In situ analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats

Anne-Soisig Steunou*[†], Devaki Bhaya*, Mary M. Bateson[‡], Melanie C. Melendrez[‡], David M. Ward[‡], Eric Brecht[§], John W. Peters[§], Michael Kühl[¶], and Arthur R. Grossman*

*Department of Plant Biology, The Carnegie Institution, 260 Panama Street, Stanford, CA 94305; Departments of [‡]Land Resources and Environmental Science and [§]Chemistry and Biochemistry, Montana State University, Bozeman, MT 59715; and [¶]Marine Biological Laboratory, Institute of Biology, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør, Denmark

Genome sequences of two *Synechococcus* ecotypes inhabiting the Octopus Spring microbial mat in Yellowstone National Park revealed the presence of all genes required for nitrogenase biosynthesis. We demonstrate that *nif* genes of the *Synechococcus* ecotypes are expressed *in situ* in a region of the mat that varies in temperature from 53.5°C to 63.4°C (average 60°C); transcripts are only detected at the end of the day when the mat becomes anoxic. Nitrogenase activity in mat samples was also detected in the evening. Hitherto, N_2 fixation in hot spring mats was attributed either to filamentous cyanobacteria (not present at >50°C in these mats) or to heterotrophic bacteria. To explore how energy-generating processes of the *Synechococcus* ecotypes track natural light and O_2 conditions, we evaluated accumulation of transcripts encoding proteins involved in photosynthesis, respiration, and fermentation. Transcripts from photosynthesis (*cpcF*, *cpcE*, *psaB*, and *psbB*) and respiration (*coxA* and *cydA*) genes declined in the evening. In contrast, transcripts encoding enzymes that may participate in fermentation fell into two categories; some (*ldh*, *pdhB*, *ald*, and *ackA*) decreased in the evening, whereas others (*pflB*, *pflA*, *adhE*, and *acs*) increased at the end of the day and remained high into the night. Energy required for N_2 fixation during the night may be derived from fermentation pathways that become prominent as the mat becomes anoxic. In a broader context, our data suggest that there are critical regulatory switches *in situ* that are linked to the diel cycle and that these switches alter many metabolic processes within the microbial mat.

Synechococcus | diel regulation | fermentation | nitrogenase | Yellowstone National Park

Microbial mats are highly structured microbial communities that populate a variety of environments (1). Cyanobacteria are key players for the biogeochemistry of photosynthetic mats as they are the predominant primary producers in these systems, and many can reduce molecular nitrogen (N_2) to ammonium. Cyanobacterial energy generation within microbial mats involves photosynthesis and aerobic respiration during the day and mainly fermentative metabolism during the night, when such mats become anoxic.

The microbial mat communities of alkaline siliceous hot springs in Yellowstone National Park have been studied in great detail (2–4). The mats are strictly prokaryotic in composition and represent stable communities containing photoautotrophic, photoheterotrophic, chemoautotrophic, and heterotrophic organisms. At temperatures between 50°C and 72°C, the 1-mm thick upper green layer of the mat contains unicellular cyanobacteria (*Synechococcus* spp.) (2, 4) embedded in a matrix of exopolymers and filamentous *Chloroflexus/Roseiflexus*-type phototrophic bacteria (5, 6). Thermophilic *Synechococcus* are an ecologically diverse group of organisms distributed along horizontal thermal and vertical light and O_2 gradients in the mats (3, 4, 7, 8). Recently, genomes of two thermophilic *Synechococcus* isolates (designated *Synechococcus*

OS-A and OS-B′), representing ecologically specialized populations in the microbial mat of Octopus Spring (9), were sequenced (A.-S.S., D.B., M.M.B., M.C.M., D.M.W., M.K., A.R.G., J. Heidelberg, and F. Cohan, unpublished data). Surprisingly, both genomes harbor a conserved *nif* gene cluster, suggesting that these organisms can fix N_2. Hitherto, filamentous cyanobacteria growing at <50°C were the only thermophilic cyanobacteria implicated in N_2 fixation in geothermal systems (10, 11). Few diazotrophic bacteria are thermophilic, and the highest temperature at which nitrogenase activity has been observed (64°C) was in the thermophilic, anaerobic archaeon *Methanococcus thermolithotrophicus* (12).

The arrangements of *nif* genes on the *Synechococcus* OS-A and OS-B′ chromosomes are essentially identical (the accession nos. for OS-A and OS-B′ genes are CP000239 and CP000240, respectively), encoding the catalytic nitrogenase subunits and the subunits involved in nitrogenase maturation/stability and in synthesis of the FeMo-cofactor (13–18). Nitrogenase is extremely O_2 sensitive (19), and the problem of nitrogenase O_2 sensitivity is acute in cyanobacteria as they perform oxygenic photosynthesis. To solve this problem, many filamentous cyanobacteria develop heterocysts, differentiated cells that exclude O_2 (20, 21), have reduced linear photosynthetic electron transport (22), and have increased respiratory activity (23); these features of heterocysts help establish an environment favorable for N_2 fixation (24). Nonheterocystous diazotrophic cyanobacteria can temporally (25) and spatially (26) separate photosynthesis from N_2 fixation. For example, photosynthetic activity may be high during the day when cells harvest light energy for CO_2 fixation, whereas N_2 fixation occurs in the evening when there is little photosynthetic activity and respiration consumes most intracellular O_2. Photosynthetic, respiratory, and nitrogenase activities in some cyanobacteria may also be under circadian control (27, 28).

During the day, *Synechococcus* spp. in the Octopus Spring mat synthesize and excrete photorespiratory glycolate, which can feed heterotrophic and photoheterotrophic bacteria (29). Most of the photosynthate synthesized during the day is stored as glycogen, which can be converted to acetate during the night (30). Acetate that accumulates in the mat overnight can be assimilated by heterotrophic and photoheterotrophic bacteria as the sun rises (31, 32). This observation is consistent with the finding that many cyanobacteria can ferment polyglucose under anoxic conditions (33). A pronounced switch from daytime hyperoxic conditions in the mat to anoxia as evening approaches suggests that marked

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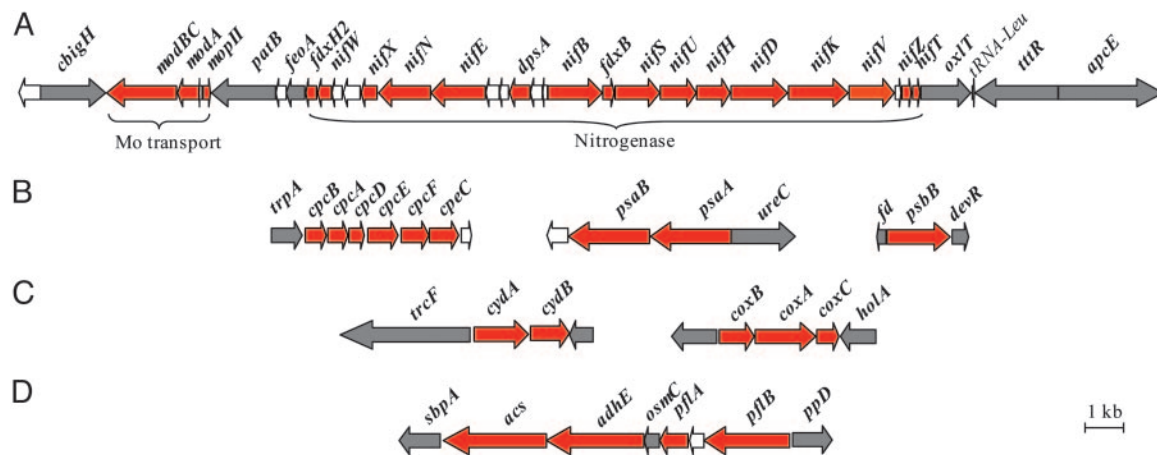
Fig. 1. Clusters of genes involved in nitrogenase synthesis and energy metabolism in *Synechococcus* OS-B′. Red arrows, genes involved in N₂ fixation (*A*), photosynthesis (*B*), respiration (*C*), and fermentation (*D*); gray arrows, genes not associated with these functions; white arrows, genes encoding hypothetical proteins. The functions of many of the different gene products are described in Table 1.

changes in cellular metabolism track the diel cycle and light conditions of the environment.

Here we explore *in situ* conditions that lead to the accumulation of nitrogenase transcripts and activity in *Synechococcus* populations inhabiting the alkaline hot springs of Yellowstone National Park. We also examine *in situ* expression of genes encoding proteins associated with key energy-generating processes in cyanobacteria, including photosynthesis, respiration, and fermentation. The results demonstrate that the levels of transcripts from many of these genes, as well as nitrogenase activity, are controlled over the diel cycle, tracking both oxic and light conditions.

Results

Nitrogenase Gene Cluster. Genes encoding proteins for N₂ fixation are clustered on the genomes of *Synechococcus* OS-A and OS-B′ (Fig. 1*A*). Genes required for nitrogenase biogenesis are on a 23-kbp region (from *modBC* to *nifT*), which is presented as part of a larger 32.5-kbp genomic region (Fig. 1*A*; from *cbigH* to *apcE*) that exhibits identical gene order in *Synechococcus* OS-A and OS-B′. Deduced amino acid sequences of the nitrogenase polypeptides of *Synechococcus* OS-A and OS-B′ are between 98% and 100%

identical (Table 2, which is published as supporting information on the PNAS web site), which is very high relative to overall identity (88%) among putative orthologs in the two *Synechococcus* ecotypes. The best identities of the *Synechococcus* OS-A and OS-B′ Nif polypeptides are mostly with putative orthologs present in *Anabaena*, *Nostoc*, and *Fischerella* strains, ranging from just >50–86% (Table 2).

Nitrogenase Transcripts. To examine *in situ* expression of *nif* genes, we used primer pairs [for RT-PCR and quantitative PCR (qPCR)] that would specifically detect *nifH*, *nifD*, and *nifK* transcripts (Table 1). Other primer pairs were designed to detect transcripts encoding photosynthesis, respiration, and fermentation proteins; the genes for some of these transcripts are present in small gene clusters (Fig. 1 *B–D*). Many of the photosynthesis, respiratory, and fermentation genes are not as similar between *Synechococcus* OS-B′ and OS-A ecotypes as are the *nif* genes (Table 1; e.g., *ald* and *cpcF*). However, primer pairs that were designed specifically for *Synechococcus* OS-B′ (except for cpcF primers) are likely to anneal to transcripts from other related *Synechococcus* ecotypes (3) within the mat.

Samples were collected from the Octopus Spring mat at six

Table 1. Primers used for RT-PCR and qPCR analysis of *Synechococcus* OS-B′ transcript levels

Gene	Function	Forward primer	Identity, %	Reverse primer	Identity, %
nifH	nitrogenase iron protein	GGAAGAAAACGGAGCCTAC	94.7	CGCCAGAGTAGGCGTATTTC	100
nifD	nitrogenase molybdenum-iron protein α chain	GCAGTGGCCAAGAGAGTTTC	100	CAATCAGGGCCACATCATAAG	100
nifK	nitrogenase molybdenum-iron protein β chain	CTTCTGCAGTGCAAGCAAAC	100	CATGGACAAAGGGCAGAGTTC	100
psbB	PSII chlorophyll binding protein	CTGCCCAAGTCTCTGCTCTTC	90	CAGGATAACCGGGAAGGTCTC	85
psaB	PSI reaction center protein	CACGTAGGCTGGGACAACTTC	95.2	GTCCGTCAGCCACAGAGAC	100
cpcE	phycocyanobilin lyase α subunit	CTGGGAAGCCCTTATCGAG	84	GTCGTAGGCTGGAGTTGGAG	75
cpcF	phycocyanobilin lyase β subunit	CTCAACTGGCAGCAGAATTG	85	CGTCAATATGGCTCAGCAAG	55
coxA	cytochrome *c* oxidase subunit I	GAACTTCCTGCTGAAGCTTG	80	GTAAAGGCACCGTTGTACCG	90
cydA	cytochrome *d* quinol oxidase subunit I	CAACCCCTTCATGGTCAACAG	80	CGCTCAAATGGCCGATATAC	90
pflB	pyruvate formate lyase	GGCGTTTTTGACGCCTATAC	85	GGTCTCTTCGTCCATCGTGTC	94.1
adhE	CoA-linked acetaldehyde dehydrogenase-alcohol dehydrogenase	CAATGCCCTGATGATTACCC	100	GTTTCCGCTTGAGTTCTTCG	80
acs	acetyl-CoA synthase	GAAAAAGTGCAGCAAACCCTTG	68	GGTGAGGGTGTGGGAGTAGAG	100
pflA	pyruvate formate lyase activating enzyme	GCACACGGCTTTGGATACTTC	90	GGAACCAGCACAAAACGAATC	100
ldh	L-lactate dehydrogenase	CCTTTTGGTGGTGAGCAATC	70	CCACCTCGCTATCTCCATG	94.7
pdhB	pyruvate dehydrogenase β subunit	GGAGAAGATGTCGGCCATTA	85	CATTGTTGGCAATTTGGTTG	95
ald	aldehyde dehydrogenase	CAGATCTGATCGAGGCTCGTG	80	GCGAAAGAGGGAGGTGTAGC	85
ackA	acetate kinase	GAGCTTAGATCGGCTGGATG	100	CAGTCCTCTTGGGCTGAGATC	85

The transcripts analyzed encode proteins associated with nitrogenase, photosynthesis, respiration, and fermentation. The gene name, protein name/function, primer sequence, and identities (%) of primer sequences to orthologous sequences of *Synechococcus* OS-A are presented. The accession nos. for all OS-A and OS-B′ genes are CP000239 and CP000240, respectively.
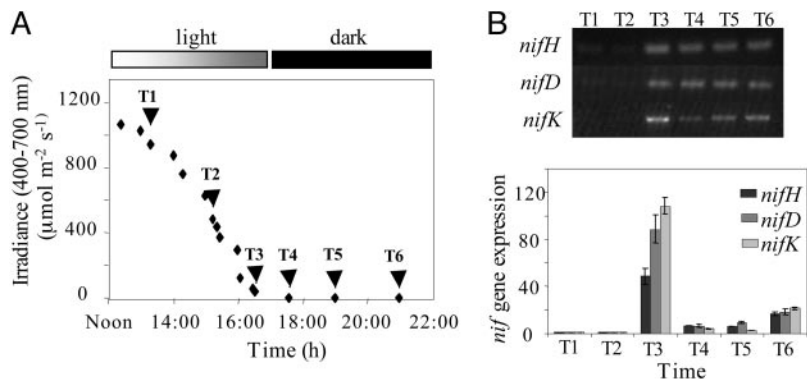
Fig. 2. Accumulation of *nif* transcripts in Octopus Spring mat samples during an afternoon to nighttime transition on Nov. 5, 2004. (*A*) Light intensity at the sampling times T1 (13:40), T2 (15:24), T3 (16:35), T4 (17:36), T5 (19:00), and T6 (21:00). (*B*) RT-PCR (*Upper*) and qPCR (*Lower*) analyses of nitrogenase transcript levels. Samples were from the top 2 mm in a region of the mat where the temperature ranged from 53.5°C to 63.4°C. Error bars on graphs indicate the mean ± SD.

different time points and photon irradiances on Nov. 5, 2004 (Fig. 2*A*). Total RNA from each sample was used to evaluate *nifH*, *nifD*, and *nifK* transcript levels by RT-PCR (Fig. 2*B Upper*). The RNA preparation exhibited no detectable contamination with genomic DNA, as assessed by performing amplification reactions in the absence of reverse transcriptase (data not shown). Almost no transcript from *nifH*, *nifD*, or *nifK* was detected at time point 1 (T1) and T2, when ambient light levels were high. Transcripts for all three *nif* genes were detected at T3, T4, T5, and T6; at T3, the irradiance had declined to ≈50 μmol·m^{-2}·s^{-1}, whereas, at T4–T6, it approached 0. Quantitative analyses of transcript levels by qPCR (Fig. 2*B Lower*) showed that *nif* transcripts were barely detected at T1 and T2, increased sharply at T3 (≈40- to ≈120-fold the T1 value), and then declined as the evening progressed (to between 5- and 25-fold the T1 value). These findings suggest that the *nif* genes are only expressed when light levels are low and the mat is near anoxic.

Nitrogenase Activity. To determine whether nitrogenase activity also increased in the mat toward evening, we performed *in situ* nitrogenase assays by using the top 2 mm of core samples from Octopus Spring (June 2005) and simultaneously monitored O$_2$ profiles and *nif* transcript levels (Fig. 3). From 16:00 to 18:00 h, when incident irradiance was mostly above 900 μmol·m^{-2}·s^{-1}, nitrogenase activity was not detected and high rates of cyanobacterial photosynthesis caused hyperoxic conditions in the upper part of the mat. As photon irradiance declined, O$_2$ concentration in the mat decreased dramatically. Irradiance at time 20:05 h was only ≈31 μmol·m^{-2}·s^{-1}, whereas O$_2$ penetration was <0.1 mm, and high nitrogenase activity (relative to daytime levels) was detected in mat samples. Sampling at later times showed that the mat remained anoxic during the night, and nitrogenase activity was sustained. Transcripts for the nitrogenase subunits were only detected as the light levels declined and the mat became anoxic, consistent with the results presented in Fig. 2; almost no *nif* transcripts were detected at 18:00 h, whereas high transcript levels were observed at 20:30 and 23:00 h (Fig. 3 *Bottom*). Nitrogenase activity was only detected at the time when nitrogenase transcripts began to accumulate. Transcripts encoding photosynthetic proteins such as PsaB showed an opposite trend relative to *nif* transcripts; they were high during the day and low in the evening (Fig. 3 *Bottom*). Nitrogenase activity and cyanobacterial *nif* gene expression were also detected in mat samples collected from 60–61°C sites in Mushroom Spring (a nearby spring with similar mat communities as in Octopus Spring) during the evening (A.-S.S., D.B., D.M.W., E.B., J.W.P., M.K., A.R.G., and S. Jensen, unpublished data).

Transcripts for Proteins Involved in Energy Metabolism. Nitrogenase requires 16 ATP per N$_2$ reduced. The ATP used for N$_2$ fixation in mats could be from photosynthesis, which would still be occurring as light levels decline and the consumption of O$_2$ by respiration

becomes more rapid than O$_2$ production. Fermentation of carbohydrates that accumulate as a consequence of photosynthetic CO$_2$ fixation in the day may also generate energy during the evening.

We used specific primer pairs (Table 1) to evaluate *in situ* transcript levels from genes encoding proteins important for photosynthesis, respiration, and fermentation during the November 2004 experiment (Fig. 4 *A–D*). Based on RT-PCR, transcripts for photosynthetic proteins showed an opposite trend relative to *nif* transcripts (compare Fig. 4*A* with Fig. 2*B*); they were high during the day and low in the evening. For genes associated with photosynthesis (*cpcF* and *cpcE*, phycocyanin lyase; *psaB*, subunit of PS I reaction center; and *psbB*, PS II antenna chlorophyll binding
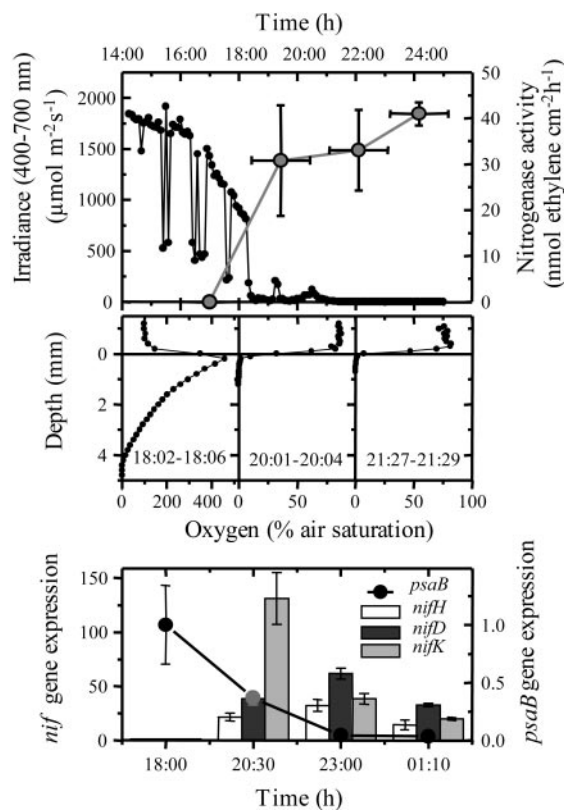
Fig. 3. *In situ* nitrogenase activity, photon irradiance, oxygen penetration, and transcript abundance measured in the Octopus Spring microbial mat on June 23, 2005. (*Top*) Incident photon irradiance (small black circles) and nitrogenase activity (large gray circles) as a function of time of day. Vertical bars represent error, whereas horizontal bars indicate incubation period. (*Middle*) Depth distribution of oxygen concentration in the microbial mat at 934, 29.3, and 0.0 μmol·m^{-2}·s^{-1}, respectively. (*Bottom*) qPCR examination of *nifHDK* and *psaB* transcript levels at times indicated. Error bars are as in Fig. 2.
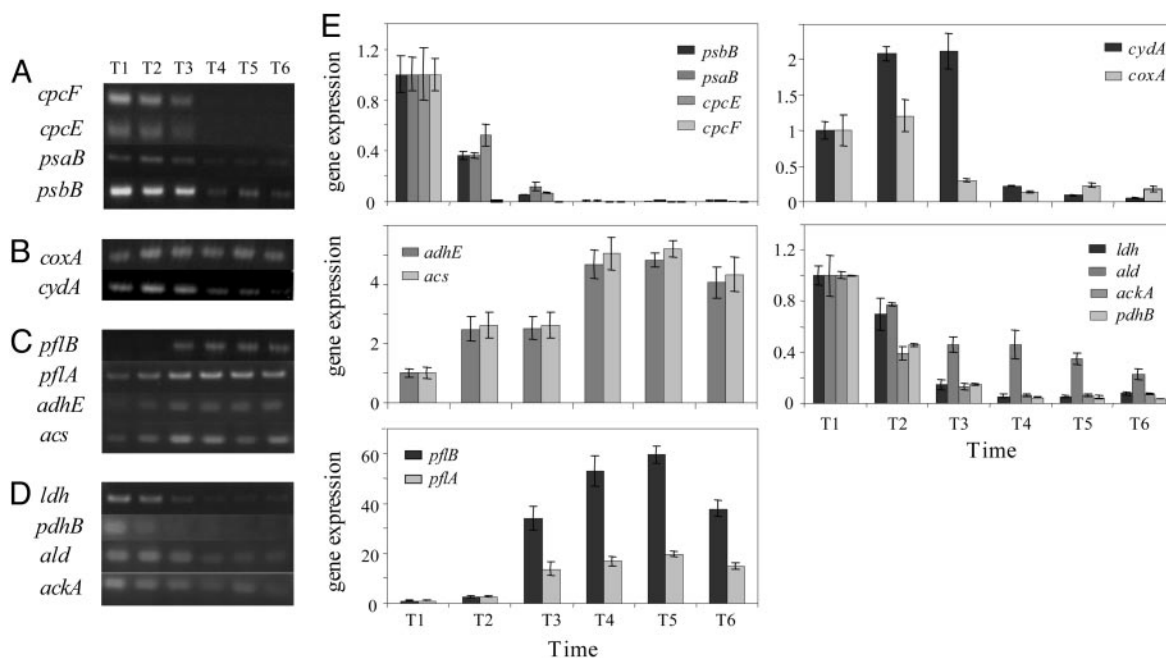
Fig. 4. Transcript levels in Octopus Spring mat samples collected on Nov. 5, 2004 at sampling times T1–T6, as in Fig. 2. RT-PCR was performed to examine accumulation of transcripts from genes involved in photosynthesis (*cpcF*, *cpcE*, *psaB*, and *psbB*) (A), respiration (*coxA* and *cydA*) (B), and fermentation (*pflB*, *pflA*, *adhE* and *acs* transcript increase during the night, whereas *ldh*, *pdhB*, *ald* and *ackA* transcripts decrease during the night) (C and D). (E) Quantification of transcript levels analyzed by RT-PCR (A–D) by using qPCR. Samples were from the top 2 mm in a region of the mat where the temperature ranged from 53.5°C to 63.4°C. Error bars are as in Fig. 2.

protein), transcripts were high at T1 and T2, decreased somewhat by T3, and then became barely detectable by T4. However, the *psbB* transcript was still detectable in mat samples collected in the evening (Fig. 4*A*, T4–T6).

Transcripts for proteins involved in respiration (*coxA*, for subunit I of cytochrome aa_3-type cytochrome *c* oxidase and *cydA*, for subunit I of the cytochrome quinol oxidase) appeared to remain nearly constant (*coxA*) or to decline slightly in the evening (*cydA*), based on RT-PCR (Fig. 4*B*). In contrast, two distinct patterns of gene expression were associated with putative fermentation genes (1, 33) (see Table 1). Transcripts encoding pyruvate formate lyase (*pflB*), pyruvate formate lyase activating enzyme (*pflA*), CoA-linked alcohol/acetaldehyde dehydrogenase (*adhE*), and acetyl-CoA synthase (*acs*) increased during the evening when the mat was anoxic (Fig. 4*C*); genes encoding these transcripts are colocalized on the genomes (Fig. 1*D*), and all or some may be cotranscribed. Transcripts for lactate dehydrogenase (*ldh*), pyruvate dehydrogenase (*pdhB*), and to some extent aldehyde dehydrogenase (*ald*) and acetate kinase (*ackA*) (genes for these proteins are not clustered on the genome) declined during the night (Fig. 4 *C* and *D*).

We also used qPCR to quantify transcript abundance. As observed by RT-PCR (Fig. 4), transcripts for photosynthesis genes (*psbB*, *psaB*, *cpcE*, and *cpcF*) were high during the day relative to the evening (Fig. 4*E Top Left*); by T4, the transcripts for these genes were barely detectable. Transcripts from genes encoding proteins involved in respiration (*coxA* and *cydA*) also declined during the evening (Fig. 4*E Top Right*). It was not clear from the RT-PCR results (Fig. 4*B*) that the transcript encoding CoxA decreased as light levels declined, but the qPCR results showed an ≈3-fold decline in transcript abundance at all times after 16:35 h (T3); because of amplification saturation, the RT-PCR does not report true abundance differences. In contrast, and in accord with RT-PCR results, transcripts for the linked set of fermentation genes (*pflB*, *pflA*, *adhE*, and *acs*; Fig. 4*E Bottom Left* and *Middle Left*) increased in the evening, whereas transcripts for the others (*ldh*, *ald*, *pdhB*, and *ackA*; Fig. 4*E Middle Right*) declined. For the former set

of fermentation-associated transcripts, once the transcript levels increased, they remained high into the night.

Discussion

The combined use of genomic information, gene expression analysis, and ecophysiological methods has provided insights into the ecology of *Synechococcus*-dominated hot spring mats. We demonstrated that the *Synechococcus nif* genes are expressed *in situ* as the mat becomes anoxic under declining photon irradiance in the late afternoon and that N₂ fixation increases in parallel with increasing transcript levels. N₂ fixation detected in earlier studies was attributed to heterocystous cyanobacteria, which only live in the lower temperature regions of the mat, or to heterotrophic bacteria (10, 12, 34). Although we cannot exclude the participation of heterotrophic bacteria in N₂ fixation at 53.5°C–63.4°C in this mat, our results demonstrate that expression of *Synechococcus nif* genes occurs in concert with considerable levels of nitrogenase activity. These results strongly suggest that the unicellular *Synechococcus* ecotypes fix N₂ at elevated temperatures within the hot spring mats. Irradiance levels of <50 μmol·m⁻²·s⁻¹ corresponded with expression of *nif* genes in the mat. At higher photon irradiances, when cyanobacterial O₂ production exceeds O₂ consumption by the various microbes in the top few millimeters of the mat, O₂ accumulates within the mat and inhibits nitrogenase activity (and possibly the expression of *nif* genes).

As seen in intertidal (35) and hypersaline cyanobacterial mats (36), the nitrogenase genes of *Synechococcus* are expressed during the night, as in the Octopus Spring mat. Together with the activity of other N₂-fixing bacteria, fixation by cyanobacteria may alleviate N-limitation in such mats. However, the absence of O₂ in the mats at these times limits energy production to that generated by fermentative metabolism. Fermentation does not produce nearly as much chemical bond energy as respiration (fermentation produces 2–4 ATPs per glucose metabolized), so the rate at which ATP is produced during the night would likely limit cyanobacterial N₂ fixation.

It is unclear how the nitrogenase genes are regulated in thermophilic cyanobacteria. In diazotrophic bacteria except for cyanobacteria, nitrogenase is often regulated by the NifLA proteins, regulatory elements that sense and respond to changing environmental O_2 levels (37). We have not detected genes encoding NifLA-like proteins on the *Synechococcus* OS-A and OS-B′ genomes, but the genomes do contain genes encoding proteins that regulate nitrogen-associated processes, including the *glnB* (for PII) and *ntcA* genes (18). The PII polypeptide can function as a global regulator of nitrogen utilization, whereas cyanobacterial NtcA appears to activate genes encoding proteins involved in the transport and assimilation of various nitrogen compounds (38). NtcA is homologous to transcriptional regulators belonging to the cAMP receptor protein family and directly binds to the promoter regions at specific sites with the consensus nucleotide sequence GTAN$_8$TAC. We localized this sequence upstream of several genes in the genomes of *Synechococcus* OS-A and OS-B′; for example, the sequence was localized upstream of genes for nitrite reductase (*nirA*), the PII protein, the ammonium transporter (*amt*), and NtcA itself. However, there is no clear consensus sequence upstream of the *nif* genes, so it is uncertain what regulatory protein modulates *nif* gene expression.

Transcripts for proteins involved in photosynthesis and respiration were abundant during the day when O_2 levels in the mat are high, but decreased, in many cases dramatically, as the mat became anoxic in the evening. Maintaining some transcripts (and proteins) for fully active photosynthetic and respiratory systems throughout the night could be advantageous for the cyanobacteria. At daybreak, an intact photosynthetic and respiratory capacity would allow for the production of moderate levels of ATP and rapid consumption of photosynthetically produced O_2, thereby sustaining N_2 fixation activity. In preliminary experiments, a strong burst of nitrogenase activity was observed during sunrise in mat samples from Mushroom Spring (A.-S.S., D.B., D.M.W., E.B., J.W.P., M.K., A.R.G., and S. Jensen, unpublished data). A similar burst of N_2 fixation in temperate microbial mats, occurring soon after sunrise, has been reported in refs. 1 and 39.

To examine the possibility that *Synechococcus* might perform fermentation in the dark, we studied expression of a number of genes implicated in cyanobacterial fermentation pathways (1, 33). It is interesting that genes possibly associated with fermentation processes during the day differ from those active in the evening. The *pflB*, *pflA*, *adhE*, and *acs* genes are clustered on the genome (Fig. 1D), and the transcripts from these genes increase in parallel during the evening (as noted in Fig. 1D, some genes may be part of a single transcriptional unit). The PflB and PflA proteins are involved in the fermentation pathway that generates acetyl-CoA and formate from pyruvate whereas AdhE converts acetyl-CoA into ethanol. The *Synechococcus* ecotypes examined in this study thus appear to have the potential to synthesize formate and ethanol during the night. Transcripts from the *ldh*, *pdhB*, and, to some extent, *adh* and *ackA* are high in the day and decline in the evening. Most of these transcripts appear to be present (especially the *ald* transcript) over the entire diel cycle, although the levels are significantly higher in the day. These transcript profiles suggest that the *Synechococcus* ecotypes may have a potential to form lactate and possibly acetate during the day and also during the transition period when the cyanobacteria switch from "light" to "dark" metabolism. These putative fermentation proteins may also be involved in metabolic pathways other than fermentation.

It is interesting to consider these results in the context of past work on the formation and fate of fermentation products in alkaline hot spring cyanobacterial mats and the nature of the interactions of organisms within mat communities. Acetate was measured as the major product of fermentation that accumulated when mat samples were shifted from light, oxic conditions to dark, anoxic conditions. Smaller amounts of propionate and even smaller amounts of butyrate and valerate were detected, but more reduced fermenta-

tion products (e.g., ethanol and lactate) did not accumulate (31). The production of acetate (and CO_2) occurred within hours after mat samples were transferred to the dark; acetate production was linked to glycogen fermentation by *Synechococcus* (30). Under conditions used for these cited experiments, the production and consumption of fermentation products by all members of the mat community would occur simultaneously. For instance, the production of CO_2 associated with fermentation of glycogen by *Synechococcus* may require the presence of a second formate-using organism, because so far we have found no evidence that the *Synechococcus* genomes contain the gene encoding formate hydrogenlyase, which converts formate to H_2 and CO_2. The generation of acetyl-CoA in fermenting *Synechococcus* cells via pyruvate formate lyase, rather than by the pyruvate dehydrogenase reaction (which generates reduced pyridine nucleotide), would help protect the NAD^+ pool. This finding highlights the issue that changes in transcript levels in one organism may not be reflected by accumulation of a specific "predicted" end product in the environment because of metabolite exchange processes that occur within the mat consortium. Furthermore, the failure to observe lactate accumulation may be a consequence of the utilization of this metabolite by other organisms in the mat community (31). The production of reduced fermentation products like lactate and ethanol can be decreased by syntrophic interactions between fermentative and H_2-consuming organisms (e.g., acetogenic and sulfate-reducing bacteria and methanogenic archaea) in these mats (31). Interspecies H_2 transfer allows fermentative microorganisms to reoxidize NADH by reducing protons to H_2, alleviating the need for regenerating NAD^+ by reduction of high-energy intermediates; more rapid regeneration of NAD^+ would allow for elevated rates of fermentation and ATP production, which in turn could stimulate N_2 fixation. However, extensive searches of the *Synechococcus* genomes have not identified genes encoding enzymes necessary for interspecies H_2 transfer (e.g., NADH:ferredoxin oxidoreductase and hydrogenase).

Our findings highlight one of the most interesting and novel aspects of microbial mats: The individuals in this consortium of organisms have metabolic capabilities that are strongly integrated into "community metabolism" (30–32). This integration in turn suggests strong evolutionary constraints because of obligate interactions among the physiologically integrated bacterial populations. To further resolve the intriguing networks of interaction and to understand how they shape and define microbial communities and the evolution of ecotypes, we are taking an interdisciplinary approach in which molecular and genomic analyses are interfaced with ecological and evolutionary questions and methodologies.

Methods

Sample Collection. Samples were collected on Nov. 5, 2004 and June 23, 2005 from the southernmost effluent channel of Octopus Spring. The temperature of Octopus Spring exhibited a temporal cycle of ≈5 min, with a 53.5–63.4°C temperature range at the sampling site, and an average temperature of 60°C over the entire sampling period. The mat was ≈1 cm thick, with *Synechococcus* populating the top 1-mm layer (other cyanobacteria were not observed by microscopic analysis). Cores (1 cm in diameter) were collected, and the top 2 mm of each was excised, frozen in liquid N_2, and stored for nucleic acid extraction. Samples were collected at six time points during the diel cycle in November 2004 (see Fig. 2) and at four time points in June 2005 (see Fig. 3). Microprofiles of O_2 concentration were measured with a Clark-type O_2 microsensor operated by an automated profiling system (Unisense, Aarhus, Denmark) (40, 41), and the downwelling photon irradiance at each sampling time was measured by using a LI-1400 datalogger (Li-Cor, Lincoln, NE).

RNA Extraction. Mat samples that had been frozen and stored at −80°C were dispersed by vortexing them in 1 ml of 10 mM NaOAc

(pH 4.5) and 0.5 g of glass beads (150–212 μm, Sigma-Aldrich) in a 2-ml screw cap microfuge tube. Cells were harvested by centrifugation ($11,750 \times g$) for 1 min at 4°C, the cell pellet resuspended in 250 μl of 10 mM NaOAc (pH 4.5), 37.5 μl of 500 mM Na$_2$-EDTA (pH 8.0), and RNA was extracted as described in ref. 42. Isolated RNA was treated twice with 7 units per reaction of RNase-free DNase (Qiagen, Valencia, CA) for 20 min, the reaction was stopped with 1 vol of phenol:chloroform (1:1), and the RNA in the aqueous layer was precipitated during a 20-min incubation at −20°C after the addition of 0.1 vol of 10 M LiCl and 2.5 vol of 100% ethanol. RNA concentration was determined by absorption at 260 nm and with the ribogreen RNA quantification kit (Molecular Probes).

RT-PCR. Specific primers were designed to amplify regions ($\approx$200 nucleotides) of *Synechococcus* OS-B′ *nifH*, *nifK*, *nifD*, *psaB*, *psbB*, *cpcA*, *cpcF*, *cpcE*, *coxA*, *cydA*, *pflB*, *pflA*, *adhE*, *acs*, *ldh*, *pdhB*, *ald*, and *ackA* genes. The accession no. for all OS-B′ genes is CP000240. The primer pairs used are given in Table 1. Primers were annealed to 100 ng of total RNA extracted from mat samples and extended for 45 min at 55°C by using 200 units of reverse transcriptase (RT) (Superscript III, Invitrogen). Two microliters of the RT reaction was used for the subsequent PCR; each PCR contained 50 units of TaqDNA polymerase (Qiagen, Valencia, CA) and 5% dimethyl sulfoxide. The PCR program included 1 cycle of 95°C for 1 min, 30 cycles of 94°C for 10 s, 55°C for 30 s, 72°C for 30 s, and a final incubation at 72°C for 10 min. Amplified products were analyzed by electrophoresis in a 1.2% agarose gel.

qPCR. Levels of specific transcripts in total mRNA from the mat samples were quantified by RT-PCR by using the Engine Opticon system (Bio-Rad). Single-stranded cDNA, synthesized in a reverse transcriptase reaction by using DNase-treated RNA (as described above), served as the template for qPCR amplifications, which were performed by using the DyNAmo HS SYBR green qPCR kit (Finnzymes, Espoo, Finland). The specific amplification protocol was 1 cycle at 95°C for 10 min, 44 cycles at 94°C for 10 s, 56°C for 15 s, 72°C for 8 s, and a final incubation of 72°C for 10 min. We determined both the absolute (43) and relative (normalized to the T1 sample, collected at 13:30 h) levels of each specific RNA among all environmental samples.

Nitrogenase Activity. Nitrogenase activity was assayed by the acetylene reduction technique (44). Core mat samples (1.13 cm^2) were collected, and the top 2 mm was removed and placed in 10-ml serum vials that were sealed under a constant stream of argon. Two milliliters of argon-sparged Octopus Spring water was added, and the vials were incubated for 15 min in the hot spring at *in situ* temperature and photon irradiance. The nitrogenase assay was then initiated with the injection of 1 ml of O$_2$-free acetylene, and vials were incubated in the spring for an additional 2 h before each reaction was stopped with 0.2 ml of formaldehyde. Acetylene injections to initiate the assays were done at 16:00, 18:05, 21:05, and 23:00 h (15 min after each preincubation) when the irradiances were 919, 31, 0, and 0 μmol·m^{-2}·s^{-1}, respectively. Each assay was performed in triplicate. The nitrogenase activity reported was the total activity over the 2-h incubation; samples used for measuring transcript abundance were taken at the end of each incubation period, except for one sample, which was taken at 20:30 h instead of 20:05 h. Ethylene production in samples was measured by gas chromatography (Shimadzu GC-8A with a flame ionization detector).

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