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ORIGINAL ARTICLE

Temporal metatranscriptomic patterning in phototrophic Chloroflexi inhabiting a microbial mat in a geothermal spring

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Filamentous anoxygenic phototrophs (FAPs) are abundant members of microbial mat communities inhabiting neutral and alkaline geothermal springs. Natural populations of FAPs related to *Chloroflexus* spp. and *Roseiflexus* spp. have been well characterized in Mushroom Spring, where they occur with unicellular cyanobacteria related to *Synechococcus* spp. strains A and B′. Metatranscriptomic sequencing was applied to the microbial community to determine how FAPs regulate their gene expression in response to fluctuating environmental conditions and resource availability over a diel period. Transcripts for genes involved in the biosynthesis of bacteriochlorophylls (BChls) and photosynthetic reaction centers were much more abundant at night. Both *Roseiflexus* spp. and *Chloroflexus* spp. expressed key genes involved in the 3-hydroxypropionate (3-OHP) carbon dioxide fixation bi-cycle during the day, when these FAPs have been thought to perform primarily photoheterotrophic and/or aerobic chemoorganotrophic metabolism. The expression of genes for the synthesis and degradation of storage polymers, including glycogen, polyhydroxyalkanoates and wax esters, suggests that FAPs produce and utilize these compounds at different times during the diel cycle. We summarize these results in a proposed conceptual model for temporal changes in central carbon metabolism and energy production of FAPs living in a natural environment. The model proposes that, at night, *Chloroflexus* spp. and *Roseiflexus* spp. synthesize BChl, components of the photosynthetic apparatus, polyhydroxyalkanoates and wax esters in concert with fermentation of glycogen. It further proposes that, in daytime, polyhydroxyalkanoates and wax esters are degraded and used as carbon and electron reserves to support photomixotrophy via the 3-OHP bi-cycle.

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Introduction

Microbial mats in the effluent channels of the alkaline siliceous Octopus Spring and Mushroom Spring (Yellowstone National Park, USA) are among the most intensively studied natural microbial communities (Ward *et al.,* 2006) and serve as model systems for exploring general ecological principles in microbial ecology (Ward *et al.,* 2008). Molecular characterization of thermophilic microbial mat communities thriving in these springs at 50–74 °C revealed that chlorophototrophs are the dominant community members. Specifically, the most abundant organisms consist of cyanobacteria related to cultivated *Synechococcus* spp. strains A and B′ (Ward *et al.*, 1990; Ferris *et al.*, 1996; Allewalt *et al.*, 2006; Bhaya *et al.*, 2007) and filamentous anoxygenic phototrophs (FAPs) related to *Roseiflexus* spp. and *Chloroflexus* spp. (Nübel *et al.*, 2002; van der Meer *et al.,* 2010). Past work has suggested that *Synechococcus* spp. are the primary producers responsible for most inorganic carbon fixation. They also produce low-molecular weight organic

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compounds as byproducts of their metabolism, and it has been shown that FAPs assimilate these compounds photoheterotrophically (Sandbeck and Ward 1981; Anderson *et al.* 1987; Bateson and Ward 1988; Nübel *et al.*, 2002). Metabolites excreted by cyanobacteria in these mats fluctuate between daytime production of glycolate (a byproduct of photorespiration under conditions of O_2 supersaturation during the day; Bateson and Ward 1988) and nighttime production of acetate and propionate (both produced, in part, by cyanobacterial or other bacterial fermentation under anoxic conditions; Anderson *et al.*, 1987, Nold and Ward 1996, van der Meer *et al.*, 2005, 2007). FAPs are thought to perform photoheterotrophic metabolism for the uptake of low-molecular weight carbon sources both in culture and *in situ* (Madigan *et al.*, 1974; Pierson and Castenholz, 1974; Sandbeck and Ward, 1981; Anderson *et al.*, 1987; Hanada *et al.*, 2002; van der Meer *et al.*, 2003; van der Meer *et al.*, 2005, 2010). Given that *Chloroflexus aurantiacus* strain OK-70-fl can be grown photoautotrophically on a minimal medium sparged with H_2 and with CO_2 as the sole source of carbon (Holo and Sirevåg 1986; Strauss *et al.*, 1992), it is of interest whether, and when, uncultivated phototrophic Chloroflexi can perform inorganic carbon fixation. There is evidence that *Roseiflexus* spp. and *Chloroflexus* spp. in these mats might fix inorganic carbon *in situ* when light and electron donors, such as H_2 and H_2S, are available at dawn and possibly at dusk (van der Meer *et al.*, 2005; Klatt *et al.*, 2007). Furthermore, the 3-hydroxypropionate (3-OHP) CO_2 fixation bi-cycle that has been described for these organisms (Strauss and Fuchs, 1993; Zarzycki *et al.*, 2009) can also operate mixotrophically. During mixotrophic growth, organisms simultaneously incorporate both inorganic carbon and organic carbon sources, such as acetate and glycolate (Zarzycki and Fuchs, 2011; Bryant *et al.*, 2012).

Recent metagenomic characterizations of phototrophic microbial mat communities in Octopus Spring and Mushroom Spring have revealed three additional and abundant potentially photoheterotrophic populations: '*Candidatus* Chloracidobacterium thermophilum' (Kingdom *Acidobacteria*; Bryant *et al.*, 2007, Garcia Costas *et al.*, 2012), '*Candidatus* Thermochlorobacter aerophilum' (Kingdom *Chlorobi*; Klatt *et al.*, 2011, Liu *et al.* 2011, 2012) and a novel clade of organisms related to Chloroflexi of the Class *Anaerolineae* (Klatt *et al.*, 2011). These organisms are known or predicted to be photoheterotrophs and probably utilize and compete for some of the same resources as other FAPs. The abundance of these organic compounds, combined with the availability of inorganic carbon, light as an energy source and hydrogen or sulfide as a source of electrons, are factors that shape the relative degree to which FAPs perform heterotrophic, mixotrophic or autotrophic metabolism. Furthermore, various photoheterotrophic community members might escape competition for resources and enable coexistence in these mats by temporally partitioning nutrient uptake.

In this study, metatranscriptomic sequencing of RNA extracted from mat samples taken hourly over the course of a diel period was performed. The data provided a more complete view of how chlorophototrophic members of the Chloroflexi temporally transcribe their genes in relation to changing *in situ* environmental conditions and the metabolic activities of other community members. This experiment provided high-resolution, temporal transcription profiles of genes involved in photosynthesis, central carbon metabolism and energy production of uncultivated FAPs in their natural habitat. From these transcriptional analyses, a model is proposed to describe how members of the Chloroflexi regulate their metabolism and contribute to the metabolic activities of the microbial mat community.

Materials and methods

Sampling and sequence construction

RNA was extracted and cDNA sequences were produced from microbial mat samples taken at hourly intervals from the 60 °C region of the effluent channel of Mushroom Spring over a 24-h period beginning at 1700 hours on 11 September 2009 and ending at 1600 hours the following day (Liu *et al.*, 2012). A processing error caused the sample for 1100 hours to be lost. The environmental sampling, *in situ* microenvironmental analysis, RNA extraction and cDNA synthesis have been previously described (Liu *et al.*, 2011, 2012). Additional details of these methods are described in the Supplementary Information. All transcript sequences from this study have been deposited in the Sequence Read Archive under the accession number SRP018579.

Alignment and statistical analyses of cDNA sequences

The sequences from the SOLiD 3.5 analysis were aligned to an expanded metagenomic scaffold database (including scaffolds that were >5 kb in size; see Supplementary Information) using the BWA (Burrows–Wheeler Alignment) algorithm (Li and Durbin, 2009) as previously described (Liu *et al.*, 2011). Briefly, for a sequence to be counted as mapping to a particular gene, the entire 50 bp sequence had to be fully aligned to the reference and at least half of the sequence had to be aligned to the coding region of the gene with a maximum of five mismatches per sequence ($\geqslant 90\%$ nucleotide sequence identity). This 50% read length criteria ensured that the sequence was only assigned to a single gene. Relative expression values for a given time point i (E_i) were calculated using the following formula:

$$E_i = n_i/(N_i \times p_i)$$

Here, n_i denotes the number of mRNA sequences assigned to a gene for a given time point, N_i denotes the number of total mapped sequences at that time point and p_i denotes the percentage of mRNA sequences that were assigned to that particular taxonomic cluster at that time point. Each expression value from this formula was then further normalized by the mean of the expression values for all the 23 time points for that particular gene. This allows the expression values of any selected gene at two time points to be directly compared. In cases where groups of genes involved in a common pathway were considered, the mean relative expression was taken for each time point, and the standard error of the mean was calculated by dividing the s.d. of their relative expression levels by the square root of the number of genes.

Clustering and visualization of gene expression patterns

Normalized expression levels of genes were $\log_2$ transformed, centered by mean and then clustered using the *k*-means algorithm with the program Cluster (Eisen *et al.*, 1998) ($k=5$ for *Roseiflexus* spp. transcripts; $k=6$ for *Chloroflexus* spp. transcripts; runs = 1000). The resulting gene expression patterns in each cluster were visualized using Java Treeview (Saldanha, 2004). These *k*-means clusters were then assigned temporal transcription categories, such as 'diurnal' patterns when they exhibited higher expression levels during the day (typically 0800–1800 hours), 'nocturnal' patterns with higher expression levels between 1800 and 0800 hours and 'constitutive' patterns when genes in the cluster exhibited expression levels that could not be unambiguously assigned to the diurnal or nocturnal groups (see Supplementary Information).

Results and discussion

Metatranscriptomes of FAP populations

The transcripts detected from FAPs at hourly time intervals provide insights into how these organisms temporally regulate their gene expression, which, in turn, suggests how these organisms respond transcriptionally to changing environmental conditions over a diel cycle. In the discussion that follows, it is explicitly acknowledged that transcript abundance may not be directly correlated with translation of mRNAs or be proportional to enzymatic activities, and thus all statements regarding the timing of particular metabolic activities are the subject of ongoing complementary metaproteomic and metabolomic analyses. The total number of transcripts that uniquely mapped to open-reading frames (ORFs) on scaffolds assigned to *Roseiflexus* spp. (11 159 969) was 30-fold higher than the total number of transcripts assigned to *Chloroflexus* spp. (365 812) (Supplementary Table S1), which was notable in comparison to the twofold difference in metagenomic scaffold sequences contributed by these two groups (Supplementary Table S2). This difference in transcript abundance between *Roseiflexus* spp. and *Chloroflexus* spp. is, in part, due to incomplete metagenomic coverage for these organisms (see Supplementary Information), as determined by the lower depth-of-coverage and the smaller mean scaffold size for *Chloroflexus* spp. compared with *Roseiflexus* spp. (Supplementary Table S2). Additionally, it is likely that there are fewer transcripts from *Chloroflexus* spp. at the temperature at which this study was conducted (60 °C) than would be the case at a higher temperature. The metagenomic scaffolds from *Chloroflexus* spp. included sequences from samples taken at 65 °C, a temperature at which *Chloroflexus* spp. have been shown to be more abundant (Nübel *et al.* 2002, Klatt *et al.*, 2007, 2011). After transcript abundance was normalized to total mRNA, it was observed that both FAP genera exhibited their lowest transcript levels in the early morning (between 0300 and 0500 hours), with an abrupt increase at dawn when the mat was exposed to sunlight (0700–1000 hours) (Supplementary Figure S1). Transcript levels then showed a slight decrease in the afternoon (1300–1500 hours) before reaching their highest levels at 1600 hours.

Despite differences in metagenomic coverage, the *Chloroflexus* spp. and *Roseiflexus* spp. metatranscriptomes were similar in that 97.7% of the *Roseiflexus*-like metagenomic ORFs and 97.6% of the *Chloroflexus*-like ORFs had at least one cDNA sequence uniquely mapped to them. Three major transcription patterns, diurnal, nocturnal and constitutive, were observed for individual genes in the metatranscriptomes of members of the Chloroflexi after the normalized relative expression values were subjected to *k*-means clustering (Supplementary Figure S2). *K*-means clusters exhibiting diurnal or nocturnal patterns were more finely categorized into 'strong' and 'weak' patterns (dependent upon the relative difference in day and night expression levels) or into other subcategories that may be physiologically meaningful (for example, a cluster of diurnal genes from *Roseiflexus* spp. that had increased transcript levels into the evening). *Anaerolineae*-like organisms had the highest proportion of diurnally expressed genes (~14:1 diurnal:nocturnal ratio (D:N)), which supported the hypothesis that this is a phototrophic bacterium that is most transcriptionally active when light is available (Klatt *et al.*, 2011; Liu *et al.*, 2011). The majority of *Chloroflexus*-like genes were also expressed diurnally (~8:1 D:N). The majority of *Roseiflexus*-like genes had constitutive expression patterns, but compared with *Chloroflexus* spp., there was a relatively higher proportion of genes with nocturnal expression. Thus, the ratio of genes with D:N patterns was lower for *Roseiflexus* spp. (~2:1 D:N). Although FAPs must be able to cope with both oxic and anoxic conditions in these mats over the diel

cycle (for example, Jensen *et al.*, 2011; see Supplementary Figure S1), the relative degree to which these organisms utilize aerobic or anaerobic metabolism is currently unknown.

Photosynthesis

Consistent with the prediction that FAPs perform light-driven CO_2 fixation during low-light periods in the evening and early morning (Revsbech and Ward, 1984; van der Meer *et al.*, 2005), initial metatranscriptomic investigations suggested that members of the Chloroflexi transcribe genes encoding their type-2 photosynthetic reaction centers (that is, *pufLM*, homologs of RoseRS_3268, Caur_1052, and Caur_1051; *pufC*, RoseRS_3269 and Caur_2089) during these times (Liu *et al.*, 2011). The much higher temporal resolution afforded by the hourly sampling in the present study surprisingly revealed that transcripts for the *pufLM* genes of both *Chloroflexus* spp. and *Roseiflexus* spp. are actually most abundant at night (Figure 1). The *pufLMC* homologs from the more distantly related *Anaerolineae*-like population also showed the highest transcript levels during night (Figure 1). These results are consistent with the patterning of transcript abundance of type-1 reaction center genes from the other anoxygenic photoheterotrophs in this mat, namely '*Ca.* C. thermophilum' and '*Ca.* T. aerophilum' (Liu *et al.*, 2012), and are opposite of the diurnal expression of cyanobacterial genes encoding structural components of the photosynthetic apparatus (Steunou *et al.*, 2006, 2008; Jensen *et al.*, 2011; Liu *et al.*, 2011, 2012). Transcripts for genes encoding the proteins of the chlorosome envelope in *Chloroflexus* spp. (*csmA*, Caur_0126; *csmM*, Caur_0139; *csmN*, Caur_0140) were also more abundant at night (Supplementary Table S3).

Bacteriochlorophyll (BChl) biosynthesis

With a few exceptions, transcripts of genes involved in the biosynthesis of BChls in FAPs were most abundant at night (Figure 1, Supplementary Figure S3); this temporal linkage with *pufLMC* transcription is logical, as these pigment molecules are required to assemble functional photosynthetic reaction centers. Although this expression of genes for the enzymes of BChl biosynthesis under anoxic conditions is consistent with the laboratory studies for *C. aurantiacus* (Oelze, 1992) and anoxygenic phototrophic proteobacteria (Ponnampalam *et al.*, 1995; Gregor and Klug, 1999; Zappa *et al.*, 2010), the *Chloroflexus* spp. and *Roseiflexus* spp. genomes lack some of the transcriptional regulatory mechanisms present in proteobacteria, such as the co-localization of most genes related to photosynthesis in a large gene cluster or homologs to the oxygen-activated transcriptional repressor PpsR/CrtJ and redox-dependent two component regulators, RegA and RegB (Wu and Bauer 2008). Some BChl biosynthesis genes are co-localized in the *Chloroflexus* spp. and *Roseiflexus* spp. genomes (van der Meer *et al.* 2010; Klatt *et al.*, 2011; Tang *et al.*, 2011; Bryant *et al.*, 2012), and their coordinated expression as exhibited in the metatranscriptome suggests that there is an undiscovered, O_2- or redox-sensitive regulatory mechanism that is common to these organisms. In contrast to these results, the incomplete set of *Anaerolineae*-like BChl biosynthesis genes (*bchXYZ*, *bchD*, *bchF*, *bchH*, *bchI*) lacked a distinct nocturnal or diurnal pattern (Figure 1).

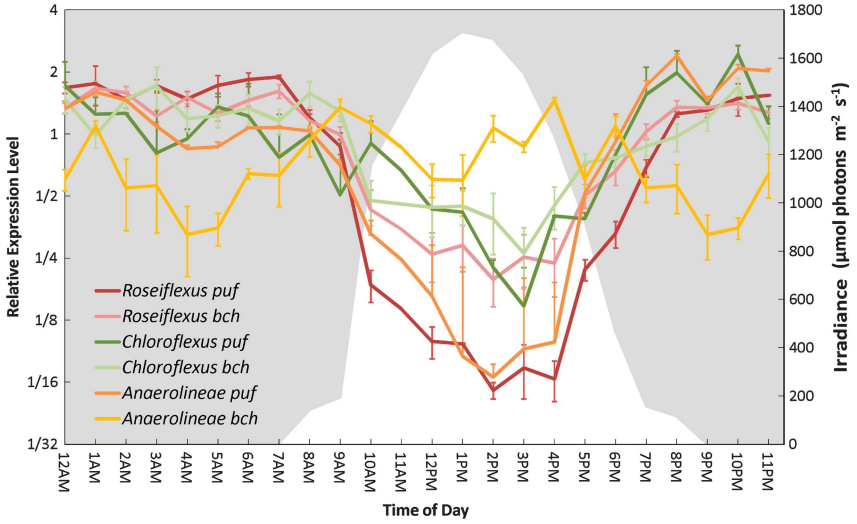

Figure 1 Expression of FAP phototrophy genes over a diel cycle. The mean relative expression level (±s.e.) is displayed for photosynthetic reaction center genes *pufLMC* (dark) and BChl biosynthesis genes (light) for *Roseiflexus* spp. (red), *Chloroflexus* spp. (green) and *Anaerolineae*-like (orange) Chloroflexi. BChl biosynthesis gene expression was the mean expression level of all BChl biosynthesis genes known in *Roseiflexus* and *Chloroflexus* spp. genomes, while for *Anaerolineae*-like Chloroflexi, the mean expression was taken from *bchH*, *bchX*, *bchY* and *bchZ* identified in previous metagenomic analyses.

The formation of the isocylic ring in BChls is catalyzed by either O_2-dependent or -independent Mg-protoporphyrin IX monomethylester oxidative cyclase enzymes. *Chloroflexus* spp. and *Roseiflexus* spp. have genes encoding both enzymes for this step of BChl biosynthesis: *acsF* (Caur_2590 and RoseRS_1905) and *bchE* (Caur_3676 and RoseRS_0942) (Bryant *et al.*, 2012). In the purple sulfur bacterium *Rubrivivax gelatinosus*, AcsF is required for the production of BChl *a* under oxic/microoxic growth conditions, while BchE is required under anoxic conditions, although the *bchE* gene is transcribed both in the presence and absence of O_2 (Ouchane *et al.*, 2004). The *acsF* and *bchE* homologs in the metatranscriptome for both *Roseiflexus* spp. and *Chloroflexus* spp. exhibited a nocturnal expression pattern, which suggested that, unlike *R. gelatinosus*, the transcription of *bchE* in FAPs may be inhibited by O_2. It is notable that the *acsF* transcripts were more abundant at night, because the activity of AcsF requires O_2 as a substrate (Pinta *et al.*, 2002). A culture-based study of *C. aurantiacus* determined that the expression of *acsF* was not significantly different between anoxic and microoxic culture conditions (Tang *et al.*, 2009). The lower abundance of *acsF* transcripts during the day could indicate that *acsF* transcription might be suppressed at high levels of O_2 that these organisms experience during the day. The consistency of nocturnal transcription patterns for genes encoding photosystem reaction centers, chlorosome envelope proteins and those involved in BChl biosynthesis all provide strong evidence that FAPs produce their phototrophic apparatus at night.

Mixotrophy and the tricarboxylic acid (TCA)/3-OHP cycles

The 3-OHP bi-cycle was discovered and characterized as an inorganic carbon fixation pathway in *C. aurantiacus* (Holo and Sirevåg, 1986, Strauss and Fuchs, 1993), and studies utilizing isotopic labeling have suggested that the FAPs in these mats incorporate inorganic carbon in the morning (van der Meer *et al.*, 2005). By contrast, the oxidative TCA cycle is of importance in chemoorganoheterotrophic metabolism, and laboratory studies have shown that all characterized FAPs have the capacity to respire organic compounds under oxic conditions. Thus, it was thought that FAPs in natural environments might primarily fix inorganic carbon during low-light conditions when H_2 and H_2S are available, that they would then switch to photoheterotrophic metabolism during the day and that they would finally perform aerobic respiration of organic compounds at night when O_2 is available near the mat surface (van der Meer *et al.*, 2005). It had even been proposed that FAPs migrate to the surface of the mat at night when O_2 is only available via diffusion from the overlying water (Doemel and Brock, 1977). Contrary to these previous proposals for natural populations, we and the others have suggested that FAPs in natural environments are more likely utilizing both the 3-OHP and the TCA cycles as mixotrophic pathways, resulting in the simultaneous incorporation of organic and inorganic carbon (Klatt *et al.*, 2007, Zarzycki and Fuchs, 2011; Bryant *et al.*, 2012). The TCA cycle is intimately linked with the 3-OHP bi-cycle; two enzymes (succinyl-CoA dehydrogenase and fumarate hydratase) and three metabolites (succinyl-CoA, fumarate and malate) are shared by these cycles, and glyoxylate forms an intermediate of both the glyoxylate bypass of the TCA cycle and the 3-OHP bi-cycle (Figure 2). Transcripts for genes encoding enzymes of the TCA cycle and the glyoxylate bypass were higher during the day for both *Roseiflexus* spp. and *Chloroflexus* spp. populations; likewise, genes for key steps in the 3-OHP bi-cycle had diurnal expression patterns for *Roseiflexus* spp. A putative operon that contains the genes encoding acetyl-CoA carboxylase, malonyl-CoA reductase and propionyl-CoA synthase (RoseRS_3199–RoseRS_3203, see Klatt *et al.*, 2007) occurs in *Roseiflexus* spp. Transcripts for these genes, which catalyze the first three steps of the 3-OHP bi-cycle, displayed a peak in relative abundance during the late morning (Figure 3a). Consistent with the prediction that FAPs fix CO_2 in the early morning (van der Meer *et al.*, 2005), the relative abundance of these 3-OHP transcripts displayed an abrupt increase in the morning at low irradiance. Unexpectedly, the relative abundance continued to increase until 1000 hours and remained higher than nighttime levels throughout the day, which suggested that the 3-OHP bi-cycle is active when the mat is highly oxic (Supplementary Figure S1). This inference is supported by recent metaproteomic evidence for the presence of peptides originating from malonyl-CoA reductase and propionyl-CoA synthase enzymes in a midday sample of a highly similar microbial community in Octopus Spring (Schaffert *et al.*, 2012).

Glyoxylate is the central metabolite that links the two cycles of the 3-OHP bi-cycle, and this metabolite could be derived from glycolate. Glycolate is produced from photorespiration by cyanobacteria during the day when the mat is highly oxic (Bassham and Kirk, 1962; Bateson and Ward, 1988), and past work has demonstrated that FAPs assimilate glycolate *in situ* (Bateson and Ward, 1988). Both *Roseiflexus* spp. and *Chloroflexus* spp. encode homologs of glycolate oxidase (*glcD*, RoseRS_3360, Caur_2132), which oxidizes glycolate to produce glyoxylate. Transcripts for *glcD* in both FAPs showed diurnal expression patterns that were consistent with the expression of other key 3-OHP bi-cycle reactions and that occurred at a time when glycolate would be available (Supplementary Table S3). Additionally, a putative Na^+ symporter predicted to enable the uptake of small organic acids such as glycolate and acetate in *Roseiflexus* spp. (homologous to RoseRS_1628) exhibited a diurnal

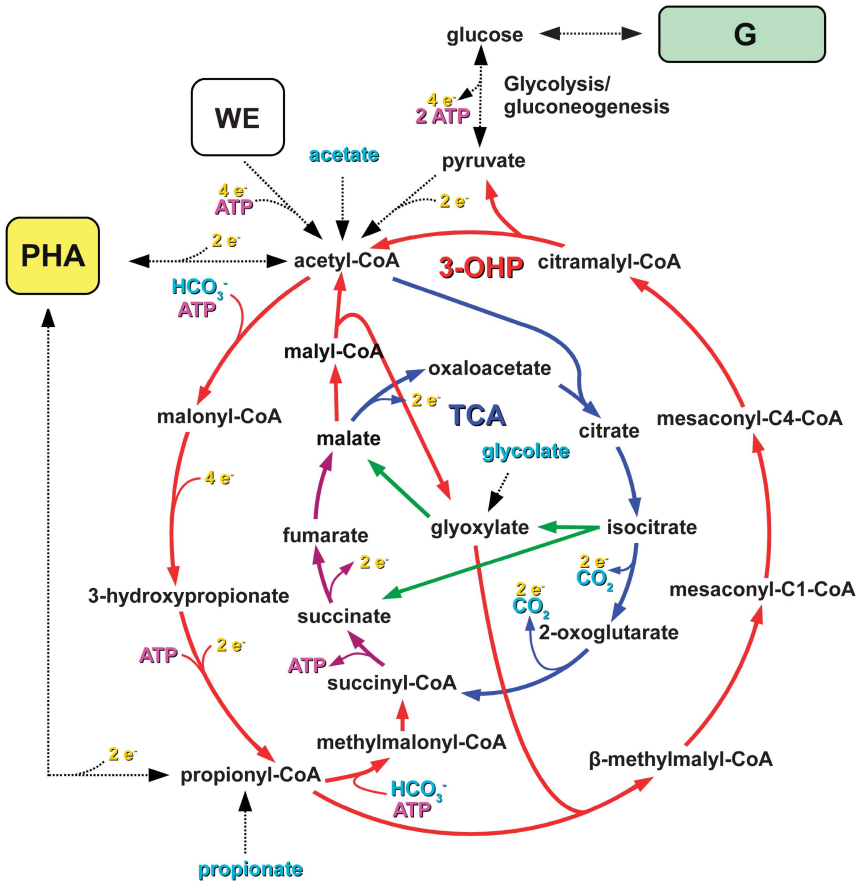

Figure 2 Model for integrated TCA and 3-OHP pathways for FAP mixotrophic metabolism. The TCA cycle (blue) operates in the oxidative direction, while the 3-OHP cycle (red) reduces inorganic carbon. Shared steps are in purple, and the glyoxylate bypass is indicated in green. Metabolites indicated in light blue are substrates that can be obtained from outside the cell. G, glycogen; PHA, polyhydroxyalkanoic acids; WE, wax esters.

expresssion pattern. The coordinated transcription patterns of key genes in the 3-OHP and TCA cycles indicated that this may be a way in which *Roseiflexus* spp. incorporate organic acids (glycolate→glyoxylate, acetate→acetyl-CoA and propionate→propionyl-CoA) while they simultaneously produce key substrates for anabolic pathways (that is, 2-oxoglutarate, succinyl-CoA and oxaloacetate) and reduce the loss of carbon as CO_2 (Figure 3a).

Heterotrophic carbon assimilation and storage

Glycolysis/gluconeogenesis. The transcript abundance of genes involved in the 3-OHP bi-cycle was compared with genes in heterotrophic pathways, such as glycolysis and gluconeogenesis, to understand when FAPs are utilizing or synthesizing glycogen, respectively. Past studies have shown that glycogen levels fluctuate in mat organisms over a diel cycle. Glycogen accumulated during the day and subsequently decreased during the night in mat fractions enriched in either *Synechococcus* spp. or FAPs (van der Meer *et al*., 2007). Scaffolds assigned to both *Chloroflexus* spp. and *Roseiflexus* spp. contain genes involved in glycogen storage and utilization (that is, Group 1 glycosyl transferases for storage and alpha-glucan phosphorylase for utilization; Supplementary Table S3). The transcription patterns of genes corresponding to unidirectional enzymatic steps in glycolysis were consistent with observations of fluctuating glycogen levels in the mat. For example, the nocturnal abundance of the gene encoding pyruvate kinase (RoseRS_1428), which catalyzes an irreversible, ATP (adenosine triphosphate)-generating, substrate-level phosphorylation step in glycolysis, was taken as evidence that *Roseiflexus* spp. catabolize glucose via glycolysis at night or in the early morning. The metagenome of *Chloroflexus* spp. did not contain a homolog of this gene (Caur_3128; Supplementary Table S6), presumably due to lower depth-of-coverage for sequences derived from these organisms. Additionally, two paralogous genes, homologous to RoseRS_1829 and RoseRS_1831 and encoding phosphofructokinase, which catalyzes an irreversible step in glycolysis, showed nocturnal expression patterns in *Roseiflexus* spp. Other genes encoding steps in glycolysis and gluconeogenesis are bidirectional and/or are used in both pathways (*Roseiflexus*

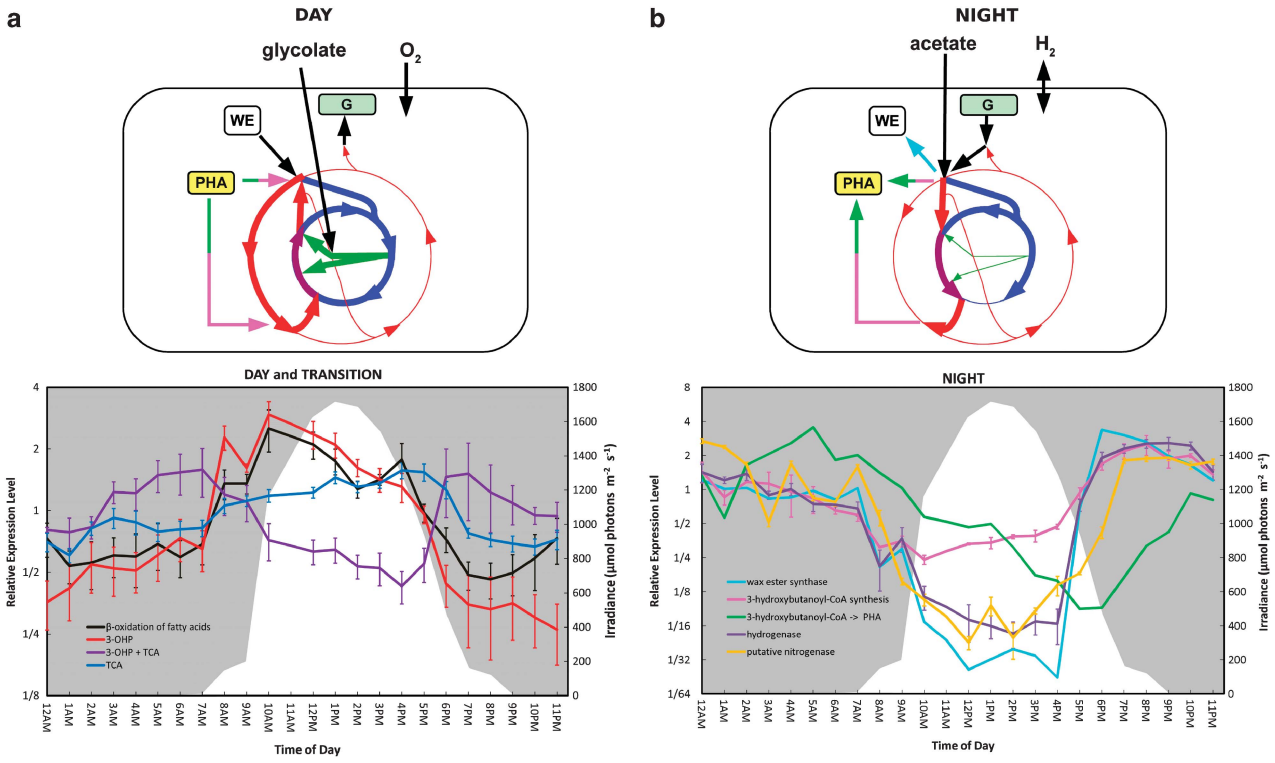

Figure 3 Models for diurnal (**a**) and nocturnal (**b**) central carbon metabolism, hydrogenase and putative nitrogenase activity in *Roseiflexus* spp. based on transcriptional results. The top panel displays a simplified diagram of Figure 3, where bold arrows indicate the predicted dominant flow of carbon through the 3-OHP/TCA cycles and related pathways. The coloring is consistent with that in Figure 2. The bottom panel shows transcription patterns for relevant genes for these pathways. (**a**) Transcripts for genes with diurnal transcription patterns, such as malonyl-CoA reductase (*mcr*) and propionyl-CoA synthase (*pcs*), were averaged for the 3-OHP bi-cycle (red). Malonyl-CoA mutase and malonyl-CoA epimerase were averaged to indicate the expression of shared components of the TCA and 3-OHP pathways (purple). The remaining genes of the TCA cycle were separately averaged (blue). The mean relative transcript levels for genes involved in the β-oxidation of fatty acids are shown in black. (**b**) Nocturnally expressed genes are shown as the mean expression values of those encoding subunits of hydrogenase (*hydABCD*) and the putative nitrogenase (*nifHBDK*). The mean expression values for genes involved in poly-hydroxybutyrate synthesis/degradation (including multiple paralogs of β-ketothiolase and acetoacetyl-CoA reductase) are represented by 3-hydroxybutanoyl-CoA synthesis. The expression values are also displayed for the single genes wax ester (WE) synthase and polyhydroxyalkanoic acid (PHA) synthase. G, glycogen.

spp. have a combined fructose-6-phosphate phosphatase/aldolase, RoseRS_2049; Say and Fuchs, 2010), and taken together, these did not exhibit strictly diurnal or nocturnal transcription patterns.

Production of acetyl-CoA. The pyruvate that is produced from glycolysis can be utilized by two different enzymes that convert it to acetyl-CoA for use in other reactions of central carbon metabolism. Pyruvate dehydrogenase (enzyme commission (EC) numbers 1.2.4.1, 2.3.1.12, and 1.8.1.4) is an enzyme complex typically found in aerobic organisms; it oxidizes pyruvate and produces acetyl-CoA, NADH and CO_2 in the gateway reaction to the TCA cycle. Pyruvate:ferredoxin oxidoreductase (PFOR, EC 1.2.7.1) can oxidize pyruvate to produce acetyl-CoA, reduced ferredoxin (or rubredoxin) and CO_2 (Yoon *et al*., 1999; Ludwig and Bryant 2011; Bryant *et al*., 2012), or it can synthesize pyruvate from acetyl-CoA, bicarbonate and reduced ferredoxin (Yoon *et al*., 2001). PFOR is typically observed in organisms with anaerobic metabolism (Buckel and Golding, 2006; Tang *et al*., 2011); for example, in cyanobacteria, pyruvate dehydrogenase is replaced by PFOR under fermentative growth conditions (dark anoxic conditions) (Ludwig and Bryant, 2011). Consistent with the absence (night) or presence (day) of O_2, the transcripts for *nifJ*/*por* (PFOR) genes of both *Chloroflexus* spp. and *Roseiflexus* spp. were most abundant at night, whereas transcripts for genes encoding pyruvate dehydrogenase were highest during the day (Supplementary Figure S4).

During either photoheterotrophic or chemoorganotrophic metabolism, FAPs take up low-molecular weight organic compounds such as acetate and propionate, and these acids must also be converted to acyl-CoA derivatives in order to be utilized by other metabolic reactions. Genes catalyzing the conversion of acetate to acetyl-CoA (acetyl-CoA synthetase, EC 6.2.1.1, RoseRS_2003) had constitutive expression patterns for both *Roseiflexus* spp. and *Chloroflexus* spp. This observation suggests that this enzyme may allow acetate to be assimilated throughout the diel cycle.

Polyhydroxyalkanoic acids (PHA). Acetyl-CoA and other acyl-CoA derivatives also serve as crucial intermediary metabolites for the biosynthesis of PHA, common carbon, electron and energy storage compounds, which are known to be produced by *C. auranticacus* (van der Meer *et al.*, 2001). Transcripts encoding key enzymes in PHA biosynthesis from *Roseiflexus* spp., including a paralog of 3-ketothiolase (RoseRS_4348), and homologs of acetoacetyl-CoA reductase (EC 1.1.1.36, RoseRS_4347) and polyhydroxyalkanoate synthase (EC 2.3.1., RoseRS_4553), were more abundant at night (Figure 3). The transcripts for these three genes were temporally offset, such that there was an increase in transcripts for 3-ketothiolase and acetoacetyl-CoA reductase in the evening (1700-2200 hours; pink line in Figure 3b) followed by an increase in transcripts for PHA synthase in the morning (peak at 0500 hours, green line in Figure 3b). These transcript patterns are consistent with the hypothesis that *Roseiflexus* spp. synthesize PHA at night, a prediction that is consistent with results of metabolomics analyses (Y-M Kim, T Metz and DM Ward, unpublished). Other ORFs homologous to genes that are known to be involved in PHA biosynthesis, such as PhaR (RoseRS_4554; McCool and Cannon 2001) and a PHA-granule associated protein (RoseRS_4398) also exhibited strong nocturnal patterns. Metagenomic coverage was more limited in the case of *Chloroflexus* spp., and transcripts for genes encoding enzymes for these latter steps were not observed (Supplementary Table S6). One important exception was the *Chloroflexus* spp. homolog of PHA synthase (Caur_3263), which also exhibited a nocturnal expression pattern. The production of PHAs at night could occur simultaneously with the fermentation of glycogen in these organisms.

The production of PHA in FAPs at night is predicted to coincide with their production of BChls, and thus they must partition their pool of acetyl-CoA to either storage polymers (PHA) or pigment molecules. It has been proposed that some anaerobic bacteria produce PHA by assimilating acetate and reducing it as described above, but they also obtain supplemental acetyl-CoA, ATP, and reducing power from stored glycogen via glycolysis (Hesselmann *et al.*, 2000). Acetyl-CoA that is produced from the oxidation of pyruvate then enters a branched TCA cycle, in which it is either converted to oxaloacetate or it is converted to 2-oxoglutarate via the first three steps of the oxidative branch of the TCA cycle. In the case of phototrophic Chloroflexi, the 2-oxoglutarate can then be used as a precursor for BChl biosynthesis, and thus the oxidative TCA cycle would be interrupted and decreased flux through 2-oxoglutarate dehydrogenase would be expected. Consistent with this hypothesis, three subunits of 2-oxoglutarate dehydrogenase (RoseRS_1675, RoseRS_3396 and RoseRS_3397) exhibited diurnal transcription patterns (that is, lower transcript levels at night; Supplementary Table S2). An additional source of 2-oxoglutarate could be propionyl-CoA by way of succinyl-CoA and 2-oxoglutarate synthase (*korB-1* and *korA-1*, EC 1.2.7.3., RoseRS_1369 and RoseRS_1370), all of which exhibited nocturnal transcription patterns that would be consistent with the timing of BChl biosynthesis (Supplementary Figure S4).

Phosphoenolpyruvate produced from glycolysis could simultaneously be converted to oxaloacetate from the reaction catalyzed by phosphoenolpyruvate carboxylase mentioned above, and it could then be reduced on the reductive branch of the TCA cycle. The reversible steps catalyzed by the enzymes of the reductive arm of the TCA cycle (malate dehydrogenase, fumarate hydratase, succinate dehydrogenase and succinyl-CoA synthase) could reductively convert oxaloacetate to succinyl-CoA. This intermediate might then enter the methylmalonyl pathway, of which the genes of the corresponding enzymes showed a nocturnal expression pattern (indicated by the shared genes of the TCA and 3-OHP cycles in Figure 3a), and would lead to the production of propionyl-CoA as a precursor for PHA biosynthesis. Alternatively, as noted above, this propionyl-CoA could also be converted to 2-oxoglutarate by KorAB for use in BChl biosynthesis.

Some of the acetyl-CoA produced from PFOR could be directly incorporated into PHA. Using this pathway, FAPs can produce both polyhydroxybutyrate (from 2 acetyl-CoA + 2 e^-) or polyhydroxyvalerate (from 1 acetyl-CoA + 1 propionyl-CoA + 2 e^-). This proposed pathway would allow FAPs to synthesize PHA at night for carbon and energy storage (Figure 3b), using electrons and acetyl-CoA released from the fermentation of stored glycogen. *Roseiflexus* spp. could also obtain acetate from cyanobacterial fermentation (Nold and Ward, 1996). Below the upper 100 μm of the mat, O_2 levels are below detection limits ($<1\,\mu M$) at night (Supplementary Figure 1), and this metabolic strategy would allow *Roseiflexus* spp. to regenerate $NADP^+$ (nicotinamide adenine dinucleotide phosphate) at those depths. If FAPs were to use a split TCA cycle at night, acetyl-CoA directed to propionyl-CoA for the production of PHA (by way of the reductive TCA cycle to succinyl-CoA) would obviate the need for an external electron acceptor and would additionally retain most of the carbon from glucose, and acetyl-CoA directed to 2-oxoglutarate could be used for BChl biosynthesis. When O_2 is plentiful during the day, the subsequent degradation of PHA (possibly by the paralogs of 3-ketothiolase that exhibited diurnal expression) would release carbon and electrons for use in the operation of the combined TCA and 3-OHP cycles. Acetate and inorganic electron donors are probably more scarce during the day because of the lack of cyanobacterial fermentation and nitrogen fixation (that is, hydrogen production) and because of competition with aerobic chemoorganoheterotrophs for organic substrates.

Wax esters. Wax esters represent another potential class of carbon and electron storage compounds produced by FAPs (Shiea *et al*., 1991, van der Meer *et al*., 2001). Their degradation to acetyl-CoA could serve as a source of electrons for either the reduction of CO_2 to biomass or for oxidative phosphorylation to produce ATP when O_2 is available as a terminal electron acceptor. Consistent with nighttime wax ester synthesis, transcripts for a *Roseiflexus* spp. gene (RoseRS_2456) homologous to wax ester synthase were most abundant at night (Figure 3b). A corresponding ortholog of this wax ester synthase was not detected in any *Chloroflexus* spp. genome, and it is currently unknown how *Chloroflexus* spp. synthesize the wax esters observed in previous studies (Knudsen *et al*., 1982). Both *Roseiflexus* spp. and *Chloroflexus* spp. exhibited constitutive expression of fatty acid biosynthesis genes. Consistent with daytime degradation of wax esters, transcripts for genes encoding enzymes for the β-oxidation of fatty acids were most abundant during the day for both *Roseiflexus* spp. and *Chloroflexus* spp. (Figure 3a). Metabolomics results are also consistent with diel cycling of *Roseiflexus* spp. wax esters (Y-M Kim, T Metz and D Ward, unpublished).

Previous inferences from isotopic labeling studies must be reinterpreted in light of the possible cycling of wax ester synthesis and degradation. Van der Meer *et al.* (2005) showed that inorganic carbon was incorporated into the wax esters of FAPs during a low-light morning period, when H_2 and sulfide were available as inorganic electron sources, but not in the afternoon when the mat is oxic. This was interpreted as being consistent with hypothesized CO_2 fixation by *Roseiflexus* spp. in the post-dawn mat, followed by midday photoheterotrophic metabolism. Alternatively, these observations could be explained by the timing of wax ester synthesis and degradation. In fact, a later study (van der Meer *et al*., 2007) suggested that incorporation of ^{13}C-bicarbonate into a mat fraction enriched in *Roseiflexus* spp. was higher at midday than in early morning (nearly equivalent to midday incorporation by a mat fraction enriched in *Synechococcus* spp.). These observations are consistent with the suggestion that *Roseiflexus* spp. have a major role in the sequestration of inorganic carbon during the entire light period through photomixotrophic CO_2 fixation.

Nitrogen and hydrogen metabolism

Chloroflexus spp. and *Roseiflexus* spp. differ in their acquisition of nitrogen, which is an essential nutrient for the biosynthesis of proteins, nucleic acids and BChls. Studies with isolates of *Chloroflexus* spp. have shown that ammonia and some amino acids can serve as nitrogen sources, but urea, nitrate and nitrite are not utilized (Hanada and Pierson, 2006). Furthermore, *Chloroflexus* spp. do not possess genes for dinitrogen fixation (Heda and Madigan, 1986). Like *Oscillochloris trichoides* (Kuznetsov *et al*., 2011), *Roseiflexus* spp. have an operon encoding *nifB* and the structural genes of nitrogenase (*nifHBDK*, RoseRS_1201 - RoseRS_1198). Although growth of *O. trichoides* on N_2 was reportedly poor, nitrogenase activity was detected by the acetylene reduction assay (Keppen *et al*., 1989; Keppen *et al*., 1994; and Tourova *et al*., 2006). Nitrogenase activity has not yet been confirmed in *Roseiflexus* spp. strains, but the overall sequence similarity of the *nifHBDK* genes to those in *O. trichoides* suggests that these genes may encode a functional nitrogenase. The absence of *nifV* and *nifEN* genes, however, suggests that homocitrate may not be a ligand in the FeMo cofactor and that the P cluster and FeMoCo (or their equivalents) might be directly assembled on the NifD/NifK heterodimer.

Transcripts for the *nifHBDK* genes in *Roseiflexus* spp. were more abundant at night (Figure 3b), but transcripts for *amtB*, encoding an ammonia transporter in *Roseiflexus* spp., exhibited a diurnal expression pattern (Supplementary Table S3). These results are similar to results for *Synechococcus* spp. in the mat, for which *nif* transcripts were also most abundant at night, and nitrogenase activity has been detected during the night and in the early morning (Steunou *et al*., 2006, 2008). Metagenomic scaffolds for *Chloroflexus* spp. contain two homologs of *amtB* (Caur_1002), and transcript levels for both were also highest during the day. Genes encoding subunits of a [Ni-Fe] hydrogenase (*hydABCD*, RoseRS_2319-RoseRS_2322) in *Roseiflexus* spp. exhibited a nocturnal pattern (Figure 3b). This suggested that H_2 produced as a by-product of nitrogen fixation by cyanobacteria and perhaps *Roseiflexus* spp. could be an important source of electrons for hypothesized light-driven CO_2 fixation in FAPs in the morning (see above), when light is available but while the mat remains anoxic (van der Meer *et al*., 2005). Clearly, further studies will be required to establish whether *Roseiflexus* spp. can actually fix dinitrogen, and virtually nothing is presently known about the *in situ* utilization of amino acids as nitrogen sources. However, the data suggest that *Chloroflexus* spp. and *Roseiflexus* spp. may not directly compete for the same nitrogen sources throughout the complete diel cycle.

Electron transport complexes

Because the data could provide information about metabolic modes employed by FAPs in these mats at different periods throughout the diel cycle, the transcript abundances for genes encoding various proteins involved in electron transport were of particular interest. Specific components of the electron transport chain may become more important at different times during the diel cycle. For example, the need for an external source of electrons might increase when FAPs couple phototrophy with

inorganic carbon fixation. In *Roseiflexus* spp., the previously mentioned nocturnal expression patterns of *hyd* genes could enable the oxidation of H_2 in order to provide electrons for inorganic carbon fixation, similar to the patterns observed for the *puf*, *bch* and the putative *nif* genes mentioned previously.

Given the environmental fluctuations in O_2 concentration that these organisms experience (Supplementary Figure S1; Ward *et al.*, 2006; Steunou *et al.*, 2008: Jensen *et al.* 2011), it is logical that they might maintain different sets of enzymes, specialized for either oxic or anoxic conditions, for some of the same reactions (Tang *et al.*, 2011; Bryant *et al.*, 2012). Genomes of *Chloroflexus* spp. contain paralogous genes encoding the soluble electron carrier, auracyanin (McManus *et al.*, 1992; van Driessche *et al.*, 1999; Tsukatani *et al.*, 2007; Cao *et al.* 2012), and both *Chloroflexus* and *Roseiflexus* spp. contain paralogs of some of the major enzyme complexes involved in the electron transport chain, namely NADH:menaquinone oxidoreductase (Complex I; van der Meer *et al.*, 2010; Tang *et al.*, 2011), and Alternative Complex III (ACIII; Yanyushin *et al.*, 2005; Gao *et al.*, 2009). The expression patterns of these genes are shown in Table 1 and are discussed below.

Respiratory electron transport complexes. *Chloroflexus* spp. contain two paralogous groups of genes encoding subunits of ACIII, which oxidizes menaquinol and donates electrons to soluble carriers such as the blue-copper protein auracyanin on the periplasmic side of the cytoplasmic membrane. These two gene sets have been named Cp, for the ACIII predicted to operate primarily for cyclic phototrophic electron transfer, and Cr, for the ACIII predicted to function in respiratory electron transfer to a terminal electron acceptor such as O_2 (Yanyushin *et al.*, 2005). Surprisingly, the transcript levels for the genes encoding Cp and Cr did not show much temporal variation, and there was no evidence to suggest that *Chloroflexus* spp. modulate the transcriptional activity of their paralogous ACIII complexes in order to produce complexes specialized for either phototrophic or respiratory electron transfer. Regardless of the lack of *in situ* transcriptional evidence, a recent proteomic study of *C. aurantiacus* showed that peptides derived from subunits of the Cp-ACIII complex occurred in higher abundance during photoheterotrophic growth compared with levels in cells grown chemoorganoheterotrophically (Cao *et al.*, 2012). It remains to be determined whether the abundance of the Cp and Cr proteins exhibit diel patterns, and ongoing metaproteomic work will determine if the same pattern is observed *in situ*. *Roseiflexus* spp. contain only one set of genes encoding a Cp-like ACIII, which thus are likely to function in both phototrophic and dark respiratory electron transfer. Because of this predicted dual function, it was unexpected that the corresponding genes of *Roseiflexus* spp. ACIII would exhibit different temporal expression patterns; however, transcripts for these genes (*actABCDEF*) were most abundant at night (Table 1).

Chloroflexus spp. and *Roseiflexus* spp. use two terminal cytochrome oxidases to perform aerobic respiration. One terminal oxidase, encoded by homologs of RoseRS_2263 to RoseRS_2266 in *Roseiflexus* spp., apparently comprises subunits I–IV of a caa_3-type cytochrome *c* oxidase (Table 1). Transcript patterns for these genes suggested that this complex is produced during the day. The other complex, encoded by RoseRS_0933 and RoseRS_0934, is predicted to be a ba_3-type cytochrome oxidase, and transcripts for these genes were most abundant at night (see Table 1). Homologs of each of the six genes in *Roseiflexus* spp. were also found in *Chloroflexus* spp., and the aa_3-type oxidase also seemed to be most abundant during the day (Table 1). However, transcripts for the ba_3-type oxidase were not detected.

Soluble electron carriers

Chloroflexus spp. genomes contain genes encoding auracyanin A and B *(aurA*, Caur_3248 and *aurB*, Caur_1950). These proteins were hypothesized to function during phototrophic (AurA) and respiratory (AurB) electron transfer, respectively (Lee *et al.*, 2009). Contrary to these predictions, a recent proteomic analysis of *C. aurantiacus* OK-70-fl revealed that peptides corresponding to AurA were more abundant in cultures grown under dark oxic conditions than when grown photoheterotrophically, while peptides from AurB showed higher abundance during photoheterotrophic growth (Cao *et al.*, 2012). The transcript levels for the *aurA* and *aurB* genes of *Chloroflexus* spp. were relatively constant over a diel cycle (Table 1); additional work is needed to verify whether there are differences in the expression of AurA *in situ*. *Roseiflexus* spp. have only one gene for auracyanin (Tsukatani *et al.*, 2007), and its transcript levels were highest during the day. Ongoing metaproteomic characterization of this community such as the work done by Schaffert *et al.* (2012) could indicate whether the abundance of proteins correlates with the observed transcription patterns and will enable direct comparisons to the proteomes of *C. aurantiacus* cells grown heterotrophically and photoheterotrophically in the laboratory (Cao *et al.*, 2012).

Alternative reactions involving CO_2

Depending upon the specific chemistry of the reactions, many enzymes other than those involved in the 3-OHP bi-cycle have the potential to either incorporate or release inorganic carbon. Analogous to the PFOR enzyme discussed above, phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) catalyzes another anaplerotic carboxylation reaction in

Table 1 Expression categories of genes involved in electron transport

Gene	Roseiflexus *spp. RS1 homolog*	Roseiflexus *expression category*[a]	Chloroflexus aurantiacus *J-10-fl homolog*	Chloroflexus *expression category*[a]
NADH menaquinone oxidoreductase (Complex I)				
nuoA	RoseRS_2089[b]	Weak night		
nuoB	RoseRS_2090[b]	[c]		
nuoC	RoseRS_2091[b]	Strong day		
nuoD	RoseRS_2092[b]	[c]		
nuoE	RoseRS_3543	Strong night	Caur_1184	NOT FOUND
nuoF	RoseRS_3542	[c]	Caur_1185	Night
nuoA	RoseRS_2989	Constitutive	Caur_1987	Constitutive
nuoB	RoseRS_2990	Weak night	Caur_1986	Night
nuoC	RoseRS_2991	Weak night	Caur_1985	Night
nuoD	RoseRS_2992	Weak night	Caur_1984	Constitutive
nuoI	RoseRS_2993	Weak night	Caur_1983	NOT FOUND
nuoH	RoseRS_2994	Constitutive	Caur_1982	Constitutive
nuoJ	RoseRS_2995	Weak day	Caur_1981	Constitutive
nuoK	RoseRS_2996	Constitutive	Caur_1980	Constitutive
nuoL	RoseRS_2997	Constitutive	Caur_1979	Day
nuoM	RoseRS_2998	Constitutive	Caur_1978	Night
nuoM	RoseRS_2999	[c]	Caur_1977	Constitutive
nuoN	RoseRS_3000	[c]	Caur_1976	1600 hours spike
nuoA	RoseRS_3678	Strong day	Caur_2896	Day
nuoB	RoseRS_3677	Strong day	Caur_2897	Day
nuoC	RoseRS_3676	Strong day	Caur_2898	Constitutive
nuoD	RoseRS_3675	Strong day	Caur_2899	Day
nuoE	RoseRS_2238	Strong day	Caur_2900	Day
nuoF	RoseRS_2237	Strong day	Caur_2901	Day
nuoG	RoseRS_2236	Strong day	Caur_2902	1600 hours spike
nuoH	RoseRS_2235	Strong day	Caur_2904	1600 hours spike
nuoI	RoseRS_2234	Constitutive	Caur_2905	1600 hours spike
nuoJ	RoseRS_2233	Weak day	Caur_2906	1600 hours spike
nuoK	RoseRS_2232	Strong day	Caur_2907	1600 hours spike
nuoL	RoseRS_2231	Strong day	Caur_2908	Day
nuoM	RoseRS_2230	Weak day	Caur_2909	Day
Alternative complex III quinone oxidoreductase				
actA (Cp)	RoseRS_4139	Weak night	Caur_0621	Constitutive
actB (Cp)	RoseRS_4140	Weak night	Caur_0622	Constitutive
actC (Cp)	RoseRS_4141	Weak night	Caur_0623	Constitutive
actD (Cp)	RoseRS_4142	Weak night	Caur_0624	NOT FOUND
actE (Cp)	RoseRS_4143	Weak night	Caur_0625	Constitutive
actF (Cp)	RoseRS_4144	Weak night	Caur_0626	Day
actG (Cp)			Caur_0627[d]	NOT FOUND
actB (Cr)			Caur_2136[d]	Day
actE (Cr)			Caur_2137[d]	1600 hours spike
actA (Cr)			Caur_2138[d]	Constitutive
actG (Cr)			Caur_2139[d]	Constitutive
SC01/SenC e^- transport?			Caur_2140[d]	Day
Auracyanin				
auracyanin A	RoseRS_2366	Weak day	Caur_3248	Constitutive
auracyanin B			Caur_1950[d]	Constitutive
Cytochrome c *oxidase (Complex IV)*				
ctaC	RoseRS_2263	Weak day	Caur_2141	NOT FOUND
ctaD	RoseRS_2264	Strong day	Caur_2142	Day
ctaE	RoseRS_2265	[c]	Caur_2143	Day
ctaF	RoseRS_2266	Constitutive	Caur_2144	NOT FOUND
cbaA	RoseRS_0934	Strong night	Caur_2426	NOT FOUND
cbaB	RoseRS_0933	Strong night	Caur_2425	NOT FOUND

[a]Expression categories were determined by labeling the dominant trends shared by clusters of genes demarcated by k-means analysis.
[b]Locus ID names are marked if they are specific to the *Roseiflexus* spp.
[c]Genes that were expected but not found in either the metagenome scaffolds or did not have a significant level of uniquely mapped transcripts are also indicated.
[d]Locus ID names are marked if they are specific to the *Chloroflexus* spp.

Chloroflexus spp. and *Roseiflexus* spp. and likely provides an additional route by which inorganic carbon is fixed in these organisms (Tang *et al.*, 2011). The transcripts for the *ppc* gene (Caur_3161), encoding phosphoenolpyruvate carboxylase for *Chloroflexus* spp. were more abundant during the day, when the transcript abundance of genes involved in the 3-OHP bi-cycle and TCA cycle were also higher (Supplementary Table S3). Transcript levels for the *ppc* gene in *Roseiflexus* spp. (RoseRS_2753) did not change much over the diel cycle.

Concluding remarks

The metabolic activities of uncultivated Chloroflexi inhabiting Mushroom Spring cannot explicitly be inferred from gene expression patterns alone. Nevertheless, such patterns provide evidence for the diel regulation of biochemical functions at the transcriptional level and provide a basis for modeling the responses of these organisms to environmental stimuli, such as light and O_2 concentration. The results presented here lead to the following hypotheses about FAP metabolism during the diel cycle.

FAPs perform photomixotrophy during the day by degrading internal carbon storage polymers such as wax esters and PHAs to obtain metabolic intermediates and electrons, by incorporating and metabolizing glycolate produced by cyanobacterial photorespiration or both. The TCA and the 3-OHP cycles are both predicted to function in central carbohydrate metabolism. The resulting ATP and electrons can be applied to daytime gluconeogenesis for the production of glycogen. During the transition between light and dark periods, FAPs are predicted to utilize photomixotrophic metabolism; in this way they could simultaneously reduce their need for an external electron acceptor (O_2) by using light-driven cyclic transport for ATP synthesis and retain organic compounds for biomass accumulation.

As light and O_2 levels decrease and light-driven electron transport diminishes, we propose that FAPs couple fermentation of their stored glycogen to the synthesis of PHAs and possibly wax esters. Hydrogenase activity during the night may act as an electron valve for the disposal of excess reductant, although most electrons are probably retained by the production of PHAs and wax esters. The branched TCA cycle operates during this time to produce either succinyl-CoA, which can be converted to propionyl-CoA and thus PHAs, or 2-oxoglutarate for BChl biosynthesis, which, in turn, is applied to the assembly of photosynthetic reaction centers and light-harvesting structures. The latter are clearly produced during the night and would be available for ATP production via cyclic electron transport at dawn. At this time, O_2 concentrations in the illuminated portions of the mat are still low (Steunou *et al.*, 2008; Jensen *et al.*, 2011), and FAPs are predicted to perform CO_2 fixation, while they incorporate exogenous organic carbon sources photomixotrophically. Fermentation and N_2 fixation from *Synechococcus* spp. (Steunou *et al.*, 2008) can produce H_2 that can be cross-fed to FAPs, and thus provide an external supply of electrons for the reductive steps of the 3-OHP bi-cycle.

The observed transcription patterns for genes encoding hydrogenase and enzymes of the 3-OHP bi-cycle contrasts with the previous hypothesis that FAPs perform light-driven CO_2 fixation in the early morning. Despite this temporal discrepancy in transcript abundance, it is possible that differences exist between transcription and translation, or perhaps post-translational regulation of these proteins would allow CO_2 fixation and hydrogenase activities to occur simultaneously. The production and degradation of carbon and energy storage polymers are predicted to be central features of FAP metabolism, which may provide carbon and electrons at times when external sources of these resources are limited. The timing of the acquisition and use of these resources may be regulated in order to reduce competition with other populations within the mat community.

Finally, it is noted that the importance of mixotrophic modes of metabolism may have implications for inferences regarding the formation of organic matter in phototrophic microbial mats and in stromatolite fossils. Past results concerning the stable isotopic composition of lipid biomarkers in these organisms and in similar hot spring environments have been interpreted to indicate that FAPs perform either strictly photoautotrophic or photoheterotrophic metabolism at different times of the diel period (van der Meer *et al.*, 2001, 2005). The metatranscriptomic patterns in this study suggest that the natural abundance levels of carbon stable isotopes in FAP lipid biomarkers could possibly result from fractionation effects arising from simultaneous incorporation of organic and inorganic carbon sources.

Conflict of Interest

The authors declare no conflict of interest.

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