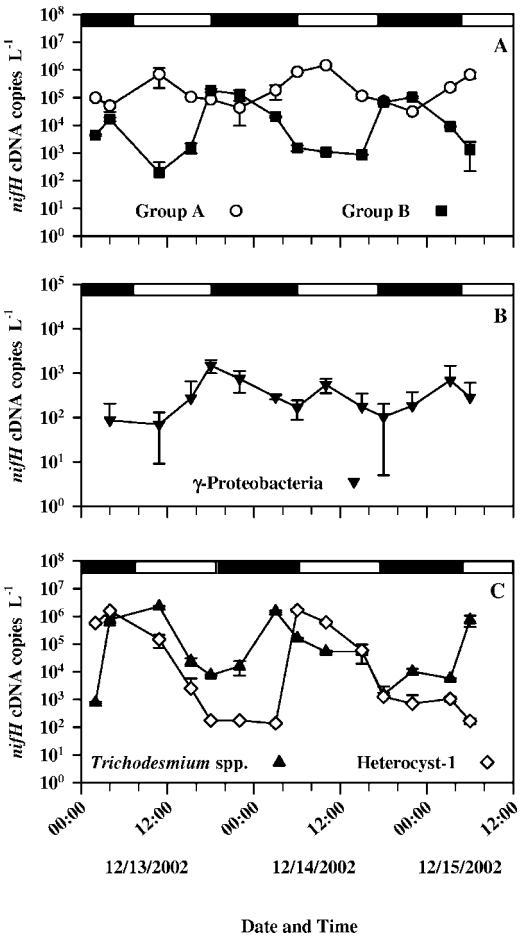


FIG. 3. Temporal patterns of *nifH* transcription (*nifH* cDNA copies liter^{-1}) by five phylotypes in the upper ocean (25 m) at Station ALOHA in December 2002. (A) Unicellular cyanobacterial transcription for group A and group B phylotypes; (B) γ-proteobacterial transcripts; (C) transcript concentrations for filamentous cyanobacterial phylotypes, including *Trichodesmium* spp. and heterocyst-1. The symbols indicate mean cDNA concentrations from triplicate QPCRs, and the error bars indicate ±1 standard deviation.

lower than any of the cyanobacterial transcript concentrations (Fig. 2E), and *nifH* expression by the γ-proteobacterial phylotype varied less than threefold between noon and midnight (Fig. 2E). The upper ocean transcript concentrations of the γ-proteobacterial phylotype ranged from ~80 to 700 *nifH* cDNA copies liter^{-1}.

Temporal dynamics in *nifH* gene expression. To evaluate higher-resolution temporal dynamics of in situ *nifH* expression, samples were collected from 25 m at ~4-h intervals over a 48-h period (Fig. 3). The group A, *Trichodesmium* spp., and heterocyst-1 cyanobacterial phylotypes all had elevated in situ *nifH* expression in the early to mid-morning. In contrast, *nifH* expression by the group B *nifH* phylotype was greatest at night (Fig. 3A). The group A *nifH* transcript concentrations varied between 3×10^4 and 2×10^6 *nifH* cDNA copies liter^{-1}, and *nifH* transcription peaked near noon (Fig. 3A) and declined throughout the afternoon and evening. Group B transcription was low throughout the day, increasing in the late afternoon. The average daytime *nifH* transcription by the group B phylotypes was ~10^2 *nifH* cDNA copies liter^{-1} and increased to ~10^5 *nifH* cDNA copies liter^{-1} in the evening. Overall, the diurnal fluctuations in *nifH* transcript concentrations appeared to be greater for the group B phylotype than for the group A phylotype; the group B *nifH* transcript concentrations varied approximately 3 orders of magnitude, while the group A *nifH* transcript concentrations varied 1 to 2 orders of magnitude over the sampling period.

Expression by the *Trichodesmium* spp. phylotypes was typically greatest in the predawn to early morning hours (average, 1×10^6 *nifH* cDNA copies liter^{-1}) and then decreased 2 to 3 orders of magnitude in the late afternoon and evening (Fig. 3B). During peak transcription, the *Trichodesmium* spp. *nifH* cDNA concentrations were approximately equivalent to the peak group A cDNA concentrations, but the *Trichodesmium* spp. *nifH* transcript concentrations varied more over a diel cycle than the group A cyanobacterial concentrations (Fig. 3C). *nifH* expression by the heterocyst-1 phylotype exhibited

TABLE 3. *nifH* gene copies of cyanobacterial phylotypes collected at a depth of 25 m in the subtropical North Pacific Ocean at Station ALOHA[a]

Plankton size class (μm)	Group A (*nifH* copies liter^{-1})	Group B (*nifH* copies liter^{-1})	*Trichodesmium* (*nifH* copies liter^{-1})
10–0.2	1.9×10^5	1.7×10^3	ND[b]
>10	2.9×10^2	ND	2.9×10^3
Total	1.9×10^5	1.7×10^3	2.9×10^3

[a] The heterocyst-1 *nifH* gene abundance phylotype was not detected in either plankton size class. DNA samples were collected at midnight on 12 December 2002 at the beginning of the in situ sampling period.
[b] ND, not detected (gene abundance less than 1 *nifH* gene copy per 25-μl PCR mixture).

the largest diel variability of all the phylotypes examined. The *nifH* transcript concentrations of the heterocyst-1 phylotype varied more than 4 orders of magnitude throughout the day ($\sim 10^2$ to 10^6 *nifH* cDNA copies liter^{-1}). *nifH* expression by the heterocyst-1 phylotype increased sharply in the early morning (0400 to 0600) and gradually declined throughout the late morning and evening (Fig. 3C).

Unlike expression by the cyanobacterial phylotypes, *nifH* expression by the γ-proteobacterial phylotype remained relatively low throughout the 48-h sampling period. Moreover, the γ-proteobacterial phylotype did not exhibit an obvious daily pattern in *nifH* expression. The average concentration of γ-proteobacterial transcripts was 4×10^2 *nifH* cDNA copies liter^{-1}, and the concentration typically varied by less than 1 order of magnitude over the sampling period (Fig. 3C).

To determine whether there were differences in the number of *nifH* transcripts per gene copy between the various phylotypes, we normalized the *nifH* cDNA concentrations to the *nifH* gene copy concentrations (measured by QPCR amplification of plankton DNA) to compare how the number of *nifH* transcripts per gene copy differed for the various phylotypes examined in this study (Table 3). The gene copy concentrations have been described by Church et al. (9). For the group A cyanobacteria, the average number of *nifH* transcripts was ~2 *nifH* cDNA copies/gene copy in the early morning, and this value declined to ~0.1 *nifH* cDNA copy/gene copy at night (Fig. 4A). The average group B transcript concentration was ~1 *nifH* cDNA copy/gene copy during the day and increased to ~10 to 100 *nifH* cDNA copies/gene copy at night (Fig. 4B). *Trichodesmium* spp. exhibited large daily fluctuations in the abundance of *nifH* transcripts; the average number of *nifH* transcripts was ~700 *nifH* cDNA copies/gene copy in the early morning and decreased to <1 *nifH* cDNA copy/gene copy in the afternoon and evening (Fig. 4C). The *nifH* gene abundance for both the heterocyst-1 and γ-proteobacterial phylotypes was below the limit of detection during this study (the gene concentrations for both these phylotypes were <1 *nifH* copy per 25-μl QPCR mixture, equivalent to <100 gene copies per liter of seawater).

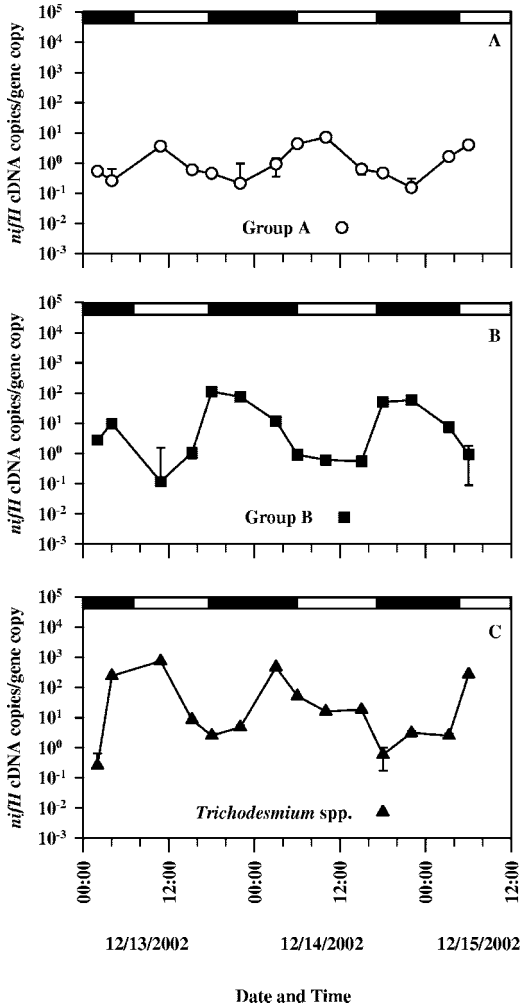


FIG. 4. Temporal patterns of cyanobacterial *nifH* transcript abundance normalized to *nifH* gene copy abundance (*nifH* cDNA copies/gene copy). *nifH* expression was normalized to the gene copy abundance for each phylotype; *nifH* gene abundance was determined for midnight on 12 December 2002. Gene abundance was normalized to *nifH* expression for unicellular group A (A), unicellular group B (B), and *Trichodesmium* spp. (C). The symbols indicate the mean number of *nifH* cDNA copies (reverse-transcribed RNA) divided by the average number of *nifH* gene copies (from DNA); the error bars indicate ±1 standard deviation (including propagated error) of the sample mean.

DISCUSSION

We evaluated the presence of selected diazotrophs actively transcribing *nifH* genes in the upper ocean at Station ALOHA in the oligotrophic North Pacific Ocean and quantified temporal patterns of *nifH* gene expression by several of these phylotypes. At the time of this study, *nifH* expression was dominated by cyanobacteria; five of the six phylotypes retrieved from RT-PCR clone libraries phylogenetically cluster with *nifH*-containing cyanobacteria. The *nifH* transcription by the cyanobacteria had pronounced diel periodicity, fluctuating with ~24-h periodicity. The daily rhythmic patterns of *nifH* expression by all of the cyanobacterial phylotypes appears to be consistent with previous laboratory and field studies that demonstrated that there was a light-dark dependence of N_2 fixation and nitrogenase gene expression in selected cyanobacteria, including *Trichodesmium* sp., *Cyanothece* sp., and *Synechococcus* sp. (7, 17, 18, 39, 49). Interestingly, we also observed that the daily patterns of *nifH* expression were unique to each phylotype,

suggesting that phylogenetically distinct N_2-fixing cyanobacteria may exhibit unique in situ physiological responses to daily fluctuations in irradiance in the upper ocean.

With the exception of the two sequence types clustering with heterocyst-forming cyanobacteria, our clone library results appear to be consistent with previous studies that examined the diversity of diazotrophic cyanobacteria in the open ocean. Both of the *nifH* sequence types clustering with unicellular *nifH*-containing cyanobacteria (groups A and B) have been retrieved from RT-PCR clone libraries at Station ALOHA and in the Sargasso Sea (13, 54). Similarly, *nifH* sequences closely related to sequences from *Trichodesmium* spp. and *K. spiralis* have been recovered from seawater samples collected in the Pacific and Atlantic oceans (19, 27, 36, 52). In addition, the sequences of a monophyletic group that clustered with *nifH*-containing γ-proteobacteria were 97 to 99% identical to sequences previously retrieved from PCR clone libraries in the Sargasso Sea (52).

Among the six different phylotypes retrieved in our RT-PCR clone libraries, we identified two novel sequence types clustering with *nifH*-containing heterocystous cyanobacteria. Heterocystous cyanobacteria in the open ocean are typically observed in associations (as endosymbionts or epibionts) with several genera of diatoms, although Carpenter and Janson (5) did observe a free-living heterocyst-forming cyanobacterium (*Anabaena gerdii*) in the South Pacific and Arabian Sea. There have been numerous reports from the tropical and subtropical Atlantic and Pacific oceans (including Station ALOHA) of diatom-associated heterocystous cyanobacteria (6, 20, 38, 47, 48). Most often, these diazotrophs are identified (by microscopy) as either *Richelia intracellularis* or *Calothrix rhizosoleniae*; however, genetic characterization of oceanic heterocystous cyanobacteria has been largely restricted to sequencing of *hetR* genes (20). To date, there are no published *nifH* sequences for *R. intracellularis*, *C. rhizosoleniae*, or *A. gerdii*, and as a result, we were unable to determine whether the heterocyst-1 and -2 *nifH* phylotypes recovered in this study stemmed from one of these commonly observed heterocystous cyanobacteria or were derived from an N_2-fixing symbiont. Zehr et al. (52) recovered two heterocystous *nifH* phylotypes (accession no. AF059624 and AF059624) from PCR clone libraries from the Sargasso Sea, but these sequences were only distantly related (76 to 84% similar) to the heterocyst-1 and heterocyst-2 phylotypes recovered from Station ALOHA (Fig. 1). Based on *nifH* sequences, we cannot determine whether the heterocyst-1 and −2 phylotypes represent different species or whether the these two phylotypes were derived from a single species that contains multiple *nifH* genes. Several heterocyst-forming cyanobacteria (including *Anabaena variabilis* and *Fischerella* sp. strain UTEX 1931) are known to possess multiple *nifH* gene copies; however, the recently completed genomes of two nonheterocystous marine cyanobacteria (*T. erythraeum* and *C. watsonii*) each contain a single *nifH* copy per genome.

We also evaluated the daily temporal patterns of *nifH* gene expression by several *nifH* phylotypes at Station ALOHA. *nifH* gene transcription by all of the cyanobacterial phylotypes exhibited clear diel patterns, and *nifH* expression oscillated with ~24-h periodicity. Consistent with laboratory and field studies of *Trichodesmium* spp. *nifH* gene expression (7, 35, 49, 55), we observed that in situ *nifH* expression by natural populations of *Trichodesmium* spp. appeared to peak in the early to mid-morning and then decrease in the late afternoon and evening. Studies using laboratory cultures of *Trichodesmium* spp. have demonstrated that both N_2 fixation and *nifH* expression are regulated by circadian control (7, 8, 49). Similarly, the diel temporal patterns of *nifH* expression by the presumed heterocystous cyanobacteria (heterocyst-1) also appear to be consistent with patterns of N_2 fixation by heterocystous cyanobacteria, in which rates of N_2 fixation appear to increase sharply in the early to mid-morning and decline by afternoon and evening (26). Localization of nitrogenase to nonvegetative heterocyst cells appears to spatially separate N_2 fixation from photosynthesis and may alleviate the need for complete temporal separation of these processes in heterocyst-forming cyanobacteria.

Perhaps the most intriguing results from this study were the distinctly different patterns of *nifH* gene expression exhibited by the two presumed unicellular *nifH* cyanobacteria. *nifH* expression by the group B phylotype peaked near midnight and remained low throughout the day. This pattern is consistent with laboratory studies that have shown that nitrogenase synthesis and N_2 fixation by nonheterocystous cyanobacteria (including *Cyanothece* sp., *Synechococcus* sp., *Gloeothece* sp., and *P. boryanum*) are restricted to the dark periods when cells are grown under cycles consisting of 12 h of light and 12 h of darkness (10, 14, 30–32, 40, 43). Our results suggest that some natural populations of unicellular cyanobacteria temporally separate N_2 fixation from photosynthetic O_2 evolution in situ. This pattern would be consistent with a strategy to minimize destruction of nitrogenase by oxygen during photosynthesis (14).

The daily pattern of in situ *nifH* expression by the unicellular group A cyanobacterial phylotype was markedly different than that observed for the group B phylotype. Group A *nifH* transcription was elevated throughout the early morning to midday, and group A *nifH* expression appeared to be ~12-h out of phase with group B *nifH* expression. Group A *nifH* transcription peaked near midday, when the rates of photosynthesis and O_2 evolution were presumably high. These distinctly different temporal patterns of *nifH* expression suggest that different groups of unicellular diazotrophic cyanobacteria may have different in situ physiological responses to daily fluctuations in irradiance and/or photosynthesis in the upper ocean.

Assuming that expression of the *nifH* gene corresponded to the initiation of cellular N_2 fixation, the mechanism(s) that enables the group A unicellular cyanobacteria to fix N_2 coincident with photosynthesis remains unknown. A number of investigators have suggested that N_2 fixation by nonheterocystous cyanobacteria depends on sufficient O_2 to maintain the aerobic respiratory processes which partially fuel the energy-demanding N_2 fixation (14, 30, 32, 42). There is also evidence that the temporal phasing of N_2 fixation in unicellular cyanobacteria depends on the growth rate; Ortega-Calvo and Stal (37) grew *Gloeothece* sp. in continuous culture with alternating light and dark cycles at relatively high growth rates (0.2 to 0.5 day^{-1}), and N_2 fixation was largely restricted to the early phases of the light period. The mechanisms which enable these unicellular diazotrophs to fix N_2 coincident with photosynthesis remain unknown.

By normalizing the temporal patterns of *nifH* gene expression to measured gene abundance, our results suggest that the

numbers of *nifH* transcripts produced throughout the day by the different diazotrophic phylotypes were highly variable. The number of *nifH* transcripts produced by the *Trichodesmium* spp. and group B phylotypes varied by 3 to 4 orders of magnitude over the course of a day, while group A *nifH* transcription was somewhat more stable, changing by 1 to 2 orders of magnitude over a day. It is not known how variations in the number of *nifH* transcripts produced per gene copy influence N_2 fixation by these phylotypes. Nitrogenase is regulated at both the transcriptional and posttranslational levels (10), and *nif* transcription likely depends on various factors, such as light, O_2, nitrogen availability, or circadian regulation. The unique patterns of *nifH* expression which we observed may reflect distinct in situ physiological responses to daily fluctuations in irradiance. *nif* transcripts and nitrogenase proteins appear to undergo rapid degradation in vivo (10), suggesting that transcript accumulation likely occurs only during periods of active translation, which presumably coincides with periods of N_2 fixation.

Our observations suggest that various groups of open ocean cyanobacteria have evolved different physiological adaptations that permit them to fill some or all of their cellular nitrogen demands by fixation of N_2. Such adaptations likely include temporal (group B phylotype) and spatial (heterocyst-1) separation of nitrogenase from O_2. Our results suggest that in the nitrogen-impoverished waters of the subtropical North Pacific Ocean several different bacterial groups help regulate biological N_2 fixation in the upper ocean and that the specialized activities of different diazotroph groups may permit fixed nitrogen inputs at various times of the day and night.

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