Lawrence Berkeley National Laboratory
Lawrence Berkeley National Laboratory

Title
Identification of a novel cyanobacterial group as active diazotrophs in a coastal microbial mat using NanoSIMS analysis

Permalink
https://escholarship.org/uc/item/5vm1q17g

Author
Woebken, D.

Publication Date
2012-01-30

DOI
DOI:10.1038/ismej.2011.200

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Identification of a novel cyanobacterial group as active diazotrophs in a coastal microbial mat using NanoSIMS analysis

Dagmar Woebken[1,2,*], Luke C. Burow[1,2], Leslie Prufert-Bebout[2], Brad M. Bebout[2], Tori M. Hoehler[2], Jennifer Pett-Ridge[3], Alfred M. Spormann[1], Peter K. Weber[3], Steven W. Singer[4*]

[1]Departments of Chemical Engineering, and of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305; [2]Exobiology Branch, NASA Ames Research Center, Moffett Field, CA 94035; [3]Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, CA 94550; [4]Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Running Title: Novel cyanobacterial diazotrophs in a coastal microbial mat

*Correspondence:

Dagmar Woebken, Department of Civil and Environmental Engineering, Stanford University, 318 Campus Drive, Stanford, CA 94305, Phone: 650-724-5809, Fax: 650-724-4927, E-mail: dwoebken@gmail.com

Steven Singer, Earth Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mail Stop 90R-1116, Berkeley, CA 94720, Phone: 510-486-5556, Fax: 510-486-6406, E-mail: SWSinger@lbl.gov

Abstract

N_2 fixation is a key process in photosynthetic microbial mats to support the nitrogen demands associated with primary production. Despite its importance, groups that actively fix N_2 and contribute to the input of organic N in these ecosystems still remain largely unclear. To investigate the active diazotrophic community in microbial mats from the Elkhorn Slough estuary, Monterey Bay, CA, we conducted an extensive combined approach including biogeochemical, molecular and high-resolution secondary ion mass spectrometry (NanoSIMS) analyses. Detailed analysis of dinitrogenase reductase (*nifH*) transcript clone libraries from mat samples that fixed N_2 at night indicated that cyanobacterial *nifH* transcripts were abundant and formed a novel monophyletic lineage. Independent NanoSIMS analysis of $^{15}N_2$-incubated samples revealed significant incorporation of ^{15}N into small, non-heterocystous cyanobacterial filaments. Mat-derived enrichment cultures yielded a unicyanobacterial culture with similar filaments (named ESFC-1) that contained a *nifH* gene sequence grouping with the novel cyanobacterial lineage identified in the transcript clone libraries. The 16S rRNA gene sequence recovered from this enrichment allowed for the identification of related sequences from Elkhorn Slough mats and revealed great sequence diversity in this cluster. Furthermore, by combining $^{15}N_2$ tracer experiments, fluorescence *in situ* hybridization and NanoSIMS, *in situ* N_2 fixation activity by the novel ESFC-1 group was demonstrated, suggesting that this group may be the most active cyanobacterial diazotroph in the Elkhorn Slough mat. ESFC1-related pyrotag sequences were recovered from mat samples throughout 2009, demonstrating the prevalence of this group. This work illustrates that combining standard and single cell analyses can link phylogeny and function to identify previously unknown key functional groups in complex ecosystems.

cyanobacteria/ dinitrogenase reductase (*nifH)/* microbial mats/ NanoSIMS/ N_2 fixation

Introduction

Photosynthetic microbial mats are ecosystems with high densities of functional and phylogenetic diversity resulting in a strongly coupled cycling of elements (Bebout *et al.*, 1994; Canfield and Des Marais, 1993; Ley *et al.*, 2006). Therefore, these ecosystems have been studied extensively to gain fundamental insights into global processes with implications ranging from the early evolution of microorganisms and metabolic diversity to microbial interactions and nutrient cycling (e.g. (Des Marais, 1990; Des Marais, 2003; Paerl *et al.*, 2000)). The cycling and flux of carbon, sulfur and nitrogen are particularly intertwined in these mats. The availability of nitrogen determines overall mat productivity, since photosynthetic primary production creates a high demand for fixed nitrogen, which requires high rates of N_2 fixation (Herbert, 1999). However, the distribution of N_2 fixation activity among phylogenetically and functionally diverse microorganisms in the mats remains largely unclear.

In most marine microbial mats, the uppermost layer is visually dominated by unicellular and nonheterocystous filamentous cyanobacteria, such as *Microcoleus* or *Lyngbya* spp. (D'Amelio *et al.*, 1989). N_2 fixation in these mats has a distinct diel pattern: N_2 fixation is largely absent during the day, increases after sunset, and is maximal at night or in the early morning prior to sunrise (Bebout *et al.*, 1994; Bebout *et al.*, 1987; Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b; Stal *et al.*, 1984). Daytime suppression of N_2 fixation has been attributed to high levels of O_2 from oxygenic photosynthesis, which leads to inhibition of the O_2-sensitive nitrogenase (Bothe, 1982). The mats in the Elkhorn Slough estuary, Moss Landing, CA that were the focus of this study are dominated by filamentous cyanobacteria, in particular by *Microcoleus* spp. However, the N_2 fixation activity pattern and the diazotrophic community in Elkhorn Slough mats were so far unknown.

Traditionally, cyanobacteria were thought to play a major role in total N_2 fixation in

microbial mats (Bebout *et al.*, 1993; Paerl *et al.*, 1991). A few cyanobacteria that were isolated from microbial mats fixed N_2 in cultivation-based studies and corroborated this hypothesis (Paerl *et al.*, 1991; Stal and Krumbein, 1981). However, concern of general cultivation bias has motivated researchers to pursue cultivation-independent methods to gain more comprehensive insight of the diazotrophic community in complex ecosystems (Zehr *et al.*, 1995).

N_2 fixation is mediated by the nitrogenase enzyme complex, consisting of *nifH* encoded dinitrogenase reductase, which transfers reducing equivalents to dinitrogenase, encoded by *nifD* and *nifK*, ultimately catalyzing the reduction of N_2 to NH_3. This unique process has been studied at different levels using various methods such as acetylene reduction assay and *nifH* surveys. The fortuitous transformation of acetylene to ethylene by nitrogenase makes the acetylene reduction assay a useful, indirect measure for nitrogenase activity in cultures as well as in complex communities (Stewart *et al.*, 1967). The high sensitivity of the assay has enabled measurements of rapid changes in N_2 fixation activity in response to rapidly changing environmental conditions. Employment of this technique in diel cycle studies of microbial mats has provided invaluable insights into the patterns of N_2 fixation depending on irradiance and thus O_2 concentrations, such as the above-mentioned patterns for *Microcoleus* and *Lyngbya* spp. dominated mats (e.g. (Bebout *et al.*, 1994; Bebout *et al.*, 1987; Paerl *et al.*, 1996; Stal *et al.*, 1984)). More recently, the *nifH* gene has been used as a phylogenetic and functional marker for N_2 fixation and allows investigating the phylogenetic distribution of the genetic potential for N_2 fixation in complex microbial communities. Surveys of *nifH* in microbial mats suggested that heterotrophic bacteria might also play an important role in microbial mat N_2 fixation in addition to cyanobacteria (e.g. (Omoregie *et al.*, 2004a; Severin *et al.*, 2010; Severin and Stal, 2010b; Steppe *et al.*, 1996; Zehr *et al.*, 2003; Zehr *et al.*, 1995). Analysis and quantification of *nifH* transcripts has helped to identify the fraction of

diazotrophs actively expressing this essential gene for N_2 fixation and has given insights into gene expression dynamics in the environment (Moisander *et al.*, 2006; Omoregie *et al.*, 2004b; Severin and Stal, 2010a). Previous studies (Severin and Stal, 2010a; Steunou *et al.*, 2008) have revealed discrepancies between the expression of *nifH* by diazotrophic groups and nitrogenase activity patterns measured by acetylene reduction, illustrating that gene expression does not necessarily correspond to activity.

In contrast to the above-mentioned methods, stable isotope probing with $^{15}N_2$ provides a direct and unambigious measure of N_2 incorporation activity (Montoya *et al.*, 1996). While ^{15}N incorporation is measured in bulk by isotope ratio mass spectrometry (IRMS), secondary ion mass spectrometry (SIMS) and the recently developed CAMECA NanoSIMS for high resolution SIMS have enabled the connection of ecosystems level processes to activities at the level of single cells. These technologies have been used for the stable isotope probing of the metabolic activities of cell-aggregates (Orphan *et al.*, 2001) or single cells (Finzi-Hart *et al.*, 2009; Foster *et al.*, 2011; Lechene *et al.*, 2006; Ploug *et al.*, 2010; Popa *et al.*, 2007), respectively. In combination with fluorescence *in situ* hybridization (FISH) targeting 16S rRNA, SIMS studies revealed direct linkages of phylogeny to function in natural communities (Behrens *et al.*, 2008; Halm *et al.*, 2009; Li *et al.*, 2008; Musat *et al.*, 2008; Orphan *et al.*, 2001).

In this study, we used (to the best of our knowledge) an unprecedented breadth of methods - nitrogenase activity measurements, analysis of *nifH* gene diversity and expression, $^{15}N_2$ tracer experiments, NanoSIMS, catalyzed reporter deposition (CARD)-FISH and cultivation experiments- to identify active N_2 fixing microorganisms in a complex microbial mat ecosystem. By this combined approach, we were able to characterize a novel group of diazotrophic cyanobacteria in Elkhorn Slough microbial mats, and demonstrated their ecophysiological importance in N_2 fixation.

Materials and Methods

Study site

The sampling site is located in the Elkhorn Slough estuary in Central California, USA, at 36°48’46.61”N and 121°47’4.89”W. The Elkhorn Slough is a shallow seasonal estuary that extends inland 11 km from Monterey Bay with mixed semidiurnal tides; tidal exchange and sporadic surface water input during winter rainy seasons are the main water transport mechanisms (Chapin and Johnsin, 2004).

Mat Sampling and Diel Cycle Studies Set-up

Microbial mats collected at Elkhorn Slough (10 pieces of ca. 144 cm^2 of 2 cm thickness including a 1 cm sediment layer) were sampled on 20th October 2009 and transported to a greenhouse facility transparent to ultraviolet radiation at NASA Ames Research Center within 1 to 2 hours. In the greenhouse, mat pieces were placed in acrylic aquaria transparent to ultraviolet radiation and covered with *in situ* water (circulated and aerated) for ca. 20 h before the beginning of a diel cycle study (starting at 12:00 pm and ending at 3:00 pm the following day). Two successive diel cycle studies with the same mats were carried out (21st/22nd and 23rd/24th October 2009) under natural solar irradiance, and the water temperature was kept constant at ca. 18 °C (*in situ* average).

Biogeochemical Analysis (Acetylene Reduction Assays and $^{15}N_2$ Incubations)

Nitrogenase activity was measured with the acetylene reduction assay (ARA) as previously described (Bebout *et al.*, 1993). Triplicate mat cores (10 mm diameter, 10 mm thick) were sampled every 3 hours, and subsequently incubated with acetylene for 3 hours. Triplicate water samples without mat served as negative controls. Ethylene was quantified in a Shimadzu GC-14A gas chromatograph. For measuring the depth distribution of nitrogenase

activity in the mats, triplicate mat cores of 10 mm diameter and 10 mm thickness were horizontally sectioned into 3 layers (uppermost layer 0 to 2 mm depth, second layer 3 to 6 mm and third layer of 7 to 10 mm depth) and the layers were separately incubated as mentioned above.

To measure $^{15}N_2$ incorporation, mat cores of 10 mm diameter and 10 mm thickness were transferred to a 14 ml serum vial, covered with 1ml of *in situ* water, capped with gas-tight rubber stoppers and 8 ml of the headspace was exchanged with $^{15}N_2$ gas (98+ atom% $^{15}N_2$; Cambridge Isotope Laboratories, Andover, MA, USA). Mats were incubated for 10 hours in the dark (8:30 pm until 6:30 am the next day), and subsequently, half of the mat cores were sectioned for bulk isotope analysis in the same depth intervals as mentioned above. The other portions of the sectioned cores were preserved for NanoSIMS analysis by fixation in 4% paraformaldehyde for ca. 16 h at 4 °C. Fixed cores were washed twice in 1 x phosphate buffered saline (PBS) (pH 8.0), and stored in PBS/ethanol (40/60, vol/vol) at -20 °C for further analysis. Unlabeled mats sections served as controls. Isotope ratios for $^{15}N/^{14}N$ were determined by isotope-ratio mass spectrometry (IRMS) (ANCA-IRMS, PDZE Europa Limited, Crewe, England) at the University of California-Berkeley. Additionally, mat cores were first sectioned and then incubated with $^{15}N_2$ to verify the depth distribution of N_2 fixation by IRMS.

IRMS and ARA data of vertical sections were analyzed for statistical differences using an analysis of variance with a Tukey's HSD mean separation at $p<0.05$ using the R program version 2.13.1 (http://www.r-project.org/index.html).

Companion samples for nucleic acid extraction and subsequent molecular analysis (6 independent cores of 10 mm diamater and 10 mm thickness per time point) were flash frozen in liquid nitrogen and stored at -80 ^{o}C.

Cultivation and $^{15}N_2$ Incubation of ESFC-1 Enrichment

Cyanobacteria were enriched from microbial mat samples as described previously (Prufert-Bebout and Garcia-Pichel, 1994). Media formulations and enrichment conditions are described in the Supplemental Information. After enrichment, the cyanobacterial cultures were assessed for N_2 fixation activity after 3 washes in N-free media (ASN-) and pre-incubated in ASN- for 4 days. Cultures were then transferred into 14 ml serum vials, which were filled with fresh ASN- media to eliminate gas headspace. The vials were capped with gas-tight rubber stoppers and 35 µl of $^{15}N_2$ were added, while an equal volume of medium was vented with a needle. Vials were incubated at 22 °C for 24 hours (dark/light cycle, 8 h/16 h, light intensity ca. 40 µmol photons m^{-2} s^{-1}). At the end of the incubation, the cyanobacterial biomass was rinsed in 1 x PBS and frozen at -80 °C for IRMS analysis.

Molecular analysis

Detailed information about DNA and RNA extractions can be found in Supplemental Information. Briefly, RNA and DNA of microbial mats were co-extracted from the uppermost 2 mm of 3 pooled mat cores by combining phenol-chloroform extraction with parts of the RNeasyMini and QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA), respectively. RNA was reverse transcribed into single-stranded cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

Detailed information about the construction of 16S rRNA gene/transcripts and *nifH* gene/transcript clone libraries can be found in the Supplemental Information. In summary, (1) A 16S rRNA gene clone library was constructed from the cyanobacterial enrichment culture ESFC-1 (total of 36 sequences). (2) General 16S rRNA clone libraries of the upper 2 mm of Elkhorn Slough mats were generated from mat samples collected in $12^{th}/13^{th}$ January 2009. The following clone libraries were constructed: 12th January, 9:00 pm (total of 329 sequences)

and 13th January, 7:00 am (total of 243 sequences). (3) A *nifH* gene clone library was generated from the cyanobacterial enrichment culture ESFC-1 (total of 39 sequences) (4) Clone libraries of the *nifH* genes (DNA) and transcripts (cDNA) were constructed from the uppermost 2 mm of mat cores sampled during two consecutive diels: 21st October 2009, at 10:50 pm (DNA: 93 sequences and cDNA 92 sequence) and 24th October 2009, at 3:10 am (DNA: 75 sequences and cDNA 88 sequences). Sequence analyses of the clone libraries listed above are described in Supplemental Information.

Amplicons of the 16S rRNA V8 hypervariable region were constructed from 7 time points in the year 2009 (13th January, 30th April, 1st July, 19th August, 16th September, 21st October and 13th November 2009) (see Supplemental Information for more detailed description). Analysis of the V8 amplicon sequences is described in Supplemental Information.

Sample preparation for NanoSIMS analysis

Material of the uppermost 2 mm of fixed microbial mats from the October 2009 samples ($^{15}N_2$ incubated and samples for natural abundances) were transferred with tweezers onto silicon wafers (Ted Pella, Redding, CA, USA), teased apart and attached by drying. In experiments where CARD-FISH was combined with NanoSIMS analysis, wafers were coated with VectaBond (Vector Laboratories, Burlingame, CA, USA). Wafers were mapped with reflected light and scanning electron microscopy (SEM) for orientation in the NanoSIMS. SEM images were taken to match increased $^{15}N/^{14}N$ ratios measured by NanoSIMS with microbial cells. Higher magnification images were also taken from filamentous cyanobacteria to ensure that the investigated regions of cyanobacterial filaments were free of attached microorganisms and that increased $^{15}N/^{14}N$ ratio could be attributed to the cyanobacterial bacterial filament and not to associated epibionts.

CARD-FISH for NanoSIMS analysis of ESFC-1 filaments in mat samples

Design and optimization of ESFC-1-specific oligonucleotide probes are described in Supplementary Information. CARD-FISH was conducted as decribed previously (Pernthaler *et al.*, 2002) with hybridizations conducted at 46 °C and washing at 48 °C. Hybridization was conducted on silicon wafers coated with VectaBond; embedding in agarose was omitted. Hybridizations were performed with the following probes specific for the ESFC-1 cluster: UD3_172 and UD3_177 (Supplementary Table 1), and with NON338 (Wallner *et al.*, 1993) as a negative control and EUBI-III as positive control probe (Amann *et al.*, 1990; Daims *et al.*, 1999). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Wafers were mapped with epifluorescence microscopy, ESFC-1 filaments were identified and their location on the wafer imaged. Wafers were mapped for NanoSIMS analysis as above-mentioned.

NanoSIMS

SIMS was performed at Lawrence Livermore National Laboratory (LLNL) using a Cameca NanoSIMS 50 Gennevilliers Cedex, France) as previously described (Popa *et al.*, 2007). Secondary ions $^{12}C^{14}N$ and $^{12}C^{15}N$ were detected by pulse counting to generate 10–20 serial quantitative secondary ion images (that is, layers). Samples were also imaged simultaneously by secondary electrons. Samples were sputtered to a depth of ~100 nm to achieve sputtering equilibrium before collecting data (Ghosal *et al.*, 2008). The depth of analysis during a measurement was between 50 and 200 nm. Measurements were repeated on selected cells to ensure measurement accuracy. Selected samples were also sputtered at high beam currents (~1 nA) between repeat measurements to determine if isotopic composition changed; no significant changes were found with cell depth. Natural abundance samples of the

filamentous cyanobacteria were run and analyzed, and those values were used to correct the values of $^{15}N_2$-incubated samples.

Data were processed as quantitative isotopic ratio images using custom software (LIMAGE, L. Nittler, Carnegie Institution of Washington), and were corrected for detector dead-time and image shift from layer to layer. Regions of interest (ROIs) were defined in each image, and the isotopic ratio for each ROI were calculated by averaging over all of the replicate layers. For filamentous cyanobacteria, typically 3 to 10 connected cells within a filament were analyzed, and the isotopic ratio displayed in the results section for the filamentous cyanobacteria is the average ratio of all filaments with standard error. Cells were analyzed for significant enrichment using a one-tailed Student T test with equal variance.

Data are presented as $^{15}N/^{14}N$ ratio values and $\delta^{15}N$ values (calculated after equation (1)) and are presented as means ± standard error (SE). All reported enrichment values are corrected with natural abundance values.

(1) $\delta^{15}N = (R_f/0.00367-1)*1000$

For a preliminary estimation of the contribution of small filamentous cyanobacteria and single cells to the observed ^{15}N incorporation, 30 images of each 900 to 2,500 μm^2 size were analyzed. The $^{15}N/^{14}N$ ratio of enriched cells and filaments was measured and the atom percent enrichment (APE) of the ROIs was calculated according to equation (2).

(2) $APE = [R_f/(R_f+1) - R_i/(R_i+1)]\ 100\%$

The cell area of enriched single cells and filaments were measured based on the secondary electron image, and the ^{15}N enrichment was calculated as APE per cell area or filament area. The origin of enriched signals was confirmed with SEM imaging that was conducted prior to analysis imaging.

Nucleotide Accession Numbers

16S rRNA gene sequences obtained in this study are deposited under GenBank accession numbers xxxxxxxx– xxxxxxxx. Sequences of *nifH* genes and transcripts are deposited under GenBank accession numbers xxxx-xxxx.

Results

N_2 Fixation Patterns in Elkhorn Slough Cyanobacterial Mats

Cyanobacterial mat samples were collected near the mouth of the Elkhorn Slough on the 20th October 2009. Microscopic examination of the mats revealed that the upper green layer was dominated by filamentous, non-heterocystous bacteria, primarily by *Microcoleus spp,*; *Oscillatoria* spp., smaller filamentous cyanobacteria and *Lyngbya* spp. were also observed (Supplementary Figure 1). Amplicon pyrosequencing of the 16S rRNA genes of the upper 2 mm of these mats illustrated that this layer harbored a diverse microbial community (amplicons from at least 14 bacterial phyla were recovered) and that expressed rRNA was predominately associated with cyanobacteria (data not shown).

Nitrogen fixation has not been studied previously in Elkhorn Slough mats. To elucidate the N_2 fixation patterns in these mats, mat samples were assayed for nitrogenase activity and incorporation of fixed N_2 using acetylene and $^{15}N_2$ as substrates (Bebout *et al.*, 1993; Montoya *et al.*, 1996). Acetylene reduction assays (ARA) with complete mat cores in two successive diel cycles revealed that nitrogenase activity was more than 8-fold higher at night relative to the daytime (Figure 1). The maximum daytime nitrogenase activity was 3.7 µmol C_2H_4 m^{-2} h^{-1} on 21st/22nd October and 4.2 µmol C_2H_4 m^{-2} h^{-1} on 23rd/24th October. The nighttime activity reached 30.2 µmol C_2H_4 m^{-2} h^{-1} and 62.4 µmol C_2H_4 m^{-2} h^{-1} on the 21st/22nd and on 23rd/24th October, respectively.

Further acetylene reduction assays on layer-separated mat samples obtained in 2009 showed that more than 95% of the potential nitrogenase activity measured in the whole mat was recovered in the upper 2 mm of the ca. 1 cm-thick mats (Supplemetary Figure 2). The uppermost layer (0 to 2 mm) had significantly higher activities than the other two deeper layers ($p<0.0127$, 0 to 2 mm versus 3 to 6 mm and $p<0.0125$, 0 to 2 mm versus 7 to 10 mm) (Supplementary Figure 3). In contrast, the comparison of 3 to 6 mm versus 7 to 10 mm did not show significant differences in activity ($p<0.9999$). When incubated with $^{15}N_2$ in the dark, IRMS isotopic enrichment measurements performed on these layer-seperated mat samples confirmed that the uppermost layer was significantly enriched in ^{15}N relative to the 3 to 6 mm ($p<0.000026$), and 7 to 10 mm ($p<0.000028$) sections (Supplemetary Figure 4), while no difference was observed comparing the 3 to 6 mm and 7 to 10 mm layers ($p<0.998$). After a 10 to 11 hour incubation, the $^{15}N/^{14}N$ ratio in the upper layer (0 to 2 mm) reached 0.00424 $\pm 9.2 \times 10^{-5}$ (156.2 ±25.2‰), compared to 0.00368 $\pm 2.5 \times 10^{-6}$ (3.0 ±0.7‰) in the 3 to 6 mm sections and 0.00368 $\pm 3.5 \times 10^{-6}$ (4.1 ±1.0‰) in the 7 to 10 mm sections (Supplemetary Figure 2). Therefore, the investigation of diazotrophs in Elkhorn Slough mats focused on these uppermost 2 mm.

NanoSIMS Analysis of Elkhorn Slough Mat Microbial Community

The upper layers of the ^{15}N-labeled October 2009 nighttime samples were also analyzed by NanoSIMS in order to assess ^{15}N enrichment by different cell morphotypes. Significant and high ^{15}N enrichments were observed in small cyanobacterial filaments ($p<1.47 \times 10^{-5}$), which were ≤150 μm in length and composed of individual cells that were ca. 3 μm long and ca. 2 μm wide. Enrichment was also observed in multiple single cells ($p<0.00136$). The enriched small filaments had a $^{15}N/^{14}N$ ratio of 0.01436 ±0.00176 (2912.9 ±480.8‰, n=15), and enriched single cells had a $^{15}N/^{14}N$ ratio of 0.03519 ±0.00807 (8588.6

±2200.1‰, n=42) (Figure 2). In contrast, filaments of the abundant cyanobacterial group *Microcoleus* spp. and also of *Oscillatoria* spp. showed very low levels of enrichment ($^{15}N/^{14}N$= 0.00382 ±$2.4x10^{-5}$ or 40.9 ±6.5‰ ($p<0.00036$), and $^{15}N/^{14}N$ 0.00373 ±$1.9x10^{-5}$ or 17.1 ±5.1‰ ($p<0.005$), respectively). For a preliminary estimation of the contribution of the small filamentous cyanobacteria and single cells to the observed ^{15}N incorporation in the upper mat layer, cellular ^{15}N enrichments were analyzed in over 30 images of 900 to 2,500 μm^2 size each. Based on this screen, more than 80% of the total incorporated ^{15}N was observed in smaller cyanobacterial filaments and ca. 20% in the single cells.

Identification of an Uncharacterized nifH Phylotype in Elkhorn Slough Mats

To determine the diazotroph community in the mats and determine which community members were actively expressing the functional gene for N_2 fixation, *nifH* clone libraries were constructed from DNA and cDNA recovered from mat samples in October 2009 (21st October 2009, 10:50 pm and 24th October 2009, 3:10 am). DNA-derived *nifH* sequences recovered from both time points clustered with *nifH* cluster I and cluster III of the *nifH* tree as defined previously (Chien and Zinder, 1996; Zehr *et al.*, 2003) (Table 1). Sequences of *nifH* cluster III were found to be numerically dominant in both samples: 21st October (79.6%) and 24th October (70.7%). In contrast, the majority of the expressed *nifH* sequences recovered from cDNA from these samples were found to group with cluster I (75% and 85.2%, respectively) and a minor fraction with cluster III (25% and 14.8%, respectively). Diversity indices indicated a reduced diversity of expressed *nifH* sequences (Simpson index OTU_{95}, 1.32 and 2.01, 21st and 24th October, respectively) versus sequences from *nifH* genes (Simpson index, OTU_{95} 3.17 and 2.50).

Of the *nifH* sequences expressed in samples from the 21st and 24th of October, 52% and 23.9%, respectively, of the sequences grouped with cyanobacterial sequences in cluster I.

Almost all (97%) of these Cluster I cyanobacterial sequences formed a monophyletic lineage distinct from other cyanobacterial *nifH* sequences in the database (Figure 3a). The sequence identity within this monophyletic lineage was >94.7% on the deduced amino acid level. The only closely related sequence in publicly available databases that clustered with this lineage was a translated *nifH* sequence (DQ821979) recovered from a mixed *Lyngbya* culture enriched from mats collected in Guerrero Negro, Mexico (Moisander *et al.*, 2007). The amino acid sequences of the monophyletic lineage from Elkhorn Slough were 96.2-100% identical to this sequence. This cluster does not include a cultured cyanobacterium; the closest related cultured representatives are *Myxosarcina* sp. ATCC 29377 (U73133) and *Xenococcus* sp. PCC 7305 (U73135), which were 92.3-95.2% identical to the Elkhorn Slough lineage.

Cultivation of a Cyanobacterium Belonging to the Novel Lineage from Elkhorn Slough

The NanoSIMS analysis described above identified small filamentous cyanobacteria to be highly active in incorporating $^{15}N_2$ into biomass. Sequencing of *nifH* transcripts revealed that a substantial proportion of the expressed *nifH* sequences clustered in a novel cyanobacterial lineage. Therefore, we initiated cultivation experiments targeting small, non-heterocystous filamentous cyanobacteria in an attempt to identify the cyanobacteria associated with these expressed *nifH* sequences. One of these cultivations yielded a unicyanobacterial enrichment dominated by a filamentous cyanobacterium, named ESFC-1 (Elkhorn Slough Filamentous Cyanobacterium-1), which showed a similar morphology to the highly ^{15}N-enriched filaments visualized by NanoSIMS (Figure 2 and 4 inset micrograph). The maximal filament length observed in culture was 600 µm, and average cell sizes were 2.75 ±0.071 µm length and 1.78 ±0.023 µm width. When tested for $^{15}N_2$ assimilation by IRMS, we found that the ESFC-1 culture was highly enriched in ^{15}N after 24-hour incubation ($^{15}N/^{14}N = 0.00522 \pm 4.9 \times 10^{-06}$ or 421.2 ±1.4‰).

The *nifH* sequences of the ESFC-1 enrichment culture were recovered by PCR amplification and only one single *nifH* phylotype was identified (39 sequences analyzed). This phylotype clustered with the novel cyanobacterial lineage from Elkhorn Slough microbial mats (Figure 3b) with up to 100% sequence identity at the deduced amino acid level and up to 99.4% identity at the DNA level. Cyanobacterial 16S rRNA gene sequences recovered from the ESFC-1 culture yielded one phylotype (36 sequences analyzed, ≥ 99.6% sequence identity) (Figure 4a). The ESFC-1 16S rRNA sequence was only distantly related to sequences in publicly available databases with the closest sequence (DQ289927, 92.5% sequence identity) recovered from South Atlantic Bight sediments off the coast of Savannah, GA (Hunter *et al.*, 2006). The sequence of the closest cultured representative to ESFC-1 was *Spirulina* strain CCC Snake P. Y-85 (Y18783) (92.3% sequence identity), isolated from Yellowstone hot springs.

ESFC-1 is a Representative of a Prevalent and Diverse Cyanobacterial Group

To investigate the prevalence and diversity of the novel cyanobacterial group in Elkhorn Slough mats, pyrotag sequencing of V8 amplicons of the 16S rRNA gene from multiple time points in 2009 and nearly full-length sequencing of the 16S rRNA gene of one particular time point was obtained. ESFC-1 affiliated sequences were identified in 6 out of the 7 time points from samples collected throughout 2009. The sequences were proportionally higher in amplicon libraries generated from cDNA (0.18% to 15%) than from DNA samples (0 to 4%) (Figure 5) and substantially higher in cDNA libraries recovered from mat samples collected in January 2009. Therefore, cDNA from these samples were selected to generate nearly full-length 16S rRNA clone libraries to investigate the diversity of environmental 16S rRNA sequences that clustered with the ESFC-1 cyanobacterial group. Of the 572 near full-length 16S rRNA gene sequences recovered from the January 2009 samples, 187 (32.7%)

grouped monophyletically with the 16S rRNA gene sequence type from ESFC-1. This monophyletic clustering was observed with multiple treeing algorithms (maximum likelihood, maximum parsimony and neighbor joining) and was supported with a bootstrap value of 79% (Figure 4b). The sequence diversity within this cluster of ESFC-1 related sequences (92.5 to 100%) revealed a great diversity of ESFC-1 related cyanobacteria and suggests that this group of cyanobacteria may contain multiple species and genera (Rossello-Mora and Amann, 2001).

Investigation of in situ N_2 fixation by ESFC-1 and related cyanobacteria

To confirm that cyanobacteria of the ESFC-1 cluster were actively incorporating $^{15}N_2$ as suggested by *nifH* sequencing and our intial NanoSIMS survey of the October 2009 samples, further NanoSIMS investigations were combined with CARD-FISH. Oligonucleotide probes specific for the 16S rRNA gene of the ESFC-1 cluster identified in the January 2009 samples were designed and stringent hybridization conditions for the newly designed probes were determined as described in Supplementary Information (Supplementary Table 1 and Supplementary Figure 5 and 6). Samples from October 2009 incubated with $^{15}N_2$ were hybridized with the ESFC-1 specific probes and imaged by epifluorescence microscopy. CARD-FISH combined with NanoSIMS analysis demonstrated that filaments of the ESFC-1 cluster were enriched in ^{15}N (Figure 6). Sixty-three percent of 76 analyzed ESFC-1 ROIs were significantly enriched in ^{15}N based on a 95% confidence interval, with levels ranging from low enrichments ($^{15}N/^{14}N$ = 0.00374 or 19.3‰) to a $^{15}N/^{14}N$ ratio of 0.025 (5802.3‰).

Discussion

In this study, we used a combination of biogeochemical, molecular and NanoSIMS analysis in conjunction with targeted cultivation experiments to investigate the active diazotrophic community in microbial mats from the Elkhorn Slough estuary, CA. With this

approach, we discovered a novel filamentous cyanobacterial group, ESFC-1, that represented almost the entire fraction of the cyanobacterial expressed *nifH* genes. This expression data along with NanoSIMS analysis of ESFC-1 and other mat cyanobacteria led us to conclude that this novel group was the most active N_2-fixing cyanobacterium in the investigated mat.

Biogeochemical analysis of the mats collected in October 2009 revealed patterns of N_2 fixation activity similar to those observed previously in mats from Guerrero Negro in Mexico, Bird Shoal in North Carolina, and the island Mellum in the North Sea, which were dominated by filamentous non-heterocystous cyanobacteria; nitrogenase activity was restricted to the upper 2 mm of the mat (Stal *et al.*, 1984) and highest at night under anoxic conditions (Bebout *et al.*, 1993; Bebout *et al.*, 1987; Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b; Paerl *et al.*, 1996; Stal *et al.*, 1984). Despite these biogeochemical parallels, molecular data indicated that a previously unrecognized diazotroph was present and highly active in Elkhorn Slough mats.

Overall, the *nifH* gene sequences that we obtained for the Elkhorn Slough mats grouped with the *nifH* clusters I and III. This pattern was previously observed in mats from Guerrero Negro and the island Schiermonnikoog in the Wadden Sea (Moisander *et al.*, 2006; Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b; Severin *et al.*, 2010). A reduction in *nifH* transcript diversity compared to *nifH* gene diversity was observed in clone libraries recovered from Elkhorn Slough as was previously in mats from Guerrero Negro (Moisander *et al.*, 2006). These observations support the hypothesis that only a subset of the microbial community with the genetic potential for N_2 fixation actively expresses *nifH* in these cyanobacterial mats. Interestingly, a high number of the transcripts from Elkhorn Slough in October 2009 formed a novel monophyletic sequence cluster, suggesting that a new cyanobacterial group might be an important diazotroph in these mats. However, N_2 fixation is regulated on multiple levels ranging from transcription (Chen *et al.*, 1998) to post-

translational protein modification (Kim *et al.*, 1999), so the detection of *nifH* gene transcripts does not necessarily imply active N_2 fixation *in situ*.

NanoSIMS investigations provided additional data to support the assignment of a highly active diazotrophic cyanobacterial group in Elkhorn Slough mats predicted by marker gene analysis. NanoSIMS of $^{15}N_2$-incubated Elkhorn Slough mat samples from October showed that among the filamentous cyanobacteria, a particular morphotype that had cells of ca. 2 µm width and ca. 3 µm length was highly enriched in ^{15}N. We hypothesized that these cyanobacterial filaments might harbor the novel and highly expressed *nifH* sequences. To identify these cyanobacteria, we conducted targeted cultivation experiments for diazotrophic cyanobacteria searching for the suspected morphotype, and indeed, we obtained a similar morphotype (ESFC-1) in one enrichment that fixed $^{15}N_2$ in culture. Cyanobacteria in this culture harbored the novel *nifH* sequence type and enabled us to link the *nifH* sequence to the corresponding 16S rRNA gene sequence. These data also enabled us to design specific oligonucleotide probes for CARD-FISH/NanoSIMS experiments to examine ^{15}N incorporation into the ESFC-1 populations in $^{15}N_2$ incubated Elkhorn Slough mats. These studies revealed that ca. 60% of the ESFC-1- related filaments actively incorporated ^{15}N. However, ^{15}N enrichment in these populations was highly variable, with a small fraction of the ESFC-1 community dominating the total ^{15}N incorporation. Large variations in ^{15}N enrichments have previously been noted in NanoSIMS investigation of *Chlorobium phaeobacteroides*-related cells in Lake Cadagno (Switzerland) (Halm *et al.*, 2009), *Aphanizomenon* sp. in the Baltic Sea (Ploug *et al.*, 2010) and shipworm symbionts (Lechene *et al.*, 2007). This variability may be due to spatial heterogeneity in the local environment, such as small-scale variations in nutrient concentrations, diffusion rates, or redox potentials. The microbial mat environment is highly heterogeneous (Des Marais, 2003; Jørgensen *et al.*, 1983), and as filaments experience different environmental conditions, they may differ in their

physiology and N_2 fixation activity, resulting in different ^{15}N enrichment levels. We noticed that even adjacent filaments (Figure 6) differed in the degree of ^{15}N-enrichment, suggesting that the local environment may not be solely responsible for the variable activity of the filaments, but also could be due to strain or species-level variation in ESFC-1 populations. The functional significance of this fine scale variation has been extensively studied in low-diversity acid mine drainage biofilms and wastewater treatment reactors (Denef *et al.*, 2010). The high level of diversity in the ESFC-1 clade at the 16S rRNA gene level supports this hypothesis. These data also illustrate the power of single-cell techniques to reveal functional heterogeneity at the single cell level within closely related populations.

Amplicon pyrosequencing of the V8 region across multiple samples throughout the year 2009 revealed that the ESFC-1-cluster is prevalent in Elkhorn Slough mats. The ESFC-1-related sequences were much less abundant in the total microbial community (DNA) compared to the active community (cDNA), representing an example of a low abundant member of a microbial community that may perform an important ecosystem function, as was observed in NanoSIMS investigations of phototrophic bacteria in a meromictic lake (Halm *et al.*, 2009; Musat *et al.*, 2008). An ESFC-1 related *nifH* gene sequence has also been detected in a mixed *Lyngbya* culture enriched from mats collected in Guerrero Negro, Mexico (Moisander *et al.*, 2007) suggesting that ESFC-1-related cyanobacteria may be present in other microbial mats, but may not have been identified due to low abundance at the time of sampling. The pyrosequencing data were supported by near full-length 16S rRNA genes from the January 2009 cDNA samples, in which ca. 33% of all cDNA sequences (187 total sequences) formed a broad monophyletic clade with a degree of diversity that may represent multiple genera. Deep sequencing of mat samples from other environments may identify ESFC-1 related populations and help broaden our understanding of the ecophysiology of this novel cyanobacterial group.

Interestingly, we found no evidence that *Microcoleus* spp., which are ubiquitous and abundant members of marine microbial mats globally, had an important role in N_2 fixation in the Elkhorn Slough mats. *Microcoleus chthonoplastes*, the type strain for mat-dwelling *Microcoleus* spp., was for many years not thought to be a diazotrophic cyanobacterium. However, the recently completed genome of *M. chthonoplastes* PCC 7420 revealed a *nif*-gene cluster (Bolhuis *et al.*, 2010), which grouped the *nifH* gene with δ-proteobacterial genes in cluster III. In the *nifH* clone library of DNA samples from one of the two October time points (24th October 2009), we identified three sequences that grouped closely with these *Microcoleus chthonoplastes* sequences in cluster III. However, no *nifH* transcripts related to *Microcoleus* were recovered in the Elkhorn Slough samples described in this study. Additionally, very low levels of ^{15}N enrichment (average $^{15}N/^{14}N$ ratio of 0.00382 $\pm 2.4 \times 10^{-5}$ or 40.9 ±6.6‰) in *Microcoleus* spp. filaments were observed in NanoSIMS analysis of $^{15}N_2$ incubated mat samples from October 2009 with the highest $^{15}N/^{14}N$ ratio being 0.00401. This trend was also noted in other samples with nighttime N_2 fixation (December 2007) in which *Microcoleus* spp. filaments had enrichment values very close to natural abundance ($^{15}N/^{14}N$ 0.00368 $\pm 2.4 \times 10^{-5}$, 3.4 ±6.6‰). These low levels of ^{15}N enrichment observed may be due to cross-feeding from active diazotrophs. In contrast, ESFC-1 filaments were observed with $^{15}N/^{14}N$ ratios up to 0.025 (5802‰). While *Microcoleus spp.* may fix N_2 under specialized conditions, we could not find evidence that *Microcoleus* spp. were actively fixing $^{15}N_2$ in the investigated Elkhorn Slough mats. This finding lends further support to our hypothesis that ESFC-1-related cyanobacteria were the dominant active cyanobacterial diazotrophs in the investigated Elkhorn Slough microbial mats.

Acknowledgements

We thank Prof. Castenholz for providing the cyanobacterial strains for probe optimization, Erich D. Fleming, Angela Detweiler, Adrienne Frisbee, Christina Ramon, and Mike Kubo for excellent technical assistance and Stephanie A. Eichorst for comments on the manuscript. We thank Jeff Cann, Associate Wildlife Biologist, Central Region, California Department of Fish and Game for coordinating our access to the Moss Landing Wildlife Area. Funding was provided by the U.S. Department of Energy (DOE) Genomic Science Program under contract SCW1039. Work at LLNL was performed under the auspices of the U.S. Department of Energy at Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. Work at LBNL was performed under the auspices of the U.S. Department of Energy at Lawrence Berkeley National Laboratory under Contract DE-AC02-05CH11231. D.W. was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft).

Supplementary information is available at The SIME Journal's website.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56***:* 1919-1925.

Bebout BM, Fitzpatrick MW, Paerl HW. (1993). Identification of the sources of energy for nitrogen fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. *Appl Environ Microbiol* **59***:* 1495-1503.

Bebout BM, Paerl HW, Bauer J, Canfield DE, Des Marais DJ. (1994). Nitrogen cycling in microbial mat communities: The quantitative importance of N-fixation and other sources of N for primary productivity. In: Stal LJ, Caumette P (eds.). Microbial Mats. Springer-Verlag: Berlin, pp 265–272.

Bebout BM, Paerl HW, Crocker KM, Prufert LE. (1987). Diel interactions of oxygenic photosynthesis and N_2 fixation (acetylene reduction) in a marine microbial mat community. *Appl Environ Microbiol* **53***:* 2353-2362.

Behrens S, Lösekann T, Pett-Ridge J, Weber PK, Ng WO, Stevenson BS *et al*. (2008). Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. *Appl Environ Microbiol* **74***:* 3143-3150.

Bolhuis H, Severin I, Confurius-Guns V, Wollenzien UIA, Stal LJ. (2010). Horizontal transfer of the nitrogen fixation gene cluster in the cyanobacterium *Microcoleus chthonoplastes*. *ISME J* **4***:* 121-130.

Bothe H. (1982). Nitrogen fixation. In: Carr NG, Whitton BA (eds.). Biology of cyanobacteria. Blackwell Scientific Publications: Oxford, pp 87-104.

Canfield DE, Des Marais DJ. (1993). Biogeochemical cycles of carbon, sulfur, and free oxygen in a microbial mat. *Geochim Cosmochim AC* **57***:* 3971-3984.

Chapin T, Johnsin K. (2004). Nitrate sources and sinks in Elkhorn Slough, California: Results from long-term continous in situ nitrate analyzers. *Estuaries* **27***:* 1-13.

Chen YB, Dominic B, Mellon MT, Zehr JP. (1998). Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. strain IMS 101. *J Bacteriol* **180***:* 3598-3605.

Chien YT, Zinder SH. (1996). Cloning, functional organization, transcript studies, and phylogenetic analysis of the complete nitrogenase structural genes (*nifHDK2*) and associated genes in the archaeon *Methanosarcina barkeri* 227. *J Bacteriol* **178***:* 143-148.

D'Amelio D, Cohen Y, Des Marais DJ. (1989). Comparative functional ultrastructue of two hypersaline submerged cyanobacterial mats Guerrero Negro, Baja California Sur, Mexico, and Solar Lake, Sinai, Egypt. In: Cohen Y, Rosenberg E (eds.). Microbial mats: Physiological ecology of benthic microbial communities. Am. Soc. Microbiol.: Washington, D.C, pp 97-113.

Daims H, Brühl A, Amann R, Schleifer KH, Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22***:* 434-444.

Denef VJ, Kalnejais LH, Mueller RS, Wilmes P, Baker BJ, Thomas BC *et al*. (2010). Proteogenomic basis for ecological divergence of closely related bacteria in natural acidophilic microbial communities. *Proc Natl Acad Sci USA* **107***:* 2383-2390.

Des Marais DJ. (1990). Microbial mats and the early evolution of life. *Trends Ecol Evol* **5***:* 140-144.

Des Marais DJ. (2003). Biogeochemistry of hypersaline microbial mats illustrates the dynamics of modern microbial ecosystems and the early evolution of the biosphere. *Biol Bull* **204***:* 160-167.

Finzi-Hart JA, Pett-Ridge J, Weber PK, Popa R, Fallon SJ, Gunderson T *et al*. (2009). Fixation and fate of C and in the cyanobacterium *Trichodesmium* using nanometer-scale secondary ion mass spectrometry. *Proc Natl Acad Sci USA* **106***:* 6345-6350.

Foster RA, Kuypers MM, Vagner T, Paerl RW, Musat N, Zehr JP. (2011). Nitrogen fixation and transfer in open ocean diatom-cyanobacterial symbioses. *ISME J* **5***:* 1484-1493.

Ghosal S, Fallon SJ, Leighton TJ, Wheeler KE, Kristo MJ, Hutcheon ID *et al*. (2008). Imaging and 3D elemental characterization of intact bacterial spores by high-resolution secondary ion mass spectrometry. *Anal Chem* **80***:* 5986-5992.

Halm H, Musat N, Lam P, Langlois R, Musat F, Peduzzi S *et al*. (2009). Co-occurrence of denitrification and nitrogen fixation in a meromictic lake, Lake Cadagno (Switzerland). *Environ Microbiol* **11***:* 1945-1958.

Herbert RA. (1999). Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiol Rev* **23***:* 563-590.

Hunter EM, Mills HJ, Kostka JE. (2006). Microbial community diversity associated with carbon and nitrogen cycling in permeable shelf sediments. *Appl Environ Microbiol* **72***:* 5689-5701.

Jørgensen BB, Revsbech NP, Cohen Y. (1983). Photosynthesis and structure of benthic microbial mats - microelectrode and SEM studies of 4 cyanobacterial communities. *Limnol Oceanogr* **28***:* 1075-1093.

Kim K, Zhang Y, Roberts GP. (1999). Correlation of activity regulation and substrate recognition of the ADP-ribosyltransferase that regulates nitrogenase activity in *Rhodospirillum rubrum*. *J Bacteriol* **181***:* 1698-1702.

Lechene C, Hillion F, Mcmahon G, Benson D, Kleinfeld AM, Kampf JP *et al*. (2006). High-resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry. *J Biol* **5***:* 20.

Lechene CP, Luyten Y, Mcmahon G, Distel DL. (2007). Quantitative imaging of nitrogen fixation by individual bacteria within animal cells. *Science* **317***:* 1563-1566.

Ley RE, Harris JK, Wilcox J, Spear JR, Miller SR, Bebout BM *et al*. (2006). Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. *Appl Environ Microbiol* **72**: 3685-3695.

Li T, Wu TD, Mazéas L, Toffin L, Guerquin-Kern JL, Leblon G *et al*. (2008). Simultaneous analysis of microbial identity and function using NanoSIMS. *Environ Microbiol* **10**: 580-588.

Moisander PH, Morrison AE, Ward BB, Jenkins BD, Zehr JP. (2007). Spatial-temporal variability in diazotroph assemblages in Chesapeake Bay using an oligonucleotide *nifH* microarray. *Environ Microbiol* **9**: 1823-1835.

Moisander PH, Shiue L, Steward GF, Jenkins BD, Bebout BM, Zehr JP. (2006). Application of a *nifH* oligonucleotide microarray for profiling diversity of N_2-fixing microorganisms in marine microbial mats. *Environ Microbiol* **8**: 1721-1735.

Montoya JP, Voss M, Kahler P, Capone DG. (1996). A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl Environ Microbiol* **62**: 986-993.

Musat N, Halm H, Winterholler B, Hoppe P, Peduzzi S, Hillion F *et al*. (2008). A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. *Proc Natl Acad Sci USA* **105**: 17861-17866.

Omoregie EO, Crumbliss LL, Bebout BM, Zehr JP. (2004a). Comparison of diazotroph community structure in *Lyngbya* sp and *Microcoleus chthonoplastes* dominated microbial mats from Guerrero Negro, Baja, Mexico. *FEMS Microbiol Ecol* **47**: 305-318.

Omoregie EO, Crumbliss LL, Bebout BM, Zehr JP. (2004b). Determination of nitrogen-fixing phylotypes in *Lyngbya* sp. and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja California, Mexico. *Appl Environ Microbiol* **70**: 2119-2128.

Orphan VJ, House CH, Hinrichs KU, Mckeegan KD, Delong EF. (2001). Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**: 484-487.

Paerl HW, Fitzpatrick MW, Bebout BM. (1996). Seasonal nitrogen fixation dynamics in a marine microbial mat: Potential roles of cyanobacteria and microheterotrophs. *Limnol Oceanogr* **41**: 419-427.

Paerl HW, Pinckney JL, Steppe TF. (2000). Cyanobacterial-bacterial mat consortia: Examining the functional unit of microbial survival and growth in extreme environments. *Environ Microbiol* **2**: 11-26.

Paerl HW, Prufert LE, Ambrose WW. (1991). Contemporaneous N_2 fixation and oxygenic photosynthesis in the nonheterocystous mat-forming cyanobacterium *lyngbya aestuarii*. *Appl Environ Microbiol* **57**: 3086-3092.

Pernthaler A, Pernthaler J, Amann R. (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68**: 3094-3101.

Ploug H, Musat N, Adam B, Moraru CL, Lavik G, Vagner T *et al*. (2010). Carbon and nitrogen fluxes associated with the cyanobacterium *Aphanizomenon* sp. in the Baltic Sea. *ISME J* **4***:* 1215-1223.

Popa R, Weber PK, Pett-Ridge J, Finzi JA, Fallon SJ, Hutcheon ID *et al*. (2007). Carbon and nitrogen fixation and metabolite exchange in and between individual cells of *Anabaena oscillarioides*. *ISME J* **1***:* 354-360.

Prufert-Bebout L, Garcia-Pichel LJ. (1994). Field and cultivated *Microcoleus chthonoplastes*: the search for clues to its prevalence in marine microbial mats. In: Stal LJ, Caumette P (eds.). Microbial Mats. Springer: Berlin, pp 111–116.

Rossello-Mora R, Amann R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25***:* 39-67.

Severin I, Acinas SG, Stal LJ. (2010). Diversity of nitrogen-fixing bacteria in cyanobacterial mats. *FEMS Microbiol Ecol* **73***:* 514-525.

Severin I, Stal LJ. (2010a). *NifH* expression by five groups of phototrophs compared with nitrogenase activity in coastal microbial mats. *FEMS Microbiol Ecol* **73***:* 55-67.

Severin I, Stal LJ. (2010b). Spatial and temporal variability in nitrogenase activity and diazotrophic community composition in coastal microbial mats. *Mar Ecol-Prog Ser* **417***:* 13-25.

Stal LJ, Grossberger S, Krumbein WE. (1984). Nitrogen fixation associated with the cyanobacterial mat of a laminated microbial ecosystem. *Mar Biol* **82***:* 217-224.

Stal LJ, Krumbein WE. (1981). Aerobic nitrogen-fixation in pure cultures of a benthic marine *Oscillatoria* (cyanobacteria). *Fems Microbiol Lett* **11***:* 295-298.

Steppe TF, Olson JB, Paerl HW, Litaker RW, Belnap J. (1996). Consortial N_2 fixation: A strategy for meeting nitrogen requirements of marine and terrestrial cyanobacterial mats. *FEMS Microbiol Ecol* **21***:* 149-156.

Steunou AS, Jensen SI, Brecht E, Becraft ED, Bateson MM, Kilian O *et al*. (2008). Regulation of *nif* gene expression and the energetics of N_2 fixation over the diel cycle in a hot spring microbial mat. *ISME J* **2***:* 364-378.

Stewart WD, Fitzgerald GP, Burris RH. (1967). In situ studies on N_2 fixation using the acetylene reduction technique. *Proc Natl Acad Sci USA* **58***:* 2071-2078.

Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14***:* 136-143.

Zehr JP, Jenkins BD, Short SM, Steward GF. (2003). Nitrogenase gene diversity and microbial community structure: A cross-system comparison. *Environ Microbiol* **5***:* 539-554.

Zehr JP, Mellon M, Braun S, Litaker W, Steppe T, Paerl HW. (1995). Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. *Appl Environ Microbiol* **61***:* 2527-2532.

Table 1: Taxonomic affiliation of *nifH* sequences from DNA and cDNA in October 2009 Elkhorn Slough microbial mat samples. 21st October 2009, 10:50 pm sample: 93 sequences (DNA) and 92 sequence (cDNA), and 24th October 2009, 3:10 am sample: 75 sequences (DNA) and 88 sequences (cDNA) were analyzed. The percantage of ESFC-1 related sequences within the cyanobacteria of the clone libraries are depicted in parantheses.

	21st October 2009		**24th October 2009**	
	DNA (%)	**cDNA (%)**	**DNA (%)**	**cDNA (%)**
Cluster I	20.4	75.0	29.3	85.2
Cyanobacteria (ESFC-1 related)	3.2 (0%)	52.5 (96%)	4.0 (100%)	23.9 (100%)
γ-proteobacteria	15.0	8.7	16.0	15.9
α-proteobacteria	2.2	-	-	-
ε-proteobacteria	-	14.1	9.3	45.5
Cluster III	79.6	25.0	70.7	14.8
Unaffiliated sequences	32.3	12.0	26.7	5.7
δ-proteobacteria	47.3	13.0	32.0	9.1
Distantly related to Clostridia	-	-	12.0	-

Figure Legends

Figure 1: Nitrogenase activity measured in a diel cycle on 21st/22nd October 2009 of Elkhorn Slough microbial mats. Acetylene reduction assays were conducted with entire mat cores of 10 mm diameter and 10 mm thickness.

Figure 2A and B. Representative NanoSIMS secondary electron (e^-) and nitrogen isotope ratio ($^{15}N/^{14}N$) images of Elkhorn Slough microbial mats from October 2009 after incubation with $^{15}N_2$ in the dark. Small filamentous cyanobacteria fixing nitrogen incorporated the ^{15}N tracer (clarified by arrows in 2B). The color scale bar represents $^{15}N/^{14}N$ ratios (0.00367 corresponds to natural abundance). Scale bars represent 5 µm.

Figure 3A: Maximum likelihood tree of cyanobacterial deduced dinitrogenase reductase (*nifH*) sequences from Elkhorn Slough microbial mats collected in October 2009 (bolded) and their closest relatives. The novel lineage represents 37% of the sequences recovered from cDNA clone libraries; DQ821979 is the only closely related sequence. Bootstrap values calculated with the PhyML algorithm are displayed in the tree. Scale bar represents 10% estimated sequence divergence.
B: Maximum likelihood tree of the ESFC-1 *nifH* phylotype (bolded) grouping with the cyanobacterial *nifH* sequences from Elkhorn Slough microbial mats and DQ821979. Representatives of OTU_{97} are depicted along with the number of sequences within each OTU in parentheses. Bootstrap values calculated with the PhyML algorithm are displayed in the tree. Scale bar represents 10% estimated sequence divergence.

Figure 4A: Maximum likelihood tree of the cyanobacterial 16S rRNA from the ESFC-1 enrichment culture (bolded) with its closest relatives, linking the new *nifH* sequence cluster

from Elkhorn Slough mats to a nearly full-length 16S rRNA gene sequence. Bootstrap values calculated with the PhyML algorithm are displayed in the tree. Scale bar represents 10% estimated sequence divergence.

Inset micrograph depicts ESFC-1 filaments from the unicyanobacterial culture. Scale bar represents 10 μm.

B: Maximum likelihood tree of nearly full-length 16S rRNA sequences from ESFC-1 (bolded) together with the related sequences from Elkhorn Slough mats collected in January 2009, illustrating the diversity of this novel group. Forty-six percent of the sequences from 12th January 2009 ("SIUS", cDNA sample, 9:00 pm, 151 of 329 sequences) and 14.8% of the sequences from 13th January 2009 ("SIUT", cDNA sample, 7:00 am, 36 of 243 sequences) grouped monophyletically with the 16S rRNA gene sequence type from ESFC-1. OTU_{98} representatives of Elkhorn Slough sequences along with the number of sequences within an OTU in parentheses are depicted in the tree. Bootstrap values calculated with the PhyML algorithm are displayed in the tree. Scale bar represents 10% estimated sequence divergence.

Figure 5. Relative abundance of ESFC-1 affiliated 16S rRNA V8 pyrotag amplicons in 2009. Sequences affiliated with ESFC-1 were found in samples from 13th January (7:00 am; total of 20,861 sequences in DNA and 9,510 sequences in cDNA samples), 1st July (4:00 am; 15,832 DNA and 8,876 cDNA sequences), 19th August (1:15 am; 6,383 DNA and 10,774 cDNA sequences), 16th September (12:00 pm; 8,289 DNA sequences), 21st October (10:50 pm; 18,458 DNA and 5,731 cDNA sequences) and 13th November (12:00 am; 6,606 DNA and 5,209 cDNA sequences).

Figure 6. NanoSIMS and epifluorescence images of filaments stained with the ESFC-1-specific CARD-FISH probe ESFC-1_177 after incubation of mat cores with $^{15}N_2$. (A)

Epifluorescence image of DAPI stained cells and filaments. (B) Epifluorescence image of filaments stained with ESFC-1-specific probe ESFC1_177. (C) Secondary electron (e^-) image of the same region. (D) Nitrogen isotope ratio ($^{15}N/^{14}N$) image depicting an ESFC1_177-stained filament highly enriched in ^{15}N ($^{15}N/^{14}N$ 0.025). Scale bar represents 5 μm.

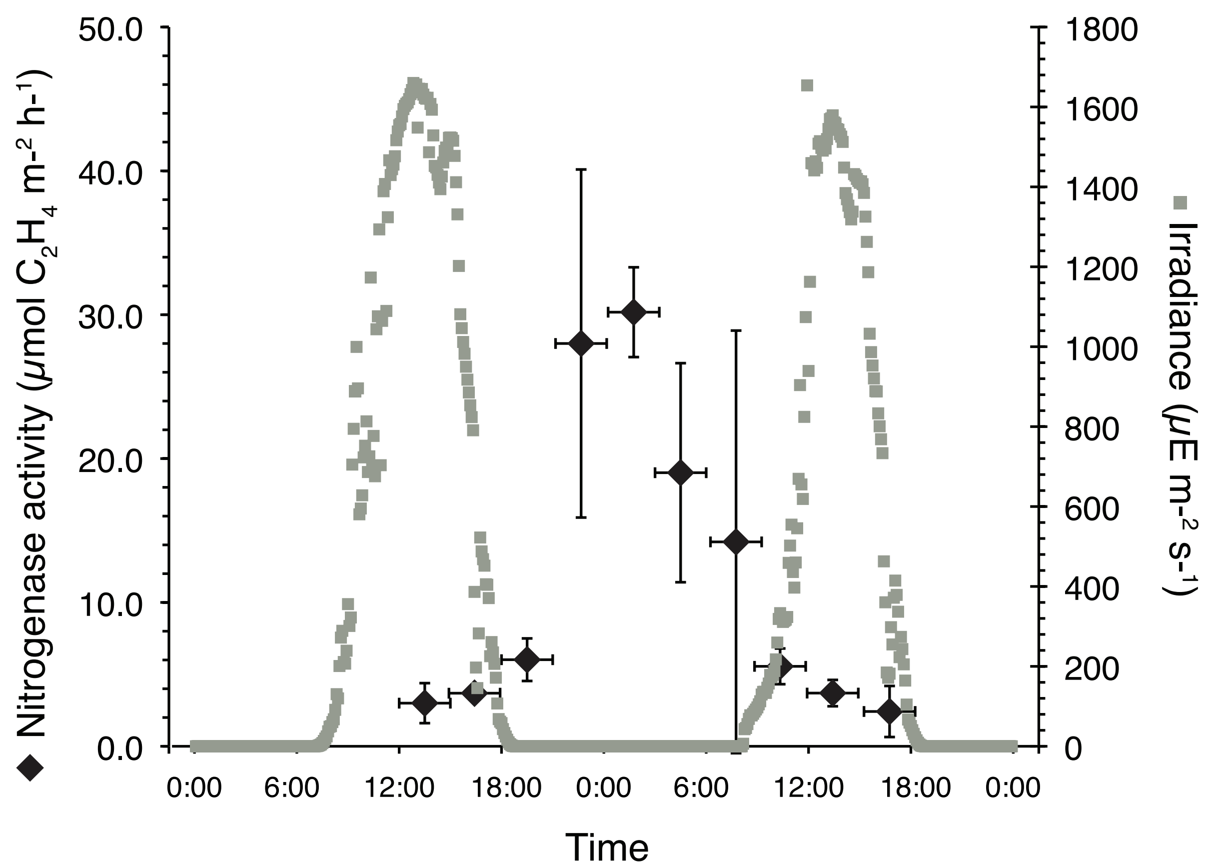
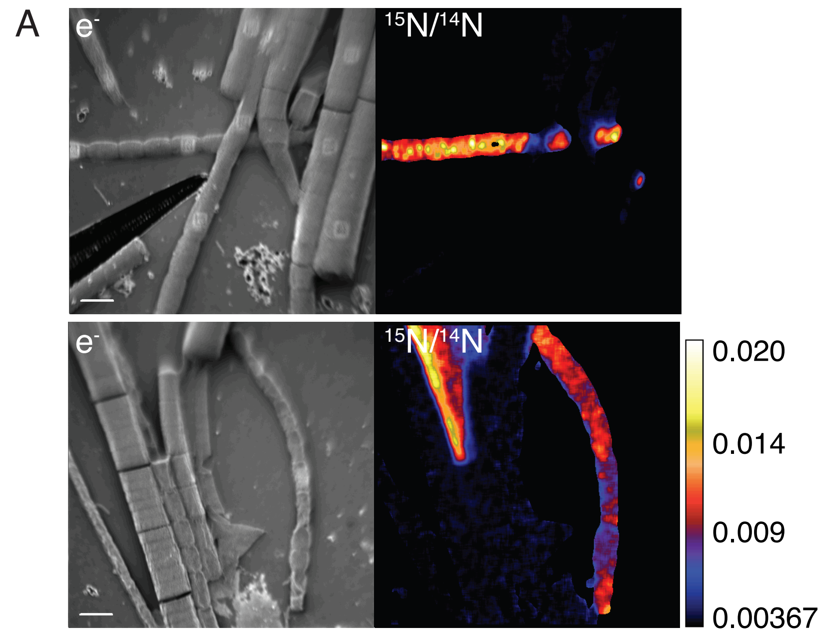


Figure 1.

A



B

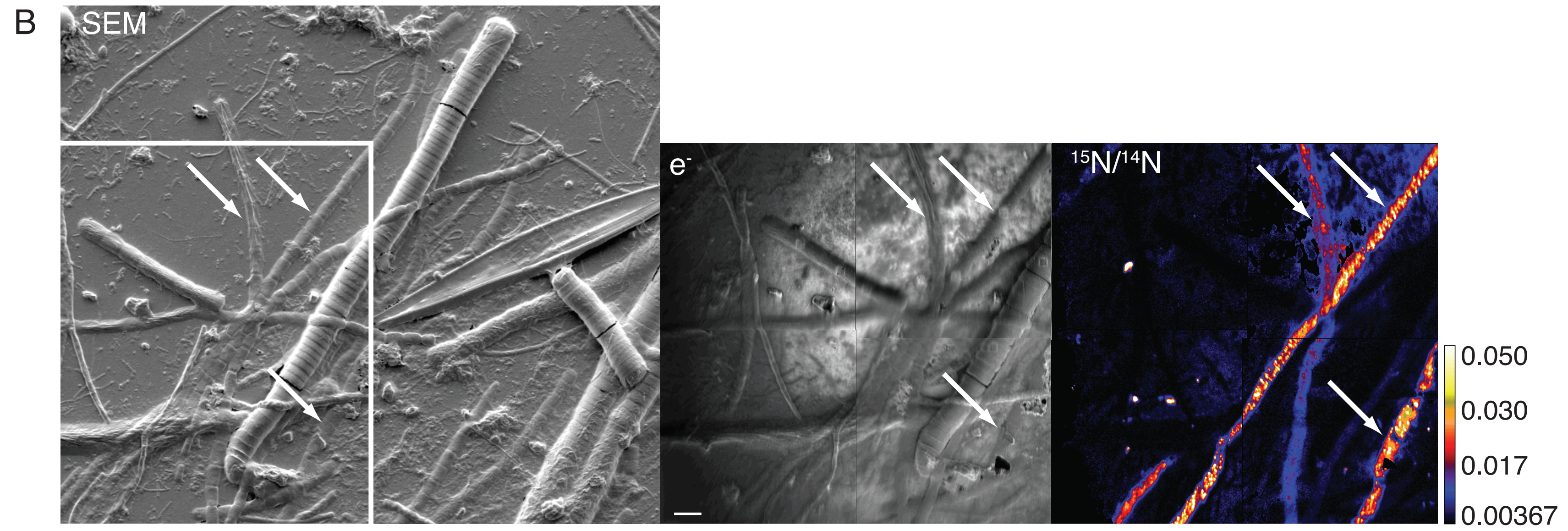


Figure 2.

A

uncultured Elkhorn Slough cyanobacteria and DQ821979
99
marine sponge symbiont (ACF72162)
marine sponge symbiont (ACF72159)
Myxosarcina sp. ATCC 29377 (U73133)
Xenococcus sp. PCC 7305 (U73135)
marine sediment (AAY85416)
microbial mat, Guerrero Negro (DQ338040)
marine sponge symbiont (ACF72226)
Halothece sp. MPI 96P605 (AY485152)
Lyngbya lagerheimii UTEX 1930 (L15550)
Calothrix sp. MCC-3A (DQ531692)
87
Scytonema sp. FGP-7A (DQ531674)
98
Nodularia spumigena FL2f (AY493375)
Nostoc commune SC (DQ531670)
Oscillatoria sp. PCC 6506 (AY768417)
Calothrix sp. ATCC 27914 (U73130)
Leptolyngbya boryana M101 (D00666)
Leptolyngbya nodulosa UTEX 2910 (FJ773236)
Pseudanabaena sp. PCC7403 (U73136)
Lyngbya wollei (EF397757)
Anabaena variabilis ATCC 29413 (AY768410)
to outgroup
97
Cyanothece sp. WH 8902 (AY620240)
Gloeocapsa sp. KO38CU6 (AB264105)
0.10

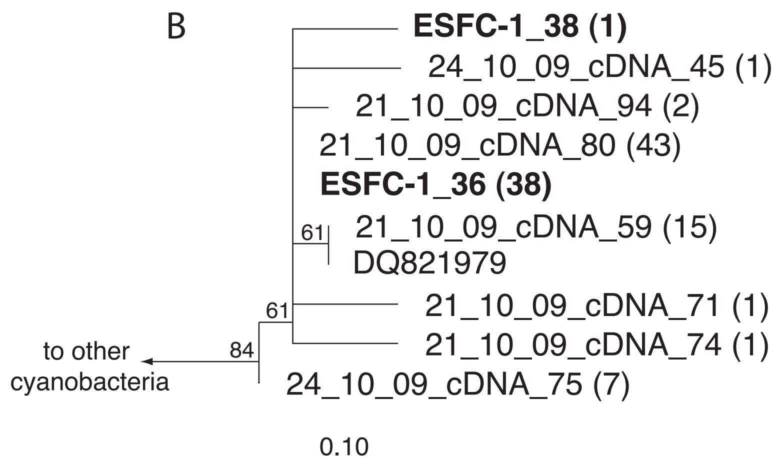
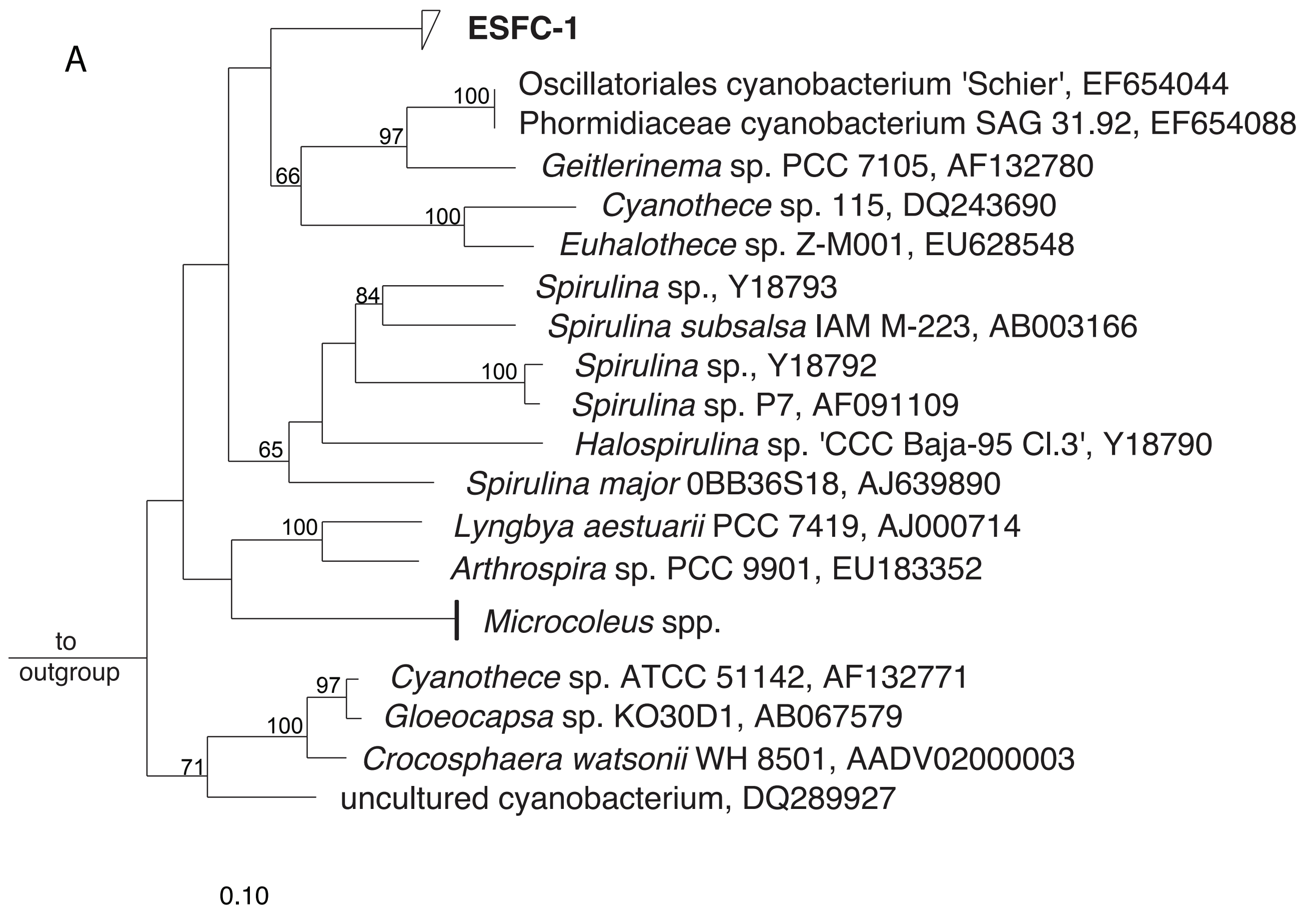
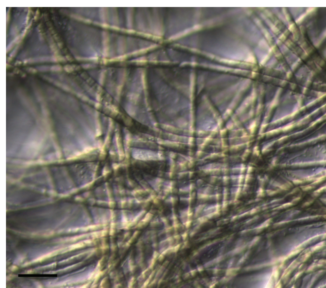


Figure 3.





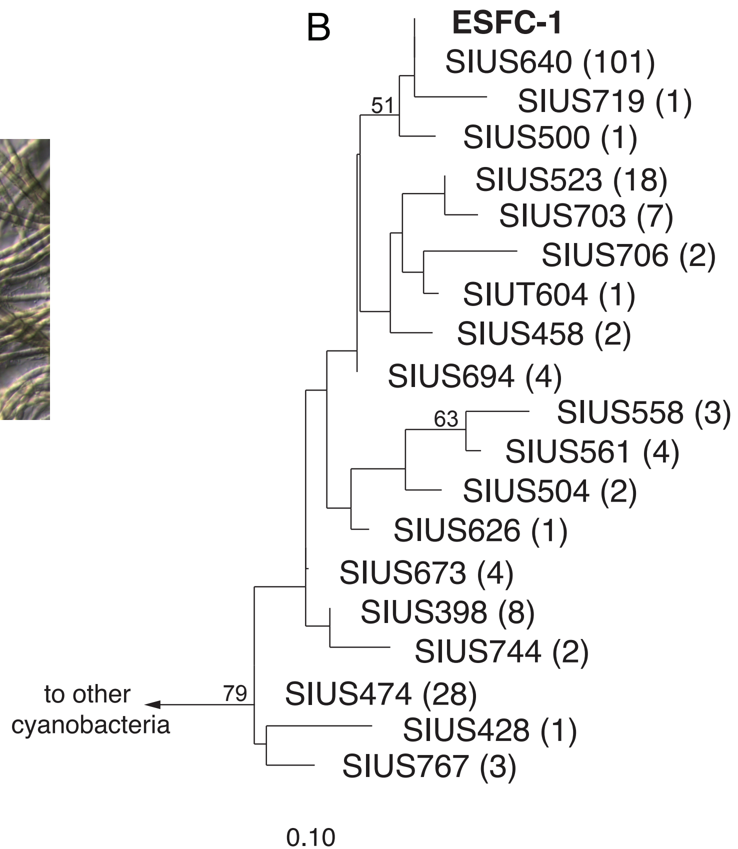
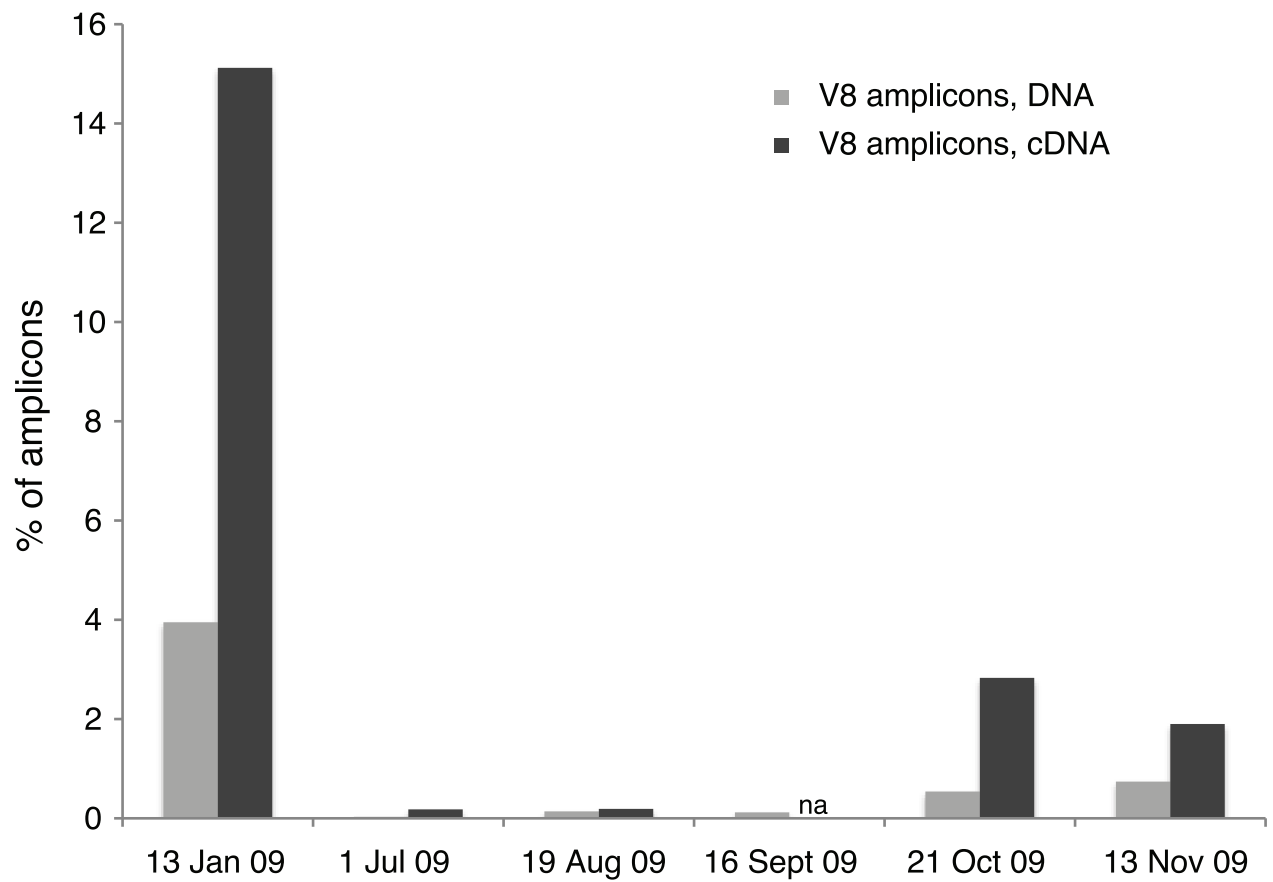


Figure 4.



na: not analyzed

Figure 5.

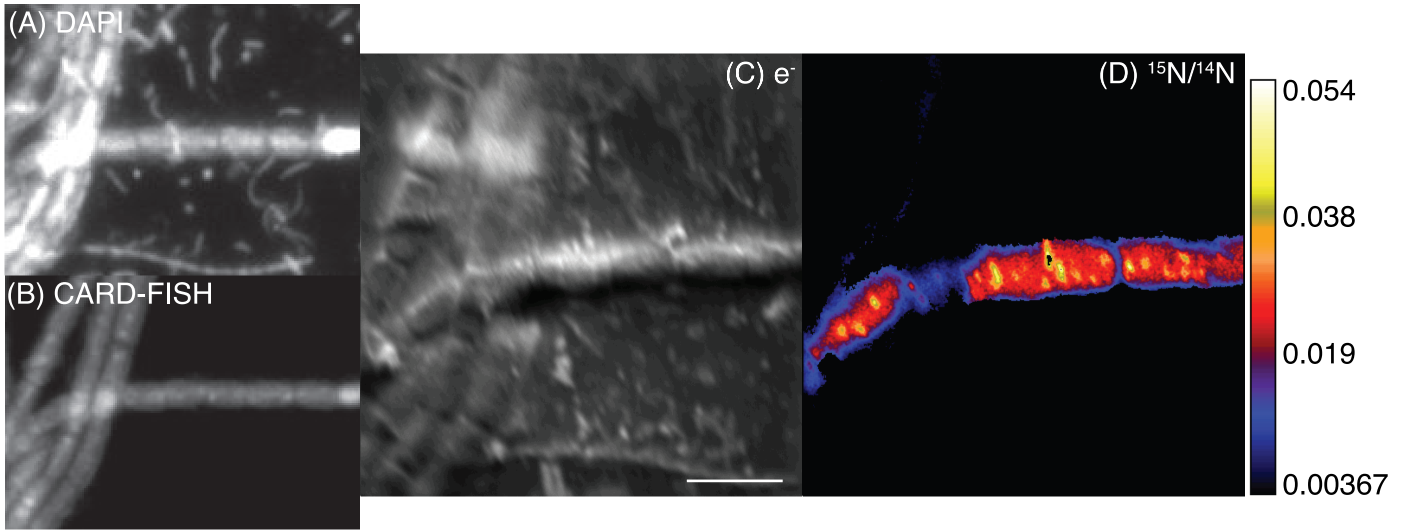


Figure 6.

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor The Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or The Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or The Regents of the University of California.

Ernest Orlando Lawrence Berkeley National Laboratory is an equal opportunity employer.