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Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean

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Fixed nitrogen (N) often limits the growth of organisms in terrestrial and aquatic biomes[1,2], and N availability has been important in controlling the CO_2 balance of modern and ancient oceans[3,4]. The fixation of atmospheric dinitrogen gas (N_2) to ammonia is catalysed by nitrogenase and provides a fixed N for N-limited environments[2,5]. The filamentous cyanobacterium *Trichodesmium* has been assumed to be the predominant oceanic N_2-fixing microorganism since the discovery of N_2 fixation in *Trichodesmium* in 1961 (ref. 6). Attention has recently focused on oceanic N_2 fixation because nitrogen availability is generally limiting in many oceans, and attempts to constrain the global atmosphere–ocean fluxes of CO_2 are based on basin-scale N balances[7–9]. Biogeochemical studies and models have suggested that total N_2-fixation rates may be substantially greater than previously believed[7,8] but cannot be reconciled with observed *Trichodesmium* abundances[8,9]. It is curious that there are so few known N_2-fixing microorganisms in oligotrophic oceans when it is clearly ecologically advantageous. Here we show that there are unicellular cyanobacteria in the open ocean that are expressing nitrogenase, and are abundant enough to potentially have a significant role in N dynamics.

Water samples were collected during several cruises to the Hawaii Ocean Time-series (HOT) station ALOHA to determine whether N_2-fixing microorganisms were present and, if so, expressing nitrogenase. Previous results indicated that diverse diazotrophs, potentially including unicellular cyanobacteria, were present at station ALOHA based on the analysis of nitrogenase genes (specifically *nifH*, which encodes the Fe protein component)[10,11]. In our study, RNA was extracted to determine if these microorganisms were expressing *nifH* under *in situ* conditions, which would indicate that they are responding to conditions of limited N and synthesizing the nitrogenase protein. Also, we looked for the putative unicellular cyanobacteria by microscopy, cultivated isolates and performed experiments to see if the microorganisms could fix N_2 both *in situ* and in culture.

Nitrogenase gene transcripts (messenger RNA) attributable to organisms other than *Trichodesmium* or cyanobacterial symbionts of large diatoms (another known diazotroph in these waters) were detected in all samples of the upper water column (0–150 m) collected in both February and May 2000, and displayed apparent spatial and diel variability (Fig. 1). The signal—detected by reverse transcription with polymerase chain reaction (RT-PCR)—at a depth of 25 m, indicating *nifH* biosynthesis, was highest at night (Fig. 1a), whereas the signal at greater depths was higher at noon than at midnight (Fig. 1b). Variation in transcript abundance during the day is a characteristic of nitrogenase expression pattern in unicellular and filamentous cyanobacteria, and is controlled by a circadian rhythm[12]. Little or no *nifH* mRNA was detected at 150 m at any time of the day, which is consistent with the presence of relatively high nitrate concentrations, which selects against N_2 fixation (NO_3^- concentration was 9.6 nM in the upper 100 m of the water column, compared with greater than 1,000 nM at 150 m; http://hahana.soest.hawaii.edu).

To identify which organisms in the 0.2–10-μm size class were expressing *nifH*, the *nifH* gene fragments that were amplified by RT-PCR were cloned and sequenced. Sequences phylogenetically related to cyanobacterial *nifH* genes were obtained from all depths (Fig. 2) and dominated the clone library (19 out of 27 clones). Eight clones clustered with sequences from proteobacteria, indicating that proteobacteria could also be fixing N_2, a system that could potentially be driven by bacterial, phototrophic metabolic pathways[13,14]. Two different cyanobacterial *nifH* sequence groups were found (Fig. 2).

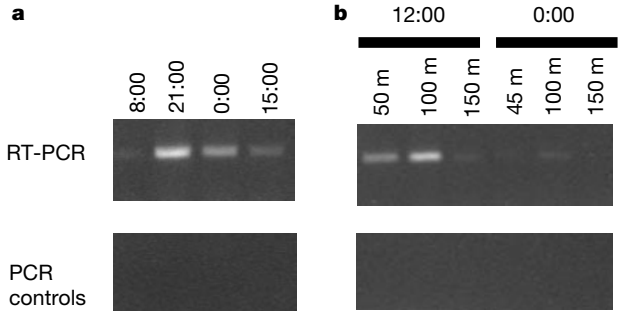

Figure 1 Expression of *nifH* genes in water samples collected at the Hawaii Ocean Time-series station ALOHA in May 2000 (HOT cruise 115). Upper gel on each panel shows amplified *nifH* fragment after RT-PCR; lower part shows the no-RT-PCR controls. No amplification was detected in samples treated with RNase. **a**, Amplification from water samples collected at 25 m at four different times of day. Highest levels of transcripts were detected at 21:00 and 0:00, with lower expression at 15:00 and 18:00. **b**, Amplification of *nifH* from water samples collected at 50, 100 and 150 m at 12:00 and 0:00.

One group of sequences (group A; Fig. 2), obtained from depths of 25, 50 and 100 m, were greater than 95% identical to cyanobacterial *nifH* sequences previously obtained by amplification of *nifH* from DNA collected at station ALOHA in 1996 and 1997 (ref. 10). The second group of sequences (group B) was found at a depth of 100 m in May and at various depths in February. The sequences recovered by RT-PCR within each group were between 93 and 99% identical to each other, which is more variability than can be accounted for by PCR error. Thus, the molecular data indicate that there are multiple strains or populations of unicellular, N_2-fixing marine cyanobacterial groups.

The cyanobacterial *nifH* genes amplified from the samples collected at station ALOHA were closely related to *nifH* genes of cultivated unicellular cyanobacteria, including the oceanic *Synechocystis* sp. strain WH 8501, previously isolated from the Atlantic Ocean (Figs 2 and 3c, d)[15,16]. The genera of 3–10-μm diameter, N_2-fixing unicellular cyanobacteria (for example *Synechocystis* sp. WH 8501, *Cyanothece* sp. ATCC 51142, and *Cyanothece* spp. Miami BG 43522 and 43511, which were previously called *Synechococcus*) have not previously been considered important components of the oceanic plankton, although phycoerythrin-containing cells of this size have been observed in oligotrophic waters at abundances much higher than we observed in July 2000 at station ALOHA[17,18].

As the evidence from the molecular sequences suggested the presence of cyanobacteria that are phylogenetically related to cultivated strains of 3–10-μm diameter cyanobacteria, we examined water samples collected at station ALOHA in July 2000 by fluorescence microscopy, and attempted to cultivate N_2-fixing cyanobacteria. Phycoerythrin-containing cells, several micrometres in diameter, were observed by fluorescence microscopy in water samples collected from HOT cruise 117 at station ALOHA in July 2000 (Fig. 3). Different morphologies of cells, in particular different sizes of cells (Fig. 3), were observed, indicating that different strains or populations may be present in the water column, which is consistent with the observed diversity of *nifH* sequences (Fig. 2). Two strains of unicellular cyanobacteria (3–5 μm in diameter) were cultivated on a medium containing no fixed N. The *nifH* gene sequences from these strains were 93% identical to cluster B sequences from station ALOHA transcripts (Fig. 2), and are very closely related to sequences from a previously cultivated isolate from the Atlantic, WH8501 (99–100% identical). The cultivated isolates fix N_2 at night in culture, which is consistent with the expression pattern observed at station ALOHA at a depth of 25 m (Fig. 1).

Water collected from a depth of 25 m in July 2000 was incubated with $^{15}N_2$ to determine if the size class containing marine unicellular cyanobacteria, but not *Trichodesmium* or *Rhizosolenia–Richelia*,

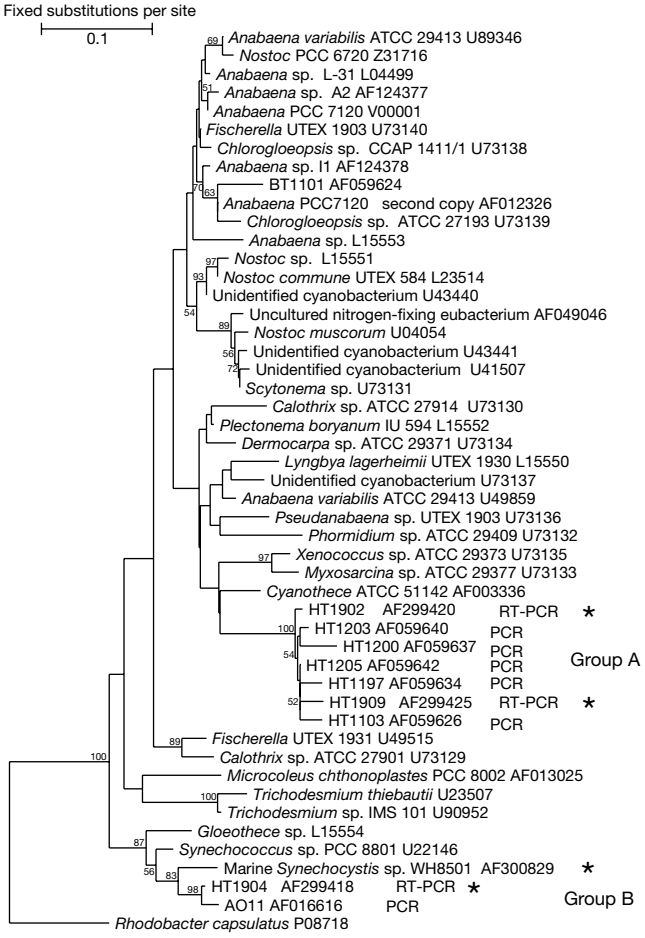

Figure 2 Phylogenetic tree showing the relationships among representative cyanobacterial *nifH* gene sequences. Transcripts detected at station ALOHA are designated RT-PCR. Nitrogenase (*nifH*) sequences amplified from station ALOHA are most closely related to *nifH* sequences from *Cyanothece*, *Myxosarcina*, *Gloeothece* and *Synechococcus* (renamed *Cyanothece*). Two different clusters of sequences were detected in May 2000. Sequence type A was found at depths of 25, 50 and 100 m; sequences of type B were detected in February and May 2000. The latter sequences are closely related to sequence AF016616, which was obtained from the equatorial North Atlantic Ocean. Sequences marked with an asterisk were determined as part of this study. The analysis was bootstrapped and bootstrap values greater than 50% (out of 100 replicates) are indicated at the respective nodes.

fixed $^{15}N_2$ under simulated *in situ* conditions. $^{15}N_2$ was fixed into particulate material in the 0.2–10-μm size fraction at rates of 10–16 pmol N l^{-1} h^{-1}. Unicellular cyanobacterial cells 3–10 μm in diameter were present during the experiment at a concentration of 52 ± 12 cells ml^{-1} (s.d.; $n = 4$). This is equivalent to a cell-specific rate of N_2 fixation in the size fraction containing the natural populations of marine *Synechocystis* at station ALOHA that is similar to the rates reported for cultures of the morphologically similar *Cyanothece* sp. ATCC 51142 grown without combined N.

Although unicellular diazotrophs in the open ocean have received little attention, there is evidence that the N_2-fixing unicellular cyanobacteria (that is, the 3–10 μm diameter morphology) may be widely distributed in marine and even freshwater environments at concentrations of up to 1,000 cells ml^{-1} (refs 17–20). The size and abundance of these diazotrophs indicate that they can equal or exceed the N_2-fixation contribution of the known diazotrophs—*Trichodesmium* and *Richelia*. For example, *Trichodesmium* is found both as microscopic free filaments called trichomes (chains of approximately 100–150 cells) and as macroscopic aggregates composed of several hundred trichomes. The mean abundance of free trichomes at station ALOHA is 5×10^4 m^{-3} (ref. 21), equivalent to an average density of 5 cells ml^{-1}. Inclusion of the cells contained in the aggregate morphology would double or triple the mean *Trichodesmium* cell abundance. On the other hand, not all cells in a *Trichodesmium* filament fix N_2 (ref. 22), and thus rates of N_2 fixation by *Trichodesmium* that are calculated from abundance data may be overestimated in some cases.

Concentrations of phycoerythrin-containing unicellular cyanobacteria cells at a diameter of 3–20 μm are present with a mean abundance of 10–50 cells ml^{-1} and can reach 1,000 cells ml^{-1} under bloom conditions[18]. For example, in April 1993, 3–20-μm diameter unicellular cyanobacteria were present at station ALOHA at an average abundance of 12 cells ml^{-1} in water samples from the surface to a depth of 187 m—there was slightly higher abundance in May[18]. In September, the unicellular cyanobacteria had an average abundance of 122 cells ml^{-1} from 0 to 150 m, with a maximum density of 928 cells ml^{-1} at 110 m (ref. 18). At these abundances, the biomass of the unicellular cyanobacteria could equal or exceed that of *Trichodesmium*. The bloom in September 1993, with an average of 122 cells ml^{-1} throughout a 150-m water column (1.22×10^6 cells m^{-3}; 150 m^3 in 150-m water column) may have been responsible for a N_2-fixation rate of 92 μmol m^{-2} d^{-1}, assuming 10 h of night-time activity at an average rate of 0.5 fmol $cell^{-1}$ h^{-1}. This is the same order of magnitude as the averaged daily N_2-fixation contribution of *Trichodesmium* (average of 137 μmol m^{-2} d^{-1} (ref. 23). However, owing to the well-documented temporal variations of N_2-fixing and non-N_2-fixing microorganisms at station ALOHA[18,21,24], determining the annual per cent contribution of N_2 fixation to new and export production will probably require a high-frequency observation programme over several years.

Our results indicate that N_2-fixing cyanobacteria, in addition to the well-known diazotroph genera *Trichodesmium* and *Richelia*, are expressing *nifH* and fixing N_2 in the subtropical North Pacific Ocean. The results reported here point to a substantially new model for N_2 fixation in the oceans. The role of unicellular cyanobacteria in N_2 fixation in the open ocean is an unstudied phenomenon of uncertain ecological significance. Even if it is eventually found that these microorganisms do not contribute fixed N at rates equivalent to *Trichodesmium* (or other diazotrophs), it is probable that these unicellular microorganisms have a very different fate in the food chain with different implications for the fate of carbon in the water column. For example, the marine N_2-fixing unicellular cyanobacteria may be more heavily consumed than *Trichodesmium*, which is toxic to some grazers[25]. The documented temporal variations in the distributions and abundances of these populations, including aperiodic blooms, suggests that climate variations may induce shifts in the relative size distribution of microbial assemblages that fix N_2. These new cyanobacterial diazotrophs may have long been, or are perhaps becoming, significant contributors to oceanic N_2 fixation, and deserve further scrutiny. The discovery that these microorganisms are present and actively expressing nitrogenase in the open ocean implies that conceptual models of the magnitude, timing and control of N_2 fixation in the ocean need to be re-evaluated[26]. □

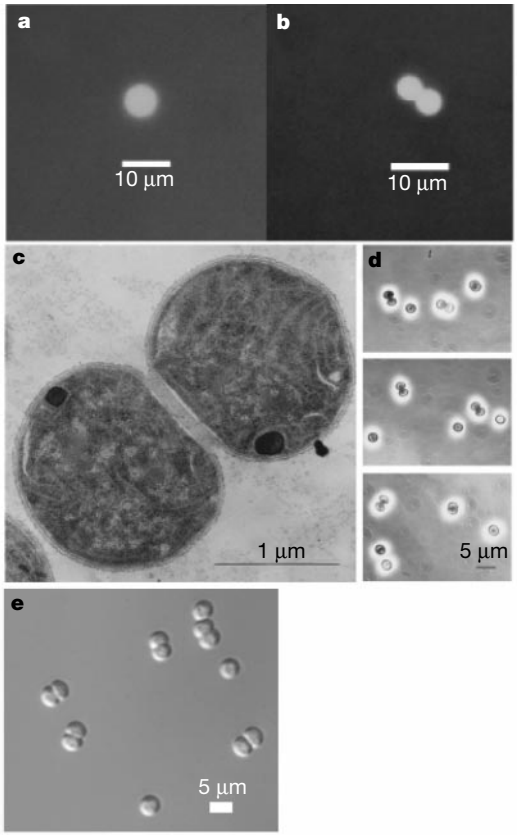

Figure 3 Photomicrographs of marine N_2-fixing unicellular cyanobacteria. **a**, Fluorescence images of phycoerythrin-containing, 7-μm diameter unicellular cyanobacteria cells collected from 25 m depth at station ALOHA in July 2000. **b**, Fluorescence images of 3-μm diameter unicellular cyanobacteria cells that have recently divided, from the same sample as **a**. **c**, **d**, Electron (**c**) and light (**d**) micrographs of marine *Synechocystis* sp. WH 8501, a unicellular cyanobacterium isolated from tropical Atlantic Ocean waters (28° S, 43° W) that is phylogenetically related to group B unicellular cyanobacteria on the basis of *nifH* sequences (see Fig. 2). Cells are spherical and 2–3 μm in diameter. **e**, Unicellular cyanobacteria in enrichment in a fixed N-free medium, cultivated from surface water collected at station ALOHA in July 2000.

Methods

Sample collection

We collected water samples from the HOT station ALOHA (22° 45′ N, 158° 0′ W) on HOT cruise 115. Samples were collected in 12-l polyvinylchloride bottles mounted on an aluminum rosette frame equipped with a SeaBird model 911 CTD and 24-place pylon. We collected samples from depths spanning the euphotic zone (25, 50, 100 and 150 m). Details of the HOT programme have been published elsewhere and are available at http://hahana.soest.hawaii.edu/hot/hot_jgofs.html.

Sample processing

Water samples were pre-filtered through plankton netting (pore size of 10 μm) to remove *Trichodesmium* and large phytoplankton, including diatoms, and then cells were collected on Millipore Durapore membrane filters (pore size of 0.22 μm). RNA was extracted from filters using the Qiagen RNeasy kit. RT-PCR was performed with the Promega RT-PCR Access protocol on RNA using degenerate primers for *nifH*[27]. The nested PCR step was performed using a second inner set of degenerate *nifH* primers. The amplified products were separated on a 1.5% agarose gel and photographed with a Bio-Rad Gel Doc photodocumentation system.

Cloning and sequencing

The amplified *nifH* fragments, approximately 359 base pairs in length, were cloned in Promega pGEM-T vector using the manufacturer's protocol. Recombinants containing the cloned insert were identified by restriction fragment analysis, and the fragment sequenced using an ABI 310 automated DNA sequencer. The translated sequences were aligned with Genetics Computer Group software, and the phylogenetic relationship determined by a distance method and neighbour joining using TREECON software[28]. The analysis was bootstrapped 100 times.

Epifluorescence microscopy

Formalin-preserved water samples (10–20 ml) were filtered onto black polycarbonate Poretics filters (pore size 0.22 μm). An Olympus BX-60 epifluorescence microscope was used with the U-WMB filter set (excitation 450–480 nm; emission greater than 515 nm). Phycoerythrin-containing cells (fluorescing yellow–orange) were enumerated in 100 fields, and cells were measured using an ocular micrometer.

^{15}N experiments

Water collected from a depth of 25 m at station ALOHA in July 2000 was transported to the marine laboratory at the Hawaii Institute of Marine Biology, where it was incubated in 2–4-l acid-washed polycarbonate bottles in running seawater, in a flume. The flume was covered with one layer of neutral-density screening to decrease light levels to roughly 50% surface irradiance (photosynthetically active radiation) approximately 600 μmol m^{-2} s^{-1}). At each time point, samples were processed for ^{15}N and microscopy, after pre-filtering through Nuclepore filters (pore size 10 μm) to remove the larger diazotrophs. Particle samples were collected by gentle pressure filtration (<10 pounds per square inch) through pre-combusted GF/F filters, which were analysed ashore by continuous-flow isotope ratio mass spectrometry using a Micromass Optima mass spectrometer that was interfaced with a CE-Elantech NA2500 elemental analyser. The analytical precision varies with sample size, but is about ± 0.2‰ (s.d. of replicate analyses) for samples containing 0.5–2 μmol of N.

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Density-dependent mortality in an oceanic copepod population

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Planktonic copepods are primary consumers in the ocean and are perhaps the most numerous metazoans on earth. Secondary production by these zooplankton supports most food webs of the open sea, directly affecting pelagic fish populations and the biological pump of carbon into the deep ocean. Models of marine ecosystems are quite sensitive to the formulation of the term for zooplankton mortality[1–4], although there are few data available to constrain mortality rates in such models. Here we present the first evidence for nonlinear, density-dependent mortality rates of open-ocean zooplankton. A high-frequency time series reveals that per capita mortality rates of eggs of *Calanus finmarchicus* Gunnerus are a function of the abundance of adult females and juveniles. The temporal dynamics of zooplankton populations can be influenced as much by time-dependent mortality rates as by variations in 'bottom up' forcing. The functional form and rates chosen for zooplankton mortality in ecosystem models can alter the balance of pelagic ecosystems[1–3], modify elemental fluxes into the ocean's interior[5], and modulate interannual variability in pelagic ecosystems[6].

The high-frequency (sampling interval 1–2 d, sustained for 80 d) time series of *Calanus finmarchicus* in the central Norwegian Sea conducted as part of the TASC (Trans-Atlantic Study of *Calanus finmarchicus*) programme shows a springtime emergence of juvenile (C5) and adult female copepods in late March (small hump at front left of Fig. 1). These individuals originate from the overwintering generation in deep water[7]. The onset of reproductive maturity and egg production occur at least 40–50 d before the phytoplankton bloom[8,9], contrary to the assumptions in many ecosystem models, although per capita rates of egg production increase at the time of

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