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Biochemical Validation of the Glyoxylate Cycle in the Cyanobacterium *Chlorogloeopsis fritschii* Strain PCC 9212*[S]

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Background: Conflicting claims exist concerning the occurrence of the glyoxylate cycle in cyanobacteria.
Results: The genes for isocitrate lyase and malate synthase were identified in *Chlorogleopsis fritschii* PCC 9212 and the purified enzymes were characterized.
Conclusion: *C. fritschii* has a functional glyoxylate cycle and can grow in the dark on acetate.
Significance: These results clarify the occurrence of the glyoxylate cycle in cyanobacteria.

Cyanobacteria are important photoautotrophic bacteria with extensive but variable metabolic capacities. The existence of the glyoxylate cycle, a variant of the TCA cycle, is still poorly documented in cyanobacteria. Previous studies reported the activities of isocitrate lyase and malate synthase, the key enzymes of the glyoxylate cycle in some cyanobacteria, but other studies concluded that these enzymes are missing. In this study the genes encoding isocitrate lyase and malate synthase from *Chlorogloeopsis fritschii* PCC 9212 were identified, and the recombinant enzymes were biochemically characterized. Consistent with the presence of the enzymes of the glyoxylate cycle, *C. fritschii* could assimilate acetate under both light and dark growth conditions. Transcript abundances for isocitrate lyase and malate synthase increased, and *C. fritschii* grew faster, when the growth medium was supplemented with acetate. Adding acetate to the growth medium also increased the yield of poly-3-hydroxybutyrate. When the genes encoding isocitrate lyase and malate synthase were expressed in *Synechococcus* sp. PCC 7002, the acetate assimilation capacity of the resulting strain was greater than that of wild type. Database searches showed that the genes for the glyoxylate cycle exist in only a few other cyanobacteria, all of which are able to fix nitrogen. This study demonstrates that the glyoxylate cycle exists in a few cyanobacteria, and that this pathway plays an important role in the assimilation of acetate for growth in one of those organisms. The glyoxylate cycle might play a role in coordinating carbon and nitrogen metabolism under conditions of nitrogen fixation.

Under natural growth conditions, all bacteria continually face changing nutrient availability, and consequently they must strategically adapt their metabolic capabilities in response to such changes. In addition to utilizing various storage compounds, the capacity to take up and use dissolved carboxylic acids, such as acetate, lactate, pyruvate, and succinate, from the surrounding environment is important for sustainable growth under many conditions (1–3). The ability to assimilate organic carbons also exists in some autotrophic bacteria, including cyanobacteria, even though most are able to synthesize all essential precursor metabolites from CO_2 (4). The assimilation of dissolved carboxylic acids by heterotrophic bacteria has been known and studied for decades (5, 6). Acetate is one of the most common and important carbon sources for many bacteria, and acetate is frequently used as a carbon source by eukaryotic microalgae (7, 8). Once acetate is transported into the cytosol, it is first converted by acetyl-CoA synthetase to acetyl coenzyme A (acetyl-CoA),[2] which can then be used by the tricarboxylic acid (TCA) cycle or the glyoxylate cycle to produce other important precursor metabolites, such as 2-oxoglutarate and oxaloacetate (9, 10).

Sir Hans Adolf Krebs, who also established the urea/ornithine cycle as well as the TCA cycle, discovered the glyoxylate cycle (11, 12). The glyoxylate cycle is usually described as a modified TCA cycle, because it shares the activities of malate dehydrogenase, citrate synthase, and aconitase with the TCA cycle (Fig. 1). However, the difference lies in the two key enzymes that are used in the glyoxylate cycle but which are not used in the TCA cycle, namely isocitrate lyase (AceA) and malate synthase (AceB), which convert isocitrate and acetyl-CoA into succinate and malate (Fig. 1). In more detail, isocitrate is split into succinate and glyoxylate by isocitrate lyase, after which glyoxylate and acetyl-CoA are condensed to form malate with the release of CoA by malate synthase. Malate is further converted to oxaloacetate by malate dehydrogenase to continue the cycle, and succinate is released as the net product. Overall, the net reaction of the glyoxylate cycle, which can be used to produce precursors for amino acid or carbohydrate biosynthesis, allows cells to convert two acetyl-CoA units into succinate and avoid the CO_2-releasing steps of the TCA cycle. Thus, the glyoxylate cycle enables cells to utilize C_2 units (*i.e.* acetyl-CoA) more efficiently for biomass production. These C_2

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[S] This article contains supplemental Table S1.
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[2] The abbreviations used are: acetyl-CoA, acetyl coenzyme A; FaRLiP, far-red light photoacclimation; ICL, isocitrate lyase; PCC, Pasteur Culture Collection; PHB, poly-3-hydroxybutyrate; TCA, tricarboxylic acid.

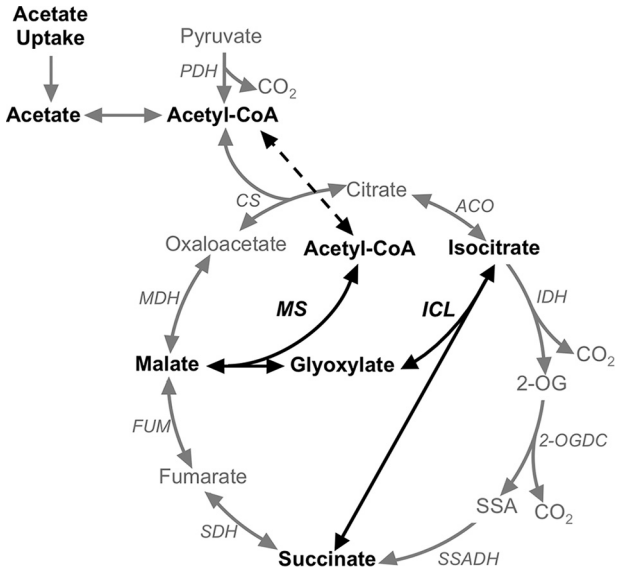

FIGURE 1. **Scheme showing the glyoxylate and TCA cycles in some cyanobacteria.** Abbreviations used were: *2-OG*, 2-oxoglutarate; *2-OGDC*, 2-oxoglutarate decarboxylase; *ACO*, aconitase; *CS*, citrate synthase; *FUM*, fumarase; *ICL*, isocitrate lyase; *IDH*, isocitrate dehydrogenase; *MDH*, malate dehydrogenase; *MS*, malate synthase; *PDH*, pyruvate dehydrogenase; *SDH*, succinic acid dehydrogenase; *SSA*, succinic semialdehyde; *SSADH*, succinic semialdehyde dehydrogenase. The *heavy arrows* show the two reactions specific for the glyoxylate cycle.

units can be derived from ethanol or acetate as the sole carbon source, and collectively these reactions are usually correlated with the ability of bacteria to assimilate acetate (13).

The glyoxylate cycle has been found in many chlorophototrophic bacteria (14, 15). Isocitrate lyase and malate synthase are found in all chlorophototrophic members of the Chloroflexi (*e.g. Chloroflexus* spp., *Oscillochloris trichoides*, and *Roseiflexus* spp.). By using the glyoxylate cycle, all of these organisms are able to photoassimilate acetate, and some can even grow heterotrophically on acetate (16, 17). In addition, the glyoxylate cycle occurs in most purple sulfur bacteria, which can also photoassimilate acetate. However, no genes encoding these enzymes have yet been identified in most purple non-sulfur bacteria (15). Heliobacteria, green sulfur bacteria, and *Chloracidobacterium thermophilum* lack isocitrate lyase and malate synthase, and thus the glyoxylate cycle is absent in these bacteria. Interesting, heliobacteria and green sulfur bacteria use a different acetate assimilation mechanism, the carboxylation of acetyl-CoA by pyruvate synthase, and thus these bacteria are thus able to assimilate both acetate and CO_2 at the same time (18, 19).

Cyanobacteria are a large group of oxygenic chlorophototrophic bacteria with highly diverse metabolic capabilities, but the occurrence of the glyoxylate cycle in these organisms has remained controversial (20). Although it has been reported that isocitrate lyase and/or malate synthase activities were detected in some cyanobacteria (21, 22), and that some cyanobacteria were able to assimilate acetate (23, 24), a recent study in *Synechocystis* sp. PCC 6803 failed to detect the enzymes of the glyoxylate cycle (25). However, a recent genome sequencing study reported that two *Cyanothece* spp. (strains PCC 7424 and PCC 7822) have an operon encoding the isocitrate lyase and malate synthase (26). However, this study did not demonstrate acetate utilization or the enzyme activities of the genes in question. Database searches showed that similar operons were also found in the genomes of two *Chlorogloeopsis* sp., strains, PCC 6912 and PCC 9212. Consistent with the presence of these two genes and thus the glyoxylate cycle in the *Chlorogloeopsis* spp., one of the organisms had been reported to assimilate acetate under both light and dark conditions (23).

Further confusing the properties of the TCA and glyoxylate cycle enzymes in cyanobacteria, no gene encoding fumarase was initially identified in the annotation of the genome of *Synechococcus* sp. PCC 7002, although a fumarase was annotated in the genome of *Synechocystis* sp. PCC 6803. BLASTP searches showed that, among all the gene products in *Synechococcus* sp. PCC 7002, the product of the open reading frame of SYNPCC7002_A2041 had the highest sequence identity (43%) to the fumarase (slr0018) from *Synechocystis* sp. PCC 6803. Although it had initially been misannotated as aspartate ammonia-lyase, it thus seemed likely that this gene encodes fumarase.

In this study, we describe the biochemical validation of the predicted fumarase (SYNPCC7002_A2041) from *Synechococcus* sp. PCC 7002, as well as for two genes in *Chlorogloeopsis fritschii* PCC 9212 that encode the key enzymes, isocitrate lyase and malate synthase, of the glyoxylate cycle. We show that *C. fritschii* PCC 9212 can take up acetate under both light and dark conditions, and that the organism grows faster when acetate is supplied in the medium. Whole cell transcription profiling showed that the mRNA levels of these two genes increased when cells were grown with acetate. Furthermore, *C. fritschii* PCC 9212 cells accumulated much higher poly-3-hydroxybutyrate (PHB) levels when cells were supplied with acetate. This observation suggested that the extra carbon supplied as acetate was mainly stored as PHB. Additionally, when the genes for isocitrate lyase and malate synthase were overexpressed in *Synechococcus* sp. PCC 7002, this cyanobacterium exhibited an enhanced capacity for acetate uptake, confirming that the glyoxylate cycle can play an important role in acetate utilization even for an organism that normally lacks this capability. Overall, this study validates the existence of the glyxoxylate cycle in cyanobacteria but demonstrates that only a small number of cyanobacteria actually have this cycle. Our studies show that the glyoxylate cycle is not a common or prominent feature of cyanobacterial metabolism, but it may nevertheless be important for acetate utilization in those few organisms that have the enzymes of this pathway.

Experimental Procedures

Strains and Growth Conditions—C. fritschii PCC 9212 was obtained from the Pasteur Culture Collection (PCC) and routinely grown in medium BG-11 at 26 °C (27). To emphasize the effects of acetate supplementation, cells were grown under constant irradiance of 50 μmol photons m^{-2} s^{-1}, which was provided by cool white fluorescent tubes, and cultures were sparged with 1% (v/v) CO_2 in air (standard growth conditions). Low CO_2 growth conditions were achieved by bubbling cultures with air while keeping all other growth conditions the same. When required, the growth medium was supplemented with 10 mM sodium acetate. The wild-type strain of *Synechococcus* sp. PCC 7002 as well as a strain overexpressing the genes

TABLE 1
Oligonucleotide primers used for PCR in this study

Name	Sequence (5′ to 3′)
MSF	TTGCGTCCATGGCAAAAGGCTTTTCCTCTG
MSR	GTGGTGCTCGAGAAAACTGACAAGATGACG
ICLF	GCGCGGCATATGAGTAAATCTACTTTTGAA
ICLR	TAGTATGGATCCCTACTCATCATCCATGCG
FUMF	CAGTCAAAACATATGAGCGCAGAT
FUMR	CGCCTTGGATCCTCCCGATTA ATT

of the glyoxylate cycle (strain *Synechococcus* 7002-glyox) were grown in liquid A^+ medium under standard conditions for this organism (28): cells were grown at an irradiance of 250 μmol photons m^{-2} s^{-1} provided by cool white fluorescent lights, at 38 °C and cultures were sparged with 1% (v/v) CO_2 in air. Low irradiance or low CO_2 growth conditions were produced by growing cells under 50 μmol photons m^{-2} s^{-1} or by sparging cultures with air under otherwise standard conditions. When required for experiments with *Synechococcus* sp. PCC 7002, 10 mM sodium acetate was added to the A^+ medium.

Acetate Concentration Measurement in Growth Medium—The concentration of acetate in the medium at different growth stages was determined by high-performance liquid chromatography (HPLC). In detail, aliquots (0.5 ml) of cell culture were removed from the growth medium at different growth stages. After centrifugation, the supernatant was filtered through 0.2-μm sterile syringe filter (VWR, Philadelphia, PA). A 20-μl aliquot of the filtered solution was loaded directly onto a Shimadzu LC-20AB HPLC system equipped with 210-nm UV detector SPD-20A. Different components in the medium were separated on a Supelcogel C610H column (Supelco, Bellefonte, PA), using 4 mM H_2SO_4 as the mobile phase. The flow rate was 0.5 ml min^{-1} and the chromatography was performed at 30 °C. Acetate concentrations were calculated on the basis of peak area using a standard curve generated from known concentrations of sodium acetate.

Cloning, Protein Purification, and Protein Identification—Open reading frames SYNPCC7002_A2041, encoding the putative fumarase of *Synechococcus* sp. PCC 7002, UYEDRAFT_02681, encoding the putative isocitrate lyase and UYEDRAFT_02682, encoding the putative malate synthase of *C. fritschii* PCC 9212 were amplified by polymerase chain reaction (PCR) with Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and separately cloned into plasmid pAQ1Ex-P_{cpcBA} (29). Primer set ICLF-ICLR was used to amplify UYEDRAFT_02681, primer set MSF-MSR was used to amplify UYEDRAFT_02682, and primer set FUMF-FUMR was used to amplify SYNPCC7002_A2041 (Table 1). An N-terminal His_{10} tag was introduced into isocitrate lyase and fumarase to facilitate subsequent protein purification. Initial attempts to add a His_{10} tag to the N terminus of malate synthase were not successful, and subsequently, a His_6 tag was successfully added to the C terminus. The resulting plasmids pAQ1Ex-P_{cpcBA}::*A2041*, pAQ1Ex-P_{cpcBA}::*U02681*, and pAQ1Ex-P_{cpcBA}::*U02682* were verified by DNA sequencing and transformed into *Escherichia coli* strain DH5-α. Cells were grown overnight in 1 liter of Luria-Bertani (LB) medium containing 50 μg ml^{-1} gentamycin, harvested by centrifugation at 4 °C at 5,000 × *g*, and washed once with 50 mM Tris-HCl buffer, pH 8.0. Cells were disrupted by three passages through a chilled French pressure cell operated at 138 MPa. Soluble lysates were obtained by centrifugation at 20,000 × *g* for 30 min and loaded onto a Ni^{2+}-NTA affinity resin (Goldbio, St. Louis, MO), which was pre-equilibrated with 10 mM imidazole in 50 mM Tris-HCl, pH 8.0, and washed with 30 mM imidazole in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl. Proteins were eluted stepwise with 50, 100, 150, 200, and 250 mM imidazole in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl. Fractions containing the recombinant proteins were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and concentrated by ultrafiltration using Centriprep columns (Millipore, Billerica, MA). Purified proteins were further analyzed by SDS-PAGE and immunoblotting with commercial antibodies (Rockland, Limerick, PA) to the poly-His tags. Proteins were also positively identified by tryptic peptide mass fingerprinting as previously described (20).

Enzymatic Assays—Fumarase activity was assayed by separately measuring the reversible interconversion of malate into fumarate, as catalyzed by the recombinant enzyme. For the conversion of malate to fumarate, the reaction mixture (0.2 ml) contained 10 mM malate, 50 mM K-phosphate, pH 7.0, and 50 μg of purified SynPCC7002_A2041. The mixture was incubated at room temperature for 1 h, and an aliquot (20 μl) of the reaction mixture was injected into the HPLC for analysis. For the conversion of fumarate to malate, the reaction mixture (0.2 ml) contained 2.5 mM fumarate, 50 mM K-phosphate, pH 7.0, and 50 μg of purified SynPCC7002_A2041. The assay mixture was incubated at room temperature for 1 h, and an aliquot (20 μl) of the reaction mixture was injected into the HPLC for analysis. Control experiments were performed in a similar manner but without the addition of the purified enzyme.

For enzyme assays with isocitrate lyase, the reaction mixture (0.2 ml) contained 2 mM isocitrate, 50 mM K-phosphate, pH 7.8, 2 mM $MgCl_2$, and 50 μg of purified UYEDRAFT_02681. The mixture was incubated at room temperature for 1 h, and then an aliquot (20 μl) of the reaction mixture was injected into the HPLC for analysis. The condensation of succinate and glyoxylate to isocitrate by isocitrate lyase was also assayed. The reaction mixture (0.2 ml) contained 1 mM glyoxylate, 1 mM succinate, 50 mM K-phosphate, pH 7.8, 1 mM $MgCl_2$, and 50 μg of purified UYEDRAFT_02681. The mixture was incubated at room temperature for 1 h, and then an aliquot (20 μl) of the reaction mixture was injected into the HPLC for analysis. Control experiments were performed similarly but without the addition of the purified enzyme.

For enzyme assays with malate synthase, the reaction mixture (0.2 ml) contained 2 mM acetyl-CoA, 2 mM glyoxylate, 2 mM $MgCl_2$, 50 mM K-phosphate, pH 7.8, and 50 μg of purified UYEDRAFT_02682. The mixture was incubated at room temperature for 1 h, and an aliquot (20 μl) of the reaction mixture was injected into the HPLC for analysis. Control experiments were performed similarly but without the addition of the purified enzyme. The elution times and concentrations of substrates and products were determined by comparison of results obtained from analyses of individual compounds.

Overexpression of Glyoxylate Cycle Genes in Synechococcus sp. PCC 7002—Open reading frames UYEDRAFT_02681 (isocitrate lyase) and UYEDRAFT_02682 (malate synthase)

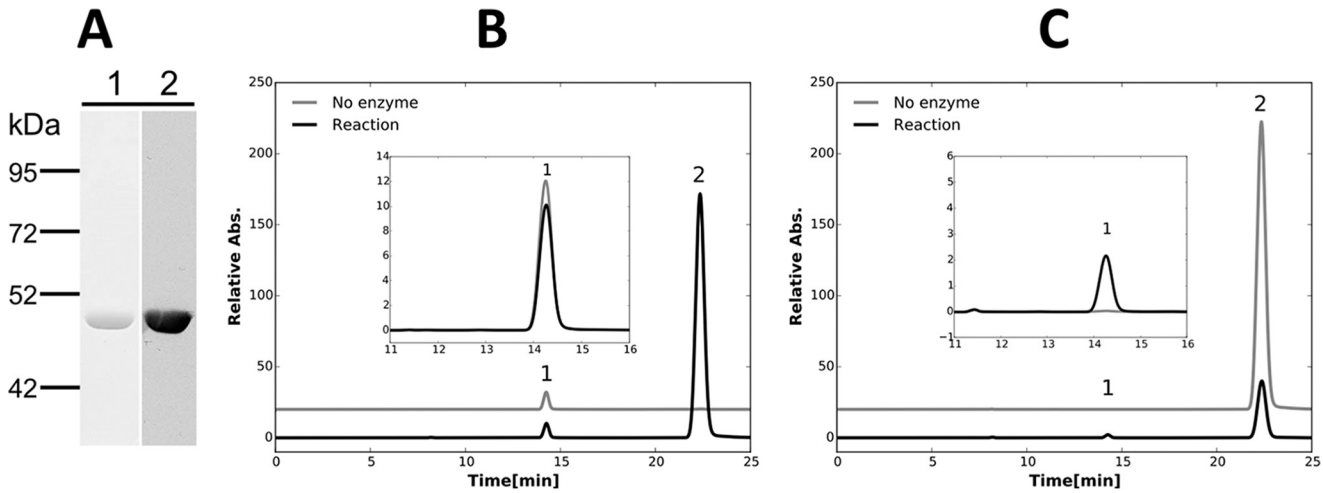

FIGURE 2. **Characterization of purified recombinant fumarase (SYNPCC7002_A2041).** *A,* SDS-PAGE (*lane 1*) and immunoblotting analysis with a commercial antibody to the poly-His_6 tag (*lane 2*) of the purified fumarase. *B,* HPLC analysis showing that malate (*peak 1*) was converted to fumarate (*peak 2*) by fumarase (SYNPCC7002_A2041). Specifically, when the product of SynPCC7002_A2041 was incubated with 10 mM malate at room temperature, 2.1 mM fumarate was formed and 2.2 mM malate was consumed. *C,* HPLC analysis showing the formation of malate (*peak 1*) from fumarate (*peak 2*) catalyzed by the purified fumarase (SYNPCC7002_A2041). When the product of SynPCC7002_A2041 was incubated with 2.5 mM fumarate at room temperature, 1.9 mM malate was formed and 2.1 mM fumarate was consumed (*C*). The differences in the peak areas for identical amounts of fumarate and malate are due to the very different molar extinction coefficients of these two compounds at 210 nm. *Insets* represent the enlarged parts of the elution curves from 11 to 16 min to illustrate the changes observed more clearly. Other details of the assay conditions are described under "Experimental Procedures."

form an apparent operon in *C. fritschii* PCC 9212, and the entire operon was amplified by PCR and inserted into the pAQ1-based expression system (29) using primer sets MSF and ICLR (Table 1). The resulting plasmid was verified by DNA sequencing and transformed into wild type *Synechococcus* sp. PCC 7002 as previously described (30). The presence of the desired genes in strain *Synechococcus* 7002-glyox was confirmed by PCR using primer set MSF and ICLR (Table 1).

PHB Extraction and Quantification—Quantification of PHB was performed as previously described (31, 32). Briefly, parallel liquid cultures (20 ml) of *C. fritschii* PCC 9212 were grown to different growth stages and at selected times, the cells were harvested by centrifugation for 10 min at 10,000 × *g*. The pellets were washed once with double-deionized water (20 ml). The resulting cell pellets were lyophilized to obtain dried cells. The dried cells and PHB standards (Sigma) were placed into glass tubes with sealed rubber caps. Chloroform (1 ml) and acidified methanol (15% v/v H_2SO_4) (1 ml) were added to each sample. The samples were heated in a 97 °C water bath for 3 h to convert the PHB into 3-hydroxybutyrate methyl ester. After methanolysis, double-deionized water (1 ml) was added to each sample. Following phase separation, the bottom chloroform phase (2 μl) was extracted and loaded directly onto a GC/MS for analysis as previously described (31). The concentrations and inferred cellular contents of PHB were calculated on the basis of a standard curve generated with known concentrations of PHB (Sigma).

Transcription Profiling—Global transcriptome profiling was performed by RNA-seq as previously described (33). The *C. fritschii* PCC 9212 was fully adapted to acetate growth conditions by serially subculturing cells three times in liquid BG-11 medium containing 10 mM acetate. The control strain was similarly grown three times on medium without acetate, and each culture was harvested at $A_{750\ nm} = 1$. Total RNA was then extracted from these two strains, and rRNA depletion was performed as described (33). The construction of cDNA libraries and Illumina sequencing (50 nucleotides, single read) were performed in the Genomic Core Facility at The Pennsylvania State University. Mapping against the *C. fritschii* PCC 9212 genome was performed using the BWA software package, allowing a maximal 4 mismatches per read. The resulting alignment files were further analyzed with self-developed scripts to extract relative expression levels for each gene. The RNA sequencing data have been deposited in the NCBI Sequence Read Archive under accession number SRP052045.

Results

Enzyme Characterization—Fig. 2*A* shows the results of SDS-PAGE and immunoblotting analysis of the purified recombinant product of open reading frame (ORF) SYNPCC7002_A2041. The purified protein had an apparent molecular weight of 50,000 and cross-reacted with commercial antibodies to the poly-His tag. Moreover, tryptic peptide mass fingerprinting showed that the major protein present in the preparation was the product of ORF SYNPCC7002_A2041 (data not shown). When the product of SynPCC7002_A2041 was incubated with 10 mM malate at room temperature, 2.1 mM fumarate was formed and 2.2 mM malate was consumed (Fig. 2*B*). When the product of SynPCC7002_A2041 was incubated with 2.5 mM fumarate at room temperature, 1.9 mM malate was formed and 2.1 mM fumarate was consumed (Fig. 2*C*). These results confirm that SynPCC7002_A2041 encodes fumarase, which catalyzes the reversible interconversion of malate and fumarate. Together with the recently identified 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase, these three enzymes closed the three gaps in the TCA cycle of *Synechococcus* sp. PCC 7002 and demonstrate that the TCA cycle is complete in most cyanobacteria (20).

The isocitrate lyase (ORF UYEDRAFT_02681) of *C. fritschii* PCC 9212 was successfully expressed and purified from *E. coli*

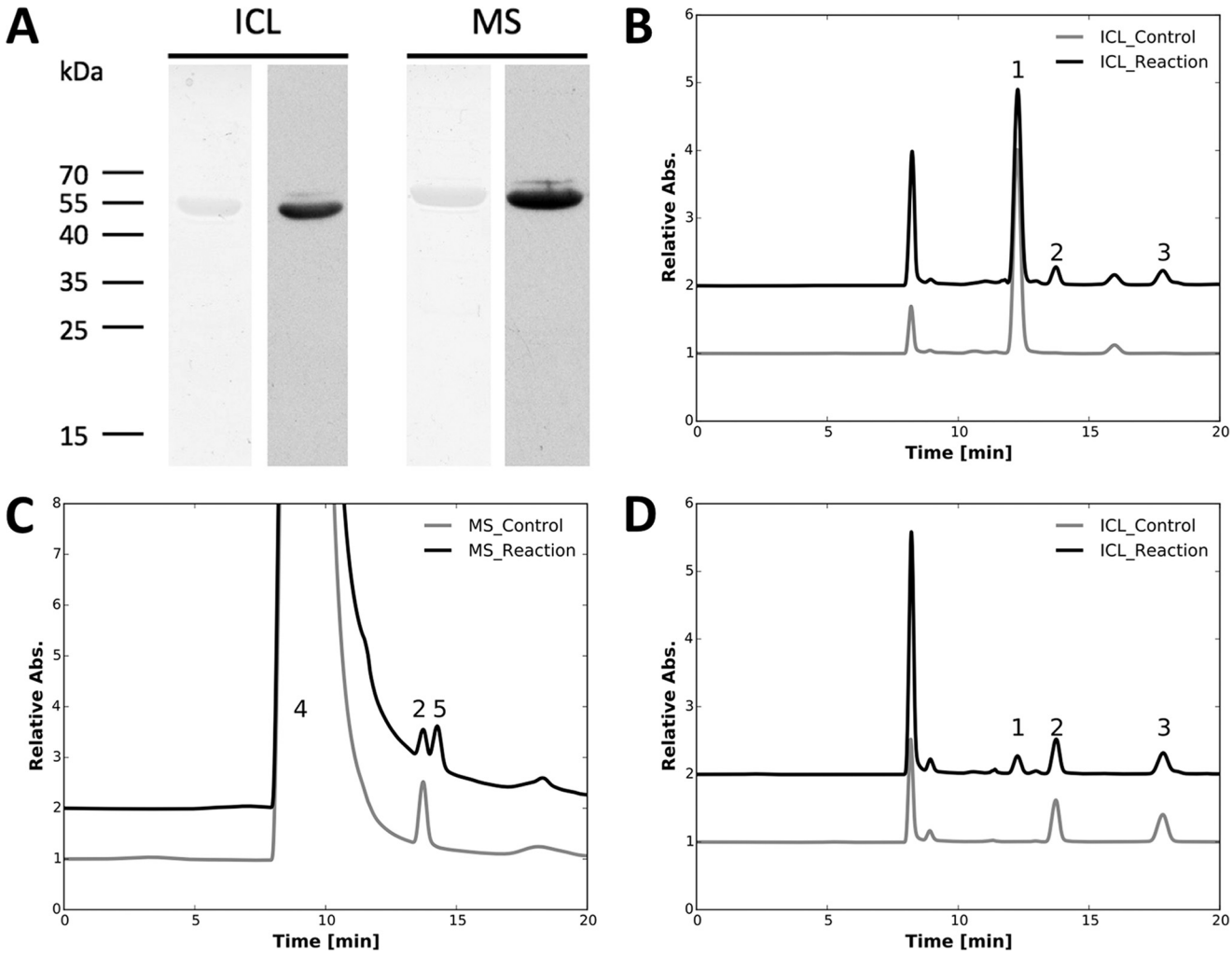

FIGURE 3. **Characterizations of purified recombinant isocitrate lyase (UYEDRAFT_02681) and malate synthase (UYEDRAFT_02682).** *A,* SDS-PAGE and immunoblotting analysis with a commercial antibody to the poly-His_6 tag for isocitrate lyase (*ICL*) and malate synthase (*MS*). *B,* HPLC analysis showing that isocitrate (*peak 1*) was converted to glyoxylate (*peak 2*) and succinate (*peak 3*) by the purified isocitrate lyase (UYEDRAFT_02681). When the protein product of UYEDRAFT_02681 was incubated with 2 mM isocitrate, 0.3 mM isocitrate was consumed and 0.25 mM succinate and 0.27 mM glyoxylate were produced. *C,* HPLC analysis showing the formation of malate (*peak 5*) from glyoxylate (*peak 2*) and acetyl-CoA (*peak 4*) catalyzed by purified malate synthase (UYEDRAFT_02682). Specifically, when the protein product from ORF UYEDRAFT_02682 was incubated with 2 mM glyoxylate and 2 mM acetyl-CoA, 1.2 mM glyoxylate and 1.1 mM acetyl-CoA were consumed, and 0.9 mM malate was produced. *D*, HPLC analysis showing production of isocitrate (*peak 1*) from glyoxylate (*peak 2*) and succinate (*peak 3*) catalyzed by the purified isocitrate lyase. Specifically, 0.15 mM isocitrate was produced, and 0.19 mM glyoxlyate and 0.15 mM succinate were consumed, when 1 mM succinate and 1 mM glyoxylate were incubated with the product of UYEDRAFT_02681. The large differences in the peak area for identical amounts of acetyl-CoA and glyoxylate are due to the different molar extinction coefficients of these two compounds at 210 nm. Detailed assay conditions are described under "Experimental Procedures."

as an N-terminally poly-His-tagged protein. The purified protein had an apparent molecular weight of 52,000 on SDS-PAGE and was positively immunoreactive with commercial antibodies to the poly-His tag (Fig. 3*A*). The purified protein was conclusively identified by tryptic peptide mass fingerprinting (data not shown). As mentioned under "Experimental Procedures," the malate synthase (ORF UYEDRAFT_02682) of *C. fritschii* PCC 9212 could not be overproduced in *E. coli* when the protein was produced with an N-terminal His tag, possibly due to protein misfolding. However, moving the poly-His tag to the C terminus resulted in the production of active, recombinant malate synthase. The recombinant protein had a molecular weight of 64,000 on SDS-PAGE and was positively immunoreactive with commercial antibodies to the poly-His tag (Fig. 3*A*). The identity of the protein was further confirmed by tryptic peptide mass fingerprinting (data not shown).

To establish that the isocitrate lyase and malate synthase had the anticipated enzymatic activities, assays were performed to characterize the enzymes. When the protein product of UYEDRAFT_02681 was incubated with 2 mM isocitrate, 0.3 mM isocitrate was consumed and 0.25 mM succinate and 0.27 mM glyoxylate were produced (Fig. 3*B*). Demonstrating that this reaction is reversible, 0.15 mM isocitrate was produced, and 0.19 mM glyoxlyate and 0.15 mM succinate were consumed, when 1 mM succinate and 1 mM glyoxylate were incubated with the product of UYEDRAFT_02681 (Fig. 3*D*). When the protein product from ORF UYEDRAFT_02682 was incubated with 2 mM glyoxylate and 2 mM acetyl-CoA, 1.2 mM glyoxylate and 1.1 mM acetyl-CoA were consumed, and 0.9 mM malate was produced (Fig. 3*C*). These biochemical results established that UYEDRAFT_02681 encodes isocitrate lyase and that UYEDRAFT_02682 encodes malate synthase. Acting together,

these two enzymes can catalyze the conversion of isocitrate and acetyl-CoA into malate and succinate (data not shown). This results in the incorporation of C_2 units into metabolic intermediates of key precursor metabolites of the central metabolism. These biochemical assays also confirm that the glyoxylate cycle is present and probably active in *C. fritschii* PCC 9212.

Growth of C. fritschii PCC 9212 with and without Acetate—Because the glyoxylate cycle is generally believed to be involved in the acetate assimilation and metabolism, we tested whether *C. fritschii* PCC 9212 could assimilate acetate under different growth conditions. As described under "Experimental Procedures," under standard growth conditions *C. fritschii* PCC 9212 grew faster when the medium was supplemented with 10 mM acetate (Fig. 4*A*). In agreement with the faster growth rate, acetate was consumed from the medium, and all of the acetate was consumed by the end of the cultivation period (Fig. 4*A*). As expected, *C. fritschii* PCC 9212 grew more slowly when cultures were sparged with air (Fig. 4*B*). Cells again grew faster when acetate was added to the growth medium but the magnitude of the stimulation was similar to that observed for cultures sparged with air containing 1% (v/v) CO_2. This result shows that acetate can stimulate growth but certainly is not able to supplant CO_2 fixation as the major route of carbon acquisition during growth under these conditions. *C. fritschii* PCC 9212 was able to grow very slowly in the dark when the medium contained acetate, but no growth was observed in the dark when acetate was eliminated from the medium (Fig. 4*C*).

Growth of Synechococcus sp. PCC 7002 with and without Acetate—We have not yet developed the ability to perform gene knock-out experiments to test the function of glyoxylate cycle in *C. fritschii* PCC 9212. Thus, we decided to study the function of the glyoxylate cycle and acetate utilization in the model cyanobacterium, *Synechococcus* sp. PCC 7002, which lacks the glyoxylate cycle. The *aceBA* operon encoding the two glyoxylate cycle genes of *C. fritschii* PCC 9212 was introduced into the pAQ1Ex expression plasmid system (29), which was subsequently transformed into *Synechococcus* sp. PCC 7002. The presence of the plasmid and the incorporation of the *aceBA* genes into *Synechococcus* sp. PCC 7002 was verified by PCR amplification of the *aceBA* operon and was further confirmed by sequencing the amplicon (Fig. 5). When the wild type and the strain carrying the *aceBA* genes, hereafter denoted as *Synechococcus* 7002-glyox, were grown under standard conditions, *Synechococcus* 7002-glyox had a slower growth rate but a faster acetate assimilation rate compared with WT (Fig. 6*A*). This indicated that the enzymes of the glyoxylate cycle were active in the recombinant strain and supported acetate assimilation. The slower growth rate may have been due to the overexpression of these two genes and the additional energy and nutrient resources required to synthesize the two foreign proteins. Furthermore, when the two strains were grown under low irradiance conditions, they had very similar growth rates and an even larger difference in acetate uptake was observed (Fig. 6*B*). This suggested that acetate was possibly more important in supplying energy for growth when light was limiting. However, no acetate uptake occurred under dark or low-CO_2 conditions for WT cells (Fig. 6, *C* and *D*). WT cells exhibited net acetate excretion under these conditions (Fig. 6, *C* and *D*), and the same was

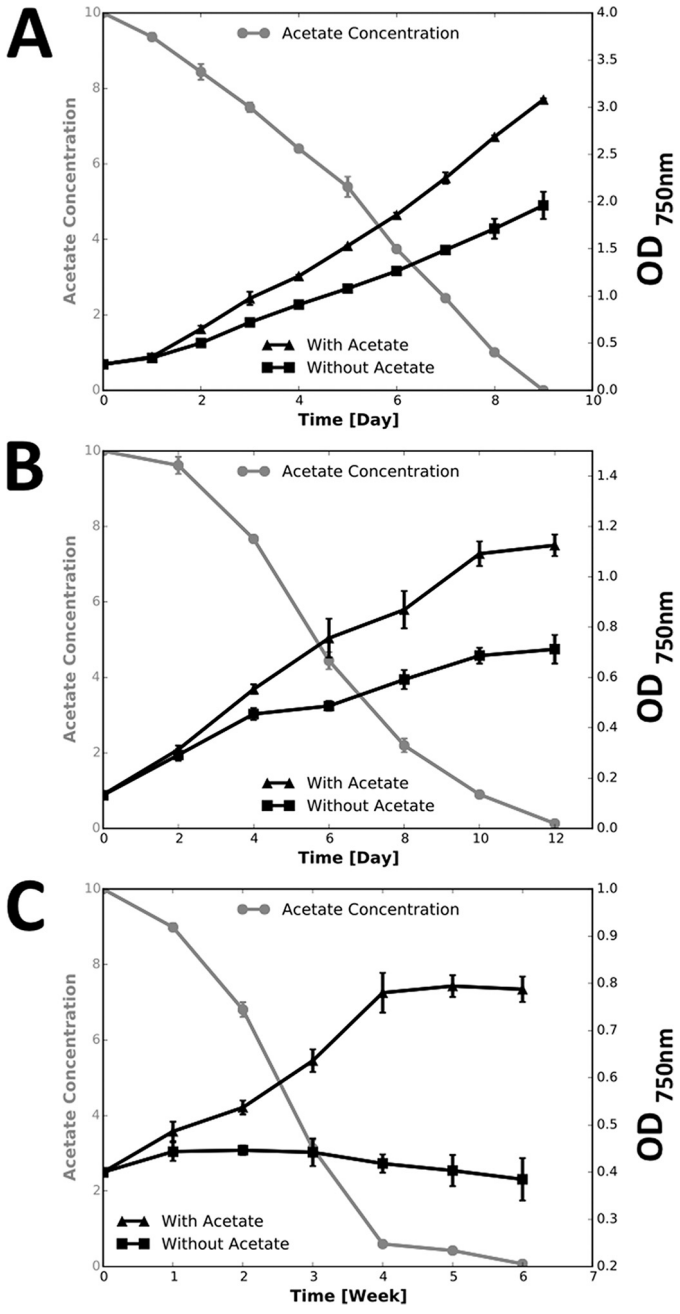

FIGURE 4. **Acetate assimilation and growth curves for *C. fritschii* PCC 9212.** *Black lines* indicate the cell density and *gray lines* indicate the acetate concentrations in the medium at different times during the batch growth cycle. *A, C. fritschii* PCC 9212 growing under standard conditions. *B, C. fritschii* PCC 9212 growing under low CO_2 conditions (cultures were sparged with air). *C, C. fritschii* PCC 9212 grown under dark conditions (1% CO_2 in air). The data shown are averages of three biological replicates, and the *error bars* show the standard deviation. Other details concerning the growth conditions are described under "Experimental Procedures."

observed for *Synechococcus* 7002-glyox under dark conditions. Acetate assimilation was still observed under low-CO_2 conditions in strain *Synechococcus* 7002-glyox (Fig. 6*D*), although the assimilation rate was much slower compared with the rates observed under standard or low-light conditions. A previous study had also shown that in *Aphanocapsa* sp. PCC 6308 and *Synechococcus elongatus* PCC 6301, the CO_2 concentration was crucial for acetate uptake and the acetate uptake rate was

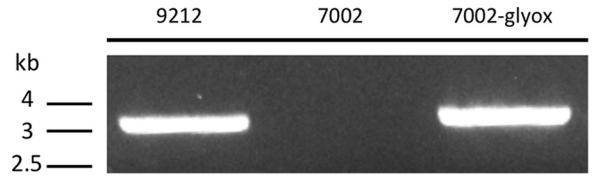

FIGURE 5. **Verification of the presence of glyoxylate cycle genes (*aceBA*) in *Synechococcus* sp. PCC 7002 strain 7002-glyox by PCR.** The template DNA was derived from wild-type *C. fritschii* PCC 9212 (*lane 9212*), from wild-type *Synechococcus* sp. PCC 7002 (*lane 7002*), and from the recombinant strain 7002-glyox (*lane 7002-glyox*), which has the *aceBA* genes from *C. fritschii* PCC 9212 inserted in plasmid pAQ1-Ex as described under "Experimental Procedures."

reduced by almost 50% in the absence of CO_2 (4). These observations confirm that acetate assimilation, the glyoxylate cycle, and CO_2 fixation are closely related metabolic processes that may possibly be coordinately regulated under different growth conditions.

Gene Neighborhood Analysis of the Glyoxylate Cycle Genes—To study the possible relationships between the glyoxylate cycle and other metabolic pathways, BLASTP analysis and gene neighborhoods surrounding the *aceBA* operon were also investigated. As mentioned before, the *aceBA* genes, encoding isocitrate lyase and malate synthase, respectively, are located in an apparent operon in *C. fritschii* PCC 9212 (Fig. 7*A*). BLASTP analysis showed that these two genes also occur in *C. fritschii* PCC 6912, *Cyanothece* sp. strains PCC 7822 and 7424, *Pleurocapsa minor* PCC 7327, *Fischerella* sp. PCC 9605, *Cyanobacterium* PCC 7702, *Mastigocoleus testarum*, and *Tolypothrix bouteillei*. Interestingly, many of these cyanobacteria (*C. fritschii* PCC 9212, *C. fritschii* PCC 6912, *P. minor* PCC 7327, *Fischerella* sp. PCC 9605, *M. testarum*) are capable of growth in far-red light and exhibit the far-red light photoacclimation (FaRLiP) response (33, 34). Furthermore, all of these strains are able to fix nitrogen; this suggests that the glyoxylate cycle may serve as an additional control point for balancing the carbon and nitrogen metabolism of these cyanobacteria.

Further examination of the genes near the *aceBA* operon in *C. fritschii* PCC 9212 indicates that there is also an apparent operon of PHB-related genes (*phaABEC*) located downstream (Fig. 7*A*). Additionally, a poly-(3-hydroxybutyrate) depolymerase gene (*phaZ*) as well as paralogous copies of acetyl-CoA acetyltransferase (*phaA*) and acetoacetyl-CoA reductase (*phaB*) are located further downstream in the same gene neighborhood. Considering that the glyoxylate cycle and PHB metabolic pathway both use the important metabolite acetyl-CoA, and considering that all of these genes are colocalized in the genome, it is highly likely that these two pathways interact closely with each other in carbon metabolism. *Synechococcus* sp. PCC 7002 does not fix nitrogen, lacks the glyoxylate cycle genes, and lacks enzymes for production and mobilization of PHB.

Global Transcription Profiling of C. fritschii PCC 9212—To investigate whether other metabolic pathways in addition to the glyoxylate cycle are involved in acetate assimilation and utilization, global transcription profiling was performed for *C. fritschii* PCC 9212 cells grown in the presence and absence of acetate. The results showed that transcripts for the isocitrate lyase and malate synthase genes increased ~1.6-fold in the presence of acetate, and further indicated that cells expressed these genes at relatively high levels even when acetate was not present in the medium. However, transcript levels for the genes involved in PHB metabolism (*phaABEC*) had similar abundance levels in cells grown with or without acetate. The different expression pattern for the *aceBA* and *phaABEC* operons suggested that the PHB metabolism genes and the glyoxylate cycle genes were probably expressed from different promoters. It should be noted that the *C. fritschii* PCC 9212 genome contains two copies of *phaA* and *phaB*; transcript levels for the second copies actually decreased about 2-fold when acetate was added to the growth medium. This could indicate that the distal *phaAB* genes might be involved in PHB degradation/utilization. Transcript levels for phosphoenolpyruvate synthase (*ppsA*) increased about 4-fold in the presence of acetate, which suggests that cells increase carbon flux toward glycolysis in the presence of acetate (Fig. 7*B*). A similar response was reported in *E. coli* cells grown in the presence of acetate (35).

Acetate Increases the Production of PHB—Because the genes for the glyoxylate cycle and for PHB metabolism were co-localized, we determined whether the PHB content of *C. fritschii* PCC 9212 cells would be affected by the addition of acetate. PHB accumulation was first tested in *C. fritschii* PCC 9212, and our results showed that PHB accumulated and represented about ~5% of the cell dry weight under standard growth conditions (Fig. 8). When acetate was supplied to the medium under the same conditions, the PHB content increased to ~15% of total cell dry weight (Fig. 8, *inset*). These results indicated that the assimilated acetate that was metabolized by the glyoxylate cycle might mainly be stored in the form of PHB. Interestingly, genes for PHB metabolism are present in many cyanobacteria that lack the glyoxylate cycle (*e.g. Synechocystis* sp. PCC 6803, *Leptolyngbya* sp. strain JSC-1). Genes for PHB metabolism are also found in other strains capable of performing FaRLiP (*e.g. P. minor* PCC 7327, *Fischerella* sp. PCC 9605) (34), suggesting that PHB biosynthesis could serve as a major carbon storage mechanism and the synthesized PHB might be used during cellular adaptation to different growth environments, such as far-red light conditions. In agreement with this hypothesis, the relative transcript abundances for genes of PHB metabolism increased ~7-fold in *Leptolyngbya* sp. strain JSC-1 when cells were shifted to far-red light conditions (33). Along with this change, transcript abundances for numerous carbon transporter genes also increased. This indicated that the cells were probably using both internal carbon stores as well as extracellular carbon sources from the environment to provide the energy and nutrients needed for cell growth. These resources could be limiting when cells are remodeling their photosynthetic apparatus to use far-red light (33). Thus, together with the PHB biogenesis and degradation pathways to balance the acetyl-CoA concentrations inside cells, the glyoxylate cycle may provide an efficient way to use storage carbon sources under conditions when energy or carbon supply is limited. These results, together with the fact that mRNA levels of many photosynthesis related genes were also regulated in the presence of acetate, suggests that the glyoxylate cycle, acetate assimilation, and PHB metabolism are all important for

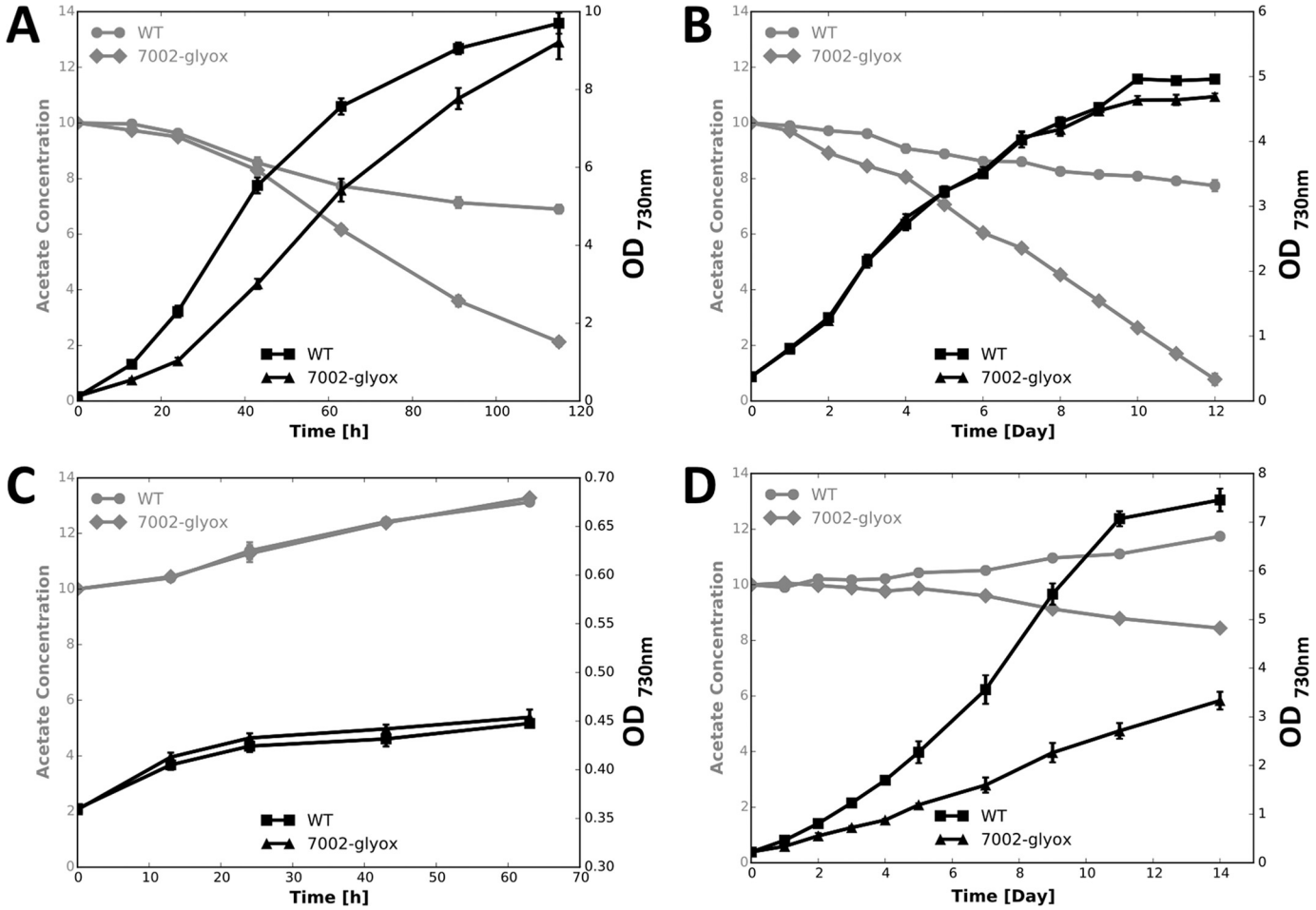

FIGURE 6. **Acetate assimilation and growth analysis of *Synechococcus* sp. PCC 7002.** *Black lines* indicate cell density and *gray lines* indicate the acetate concentrations in the medium at different growth stages. *A, Synechococcus* sp. PCC 7002 grown under standard conditions; *B, Synechococcus* sp. PCC 7002 growing under low light conditions; *C, Synechococcus* sp. PCC 7002 growing under dark conditions; *D, Synechococcus* sp. PCC 7002 growing under low CO_2 conditions. *WT*, wild type *Synechococcus* sp. PCC 7002; *7002-glyox*, *Synechococcus* sp. PCC 7002 strain with *aceBA* genes of *C. fritschii* PCC 9212 expressed from pAQ1. The data shown are averages of three biological replicates, and the *error bars* show the S.D.

the massive metabolic and physiological changes during the shift of growth condition to far-red light in FaRLiP strains.

Discussion

The glyoxylate cycle and the TCA cycle can both be used to metabolize acetate (*i.e.* acetyl-CoA), and their reactions provide essential precursor metabolites (*e.g.* 2-oxogluatarate, oxaloacetate and sometimes succinate) and reducing power (*e.g.* NADH) for cells. These two cycles share many enzymes and intermediates (Fig. 1), which makes them intrinsically interconnected. However, by using two specific enzymes, isocitrate lyase and malate synthase, the glyoxylate cycle is able to bypass the CO_2 releasing, oxidative steps of the TCA cycle (isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase or 2-oxoglutarate decarboxylase (20)). As a result, the glyoxylate cycle can more efficiently assimilate carbon from acetyl-CoA for biomass production, which could be derived from assimilated acetate, ethanol, or the degradation of fatty acids or poly-3-hydroxybutyrate. The net product of the glyoxylate cycle is succinate, which can be used to replenish TCA cycle intermediates or to generate metabolites for gluconeogenesis and other biosynthetic processes. Thus, the glyoxylate cycle provides an effective route for growth on fatty acids and C_2 compounds such as acetate and ethanol.

Since its discovery, the glyoxylate cycle has been identified and studied in many different organisms, including bacteria, archaea, protists, plants, and fungi (13, 36, 37). Although isocitrate lyase and malate synthase activities were reportedly detected in birds and amphibians (38), no genes for isocitrate lyase have been identified in animals. The nematode, *Caenorhabditis elegans,* and the protest, *Euglena gracilis,* have a single, fused gene encoding a bifunctional enzyme (39, 40). In *Chlamydomonas reinhardtii*, the glyoxylate cycle was shown to be essential for dark growth on acetate, and for efficient growth in the light when acetate is supplied (41). However, it should be noted that acetate is ineffective as a growth substrate and is even toxic for some marine algae (42). In addition to allowing the growth of bacteria on C_2 compounds, together with the β-oxidation of fatty acids, the glyoxylate cycle is also important in providing carbohydrates and biosynthetic precursors during the early stage of seedling establishment for plants (36, 43). It was reported that the β-oxidation pathway and glyoxylate cycle enzymes were induced in senescing leaves, possibly used for the breakdown of membrane lipids and gluconeogenesis (44).

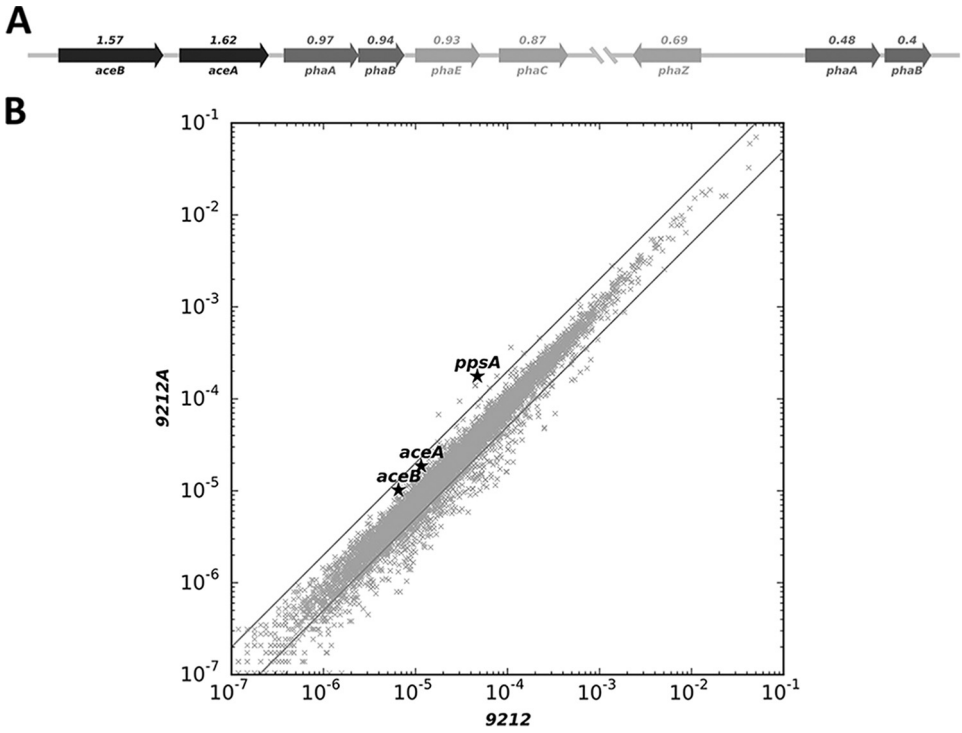

FIGURE 7. **Relative transcript abundances for mRNAs in *C. fritschii* PCC 9212 grown with or without acetate.** *A*, gene neighborhood around the glyoxylate cycle genes. *Numbers above* each gene indicate the fold-difference of mRNA abundance in cells grown with acetate compared with cells grown without acetate. *B,* scatter plot showing the relative abundance of all the mRNAs under growth conditions without acetate (*9212*) or with acetate (*9212A*). *Gray lines* indicate a 2-fold increase or 50% decrease in mRNA level. *aceA*, isocitrate lyase; *aceB*, malate synthase; *phaA*, acetyl-CoA acetyltransferase; *phaB*, acetoacetyl-CoA reductase; *phaE*, poly(R)-hydroxyalkanoic acid synthase, class III, PhaE subunit; *phaC*, poly(R)-hydroxyalkanoic acid synthase, class III, PhaC subunit; *phaZ*, poly(3-hydroxybutyrate) depolymerase; *ppsA*, phosphoenolpyruvate synthase.

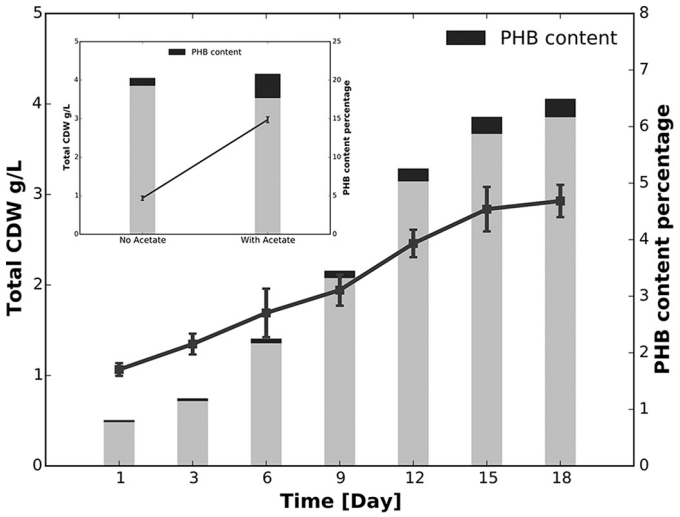

FIGURE 8. **Accumulation of PHB in *C. fritschii* PCC 9212.** PHB contents of *C. fritschii* PCC 9212 were monitored as a function of batch growth under standard conditions for *C. fritschii* PCC 9212. The *bars* indicate the total cell dry weight (*CDW*), and the *black* portions of the bars show the PHB content at different growth stages. This information is also plotted to emphasize the kinetics of PHB production. In the absence of added acetate, PHB accounted for ~5% of total CDW. The *inset* shows that CDW changed only slightly when 10 mM acetate was added to the medium, but PHB accumulated to a much higher level, ~15% of total CDW. The data shown are averages of three biological replicates, and the *error bars* show the S.D.

Despite the fact that the glyoxylate cycle is essential for the assimilation and metabolism of acetate, there are still a number of acetate-using microorganisms that lack one or both of the enzymes involved in the glyoxylate cycle. More recently, some other metabolic pathways that can be used for acetate assimilation have been identified. The glyoxylate cycle is not found in green sulfur bacteria and heliobacteria, and these bacteria instead use pyruvate synthase for acetate assimilation (45). This enzyme requires ferredoxin to supply the necessary reducing power for pyruvate synthesis and thus primarily occurs in anaerobic bacteria. CO_2 is also required for the growth of heliobacteria when acetate is supplied as the only organic carbon source (46). Another metabolic pathway that has been demonstrated to be involved in acetate assimilation is the ethylmalonyl-CoA pathway. This pathway is responsible for the production of glyoxylate, which can be further converted to phosphoenolpyruvate via the serine cycle pathway (47). The ethylmalonyl-CoA pathway is found in *Rhodobacter sphaeroides*, in which glyoxylate is condensed with acetyl-CoA to produce malyl-CoA and further hydrolyzed to malate and CoA (48). A third acetate-assimilation pathway, the methylaspartate cycle, was recently described in *Haloarcula marismortui* (49). This new pathway also results in the net synthesis of succinate but requires nearly three times as many steps as the glyoxylate cycle to generate oxaloacetate from citrate (49). Furthermore, in the methylaspartate cycle, isocitrate is first decarboxylated to 2-oxoglutarate, which is then converted to glutamate, and thus nitrogen metabolism is also linked to acetate assimilation in this cycle (50). Why certain microorganisms use very complex strategies, such as the ethylmalonyl-CoA pathway and the methylaspartate cycle, for acetate assimilation rather than the simple glyoxylate cycle remains unclear and requires further study.

Although acetate assimilation has been studied in many microorganisms, the assimilation of acetate in cyanobacteria was poorly understood and confusing. Previous studies had suggested that some cyanobacteria could use acetate as the sole carbon and energy source (22). However, the pathway(s) that were used to assimilate acetate in cyanobacteria remained unclear. As mentioned, pyruvate synthase is highly sensitive to oxygen and thus cannot function in oxygenic cyanobacteria when they grow in the light (however, pyruvate:ferredoxin oxidoreductase is used to decarboxylate pyruvate oxidatively during fermentation in the dark (51)). The ethylmalonyl-CoA pathway and the methylaspartate cycle have not yet been shown to occur in cyanobacteria. Some previous studies reported that the enzymatic activities of the glyoxylate cycle could be detected in cyanobacteria (21), and thus the glyoxylate cycle has been included in some recent FBA models for *Synechocystis* sp. PCC 6803 to investigate the possible roles of this cycle in cyanobacteria (25, 52). However, the genes encoding isocitrate lyase and malate synthase are not present in *Synechocystis* sp. PCC 6803 nor are they present in the genomes of most other cyanobacteria. Furthermore, these enzymatic activities were not identified when more refined and sensitive methods were employed with *Synechocystis* sp. PCC 6803 (25). Consistent with the absence of the detected enzyme activities, recent isotopic tracing studies also indicated that the glyoxylate cycle is not functional and that the glyoxylate cycle may mainly be used for glycine synthesis (53).

By identifying and characterizing the isocitrate lyase and malate synthase from *C. fritschii* PCC 9212, our results clearly demonstrate that the glyoxylate cycle does exist in a few cyanobacterial strains and that it plays an important role in acetate assimilation in one of those organisms. We also demonstrated that the ability to assimilate acetate could be significantly improved by introducing the *aceBA* genes to *Synechococcus* sp. PCC 7002, which normally lacks the glyoxylate cycle. However, the absence of these two genes, and thus the glyoxylate cycle, in most cyanobacteria implies that the few organisms with this pathway probably obtained the genes recently by lateral gene transfer. In addition to the intracellular metabolism of acetate, one interesting question would be to identify a potential acetate transporter (assuming that there is one). It has been reported that the *yjcG* gene is responsible for acetate transportation in *E. coli*, and another transport system for acetate may also exist (54). However, homologs of the *yjcG* gene have not been identified in cyanobacteria, and a different type of transport system might be used. Under dark aerobic conditions, microalgae use a monocarboxylic/proton transporter protein, which is a member of the Major Facilitator Superfamily, to transport acetate across the membrane (55, 56). Our transcription profiling results showed that transcript levels for several putative transporter genes increased modestly when acetate was supplied to the medium (supplemental Table S1), and further studies with these transporters might provide clues that could answer this question definitively.

Considering the importance of the glyoxylate cycle in acetate assimilation and its intrinsic link with the TCA cycle, the operation of glyoxylate cycle must be regulated properly to accommodate changes in the chemical environments of cells. Indeed, in algae the enzyme activities of isocitrate lyase have been found to increase under many different growth conditions when acetate is supplied, and the glyoxylate cycle is operated interactively with the TCA cycle and the oxidative pentose phosphate pathway (10, 56, 57). Our results showed that transcript levels for malate synthase and isocitrate lyase increased only slightly when acetate was being actively metabolized. Previous studies reported that the enzyme activities for malate synthase and isocitrate lyase did not increase when acetate was supplied to the medium, indicating that there might be regulation at other levels (23). Consistent with this hypothesis, purified isocitrate lyase from *C. reinhardtii* was shown to be inactivated by glutathionylation and reactivated by glutaredoxin, which implies that the glyoxylate cycle may be actively regulated under specific environmental conditions (58). However, the functional significance of these post-translational modifications in response to different growth conditions, as well as the possible regulation and interactions between the glyoxylate cycle and many other metabolic pathways (*e.g.* the TCA cycle, the PHB metabolism) in cyanobacteria, are not yet well understood and will require further detailed investigation.

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References

1. Kreth, J., Lengeler, J. W., and Jahreis, K. (2013) Characterization of pyruvate uptake in *Escherichia coli* K-12. *PLoS One* **8,** e67125
2. Pinchuk, G. E., Geydebrekht, O. V., Hill, E. A., Reed, J. L., Konopka, A. E., Beliaev, A. S., and Fredrickson, J. K. (2011) Pyruvate and lactate metabolism by *Shewanella oneidensis* MR-1 under fermentation, oxygen limitation, and fumarate respiration conditions. *Appl. Environ. Microbiol.* **77,** 8234–8240
3. Brown, T. D., Jones-Mortimer, M. C., and Kornberg, H. L. (1977) The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. *J. Gen. Microbiol.* **102,** 327–336
4. Ihlenfeldt, M. J., and Gibson, J. (1977) Acetate uptake by the unicellular cyanobacteria *Synechococcus* and *Aphanocapsa*. *Arch. Microbiol.* **113,** 231–241
5. Kay, W. W., and Kornberg, H. L. (1971) The uptake of C_4-dicarboxylic acids by *Escherichia coli*. *Eur. J. Biochem.* **18,** 274–281
6. Gutowski, S. J., and Rosenberg, H. (1975) Succinate uptake and related proton movements in *Escherichia coli* K12. *Biochem. J.* **152,** 647–654
7. Wright, R. T., and Hobbie, J. E. (1966) Use of glucose and acetate by bacteria and algae in aquatic ecosystems. *Ecology* **47,** 447–464
8. Gibbs, M., Gfeller, R. P., and Chen, C. (1986) Fermentative metabolism of *Chlamydomonas reinhardii* III. Photoassimilation of acetate. *Plant Physiol.* **82,** 160–166
9. Kornberg, H. (1966) The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem. J.* **99,** 1–11
10. Boyle, N. R., and Morgan, J. A. (2009) Flux balance analysis of primary metabolism in *Chlamydomonas reinhardtii*. *BMC Syst. Biol.* **3,** 4
11. Kornberg, H. L., and Krebs, H. A. (1957) Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. *Nature* **179,** 988–991
12. Kornberg, H. (2000) Krebs and his trinity of cycles. *Nat. Rev. Mol. Cell Biol.* **1,** 225–228
13. Dunn, M. F., Ramírez-Trujillo, J. A., and Hernández-Lucas, I. (2009) Ma-

jor roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* **155,** 3166–3175
14. Zhang, S., and Bryant, D. A. (2014) Learning new tricks from an old cycle: the TCA cycle in cyanobacteria, algae and plants. *Perspect. Phycol.* **1,** 73–86
15. Tang, K.-H., Tang, Y. J., and Blankenship, R. E. (2011) Carbon metabolic pathways in phototrophic bacteria and their broader evolutionary implications. *Front. Microbiol.* **2,** 165
16. Sirevåg, R. (1995) Carbon metabolism in green bacteria. in *Advances in Photosynthesis and Respiration* (Blankenship, R., Madigan, M., and Bauer, C., eds) Vol. 2, pp. 871–883, Springer, Netherlands
17. Zarzycki, J., and Fuchs, G. (2011) Coassimilation of organic substrates via the autotrophic 3-hydroxypropionate bi-cycle in *Chloroflexus aurantiacus*. *Appl. Environ. Microbiol.* **77,** 6181–6188
18. Feng, X., Tang, K.-H., Blankenship, R. E., and Tang, Y. J. (2010) Metabolic flux analysis of the mixotrophic metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*. *J. Biol. Chem.* **285,** 39544-39550
19. Madigan, M. (2006) The family heliobacteriaceae. in *The Prokaryotes* (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., eds) pp. 951–964, Springer US.
20. Zhang, S., and Bryant, D. A. (2011) The tricarboxylic acid cycle in cyanobacteria. *Science* **334,** 1551–1553
21. Eley, J. H. (1988) Glyoxylate cycle enzyme activities in the cyanobacterium *Anacystis nidulans*. *J. Phycol.* **24,** 586–588
22. Pearce, J., and Carr, N. (1967) The metabolism of acetate by the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. *J. Gen. Microbiol.* **49,** 301–313
23. Miller, J. S., and Allen, M. M. (1972) Carbon utilization patterns in the heterotrophic blue-green alga *Chlorogloea fritschii*. *Arch. Mikrobiol.* **86,** 1–12
24. Hoare, D., Hoare, S., and Moore, R. (1967) The photoassimilation of organic compounds by autotrophic blue-green algae. *J. Gen. Microbiol.* **49,** 351–370
25. Knoop, H., Gründel, M., Zilliges, Y., Lehmann, R., Hoffmann, S., Lockau, W., and Steuer, R. (2013) Flux balance analysis of cyanobacterial metabolism: the metabolic network of *Synechocystis* sp. PCC 6803. *PLoS Comput. Biol.* **9,** e1003081
26. Bandyopadhyay, A., Elvitigala, T., Welsh, E., Stöckel, J., Liberton, M., Min, H., Sherman, L. A., and Pakrasi, H. B. (2011) Novel metabolic attributes of the genus *Cyanothece*, comprising a group of unicellular nitrogen-fixing cyanobacteria. *MBio* **2,** e00214–11
27. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111,** 1–61
28. Ludwig, M., and Bryant, D. A. (2011) Transcription profiling of the model cyanobacterium *Synechococcus* sp. strain PCC 7002 by Next-Gen (SOLiD) sequencing of cDNA. *Front. Microbiol.* **2,** 41
29. Xu, Y., Alvey, R. M., Byrne, P. O., Graham, J. E., Shen, G., and Bryant, D. A. (2011) Expression of genes in cyanobacteria: Adaptation of endogenous plasmids as platforms for high-level gene expression in *Synechococcus* sp. PCC 7002. *Methods Mol. Biol.* **684,** 273–293
30. Frigaard, N. U., Sakuragi, Y., and Bryant, D. A. (2004) Gene inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium tepidum* using in vitro-made DNA constructs and natural transformation. *Methods Mol. Biol.* **274,** 325–340
31. Tsang, T. K., Roberson, R. W., and Vermaas, W. F. (2013) Polyhydroxybutyrate particles in *Synechocystis* sp. PCC 6803: facts and fiction. *Photosynth. Res.* **118,** 37–49
32. Braunegg, G., Sonnleitner, B., and Lafferty, R. M. (1978) A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol. Biotechnol.* **6,** 29–37
33. Gan, F., Zhang, S., Rockwell, N. C., Martin, S. S., Lagarias, J. C., and Bryant, D. A. (2014) Extensive remodeling of a cyanobacterial photosynthetic apparatus in far-red light. *Science* **345,** 1312–1317
34. Gan, F., Shen, G., and Bryant, D. A. (2014) Occurrence of far-red light photoacclimation (FaRLiP) in diverse cyanobacteria. *Life* **5,** 4–24
35. Oh, M.-K., Rohlin, L., Kao, K. C., and Liao, J. C. (2002) Global expression profiling of acetate-grown *Escherichia coli*. *J. Biol. Chem.* **277,** 13175–13183
36. Eastmond, P. J., and Graham, I. A. (2001) Re-examining the role of the glyoxylate cycle in oilseeds. *Trends Plant Sci.* **6,** 72–78
37. Kunze, M., Pracharoenwattana, I., Smith, S. M., and Hartig, A. (2006) A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochim. Biophys. Acta* **1763,** 1441–1452
38. Davis, W. L., Jones, R. G., Farmer, G. R., Dickerson, T., Cortinas, E., Cooper, O. J., Crawford, L., and Goodman, D. B. (1990) Identification of glyoxylate cycle enzymes in chick liver: the effect of vitamin D_3: cytochemistry and biochemistry. *Anat. Rec.* **227,** 271–284
39. Nakazawa, M., Nishimura, M., Inoue, K., Ueda, M., Inui, H., Nakano, Y., and Miyatake, K. (2011) Characterization of a bifunctional glyoxylate cycle enzyme, malate synthase/isocitrate lyase, of *Euglena gracilis*. *J. Eukaryot. Microbiol.* **58,** 128–133
40. Kondrashov, F. A., Koonin, E. V., Morgunov, I. G., Finogenova, T. V., and Kondrashova, M. N. (2006) Evolution of glyoxylate cycle enzymes in Metazoa: evidence of multiple horizontal transfer events and pseudogene formation. *Biol. Direct* **1,** 31
41. Plancke, C., Vigeolas, H., Höhner, R., Roberty, S., Emonds-Alt, B., Larosa, V., Willamme, R., Duby, F., Onga Dhali, D., Thonart, P., Hiligsmann, S., Franck, F., Eppe, G., Cardol, P., Hippler, M., and Remacle, C. (2014) Lack of isocitrate lyase in *Chlamydomonas* leads to changes in carbon metabolism and in the response to oxidative stress under mixotrophic growth. *Plant J.* **77,** 404–417
42. Wood, B. J. B., Grimson, P. H. K., German, J. B., and Turner, M. (1999) Photoheterotrophy in the production of phytoplankton organisms. *J. Biotechnol.* **70,** 175–183
43. Graham, I. A. (2008) Seed storage oil mobilization. *Annu. Rev. Plant Biol.* **59,** 115–142
44. Chen, Z.-H., Walker, R. P., Acheson, R. M., Técsi, L. I., Wingler, A., Lea, P. J., and Leegood, R. C. (2000) Are isocitrate lyase and phosphoenolpyruvate carboxykinase involved in gluconeogenesis during senescence of barley leaves and cucumber cotyledons? *Plant Cell Physiol.* **41,** 960–967
45. Pickett, M. W., Williamson, M. P., and Kelly, D. J. (1994) An enzyme and ^{13}C-NMR study of carbon metabolism in heliobacteria. *Photosynth. Res.* **41,** 75–88
46. Tang, K.-H., Yue, H., and Blankenship, R. E. (2010) Energy metabolism of *Heliobacterium modesticaldum* during phototrophic and chemotrophic growth. *BMC Microbiol.* **10,** 150
47. Korotkova, N., Chistoserdova, L., Kuksa, V., and Lidstrom, M. E. (2002) Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. *J. Bacteriol.* **184,** 1750–1758
48. Erb, T. J., Berg, I. A., Brecht, V., Müller, M., Fuchs, G., and Alber, B. E. (2007) Synthesis of C_5-dicarboxylic acids from C_2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 10631–10636
49. Khomyakova, M., Bükmez, Ö., Thomas, L. K., Erb, T. J., and Berg, I. A. (2011) A methylaspartate cycle in haloarchaea. *Science* **331,** 334–337
50. Ensign, S. A. (2011) Another microbial pathway for acetate assimilation. *Science* **331,** 294–295
51. McNeely, K., Xu, Y., Ananyev, G., Bennette, N., Bryant, D. A., and Dismukes, G. C. (2011) *Synechococcus* sp. strain PCC 7002 *nifJ* mutant lacking pyruvate:ferredoxin oxidoreductase. *Appl. Environ. Microbiol.* **77,** 2435–2444
52. Shastri, A. A., and Morgan, J. A. (2005) Flux balance analysis of photoautotrophic metabolism. *Biotechnol. Prog.* **21,** 1617–1626
53. You, L., Berla, B., He, L., Pakrasi, H. B., and Tang, Y. J. (2014) ^{13}C-MFA delineates the photomixotrophic metabolism of *Synechocystis* sp. PCC 6803 under light- and carbon-sufficient conditions. *Biotechnol. J.* **9,** 684–692
54. Gimenez, R., Nuñez, M. F., Badia, J., Aguilar, J., and Baldoma, L. (2003) The gene *yjcG*, cotranscribed with the gene *acs*, encodes an acetate permease in *Escherichia coli*. *J. Bacteriol.* **185,** 6448–6455
55. Becker, H. M., Hirnet, D., Fecher-Trost, C., Sültemeyer, D., and Deitmer, J. W. (2005) Transport activity of MCT1 expressed in *Xenopus oocytes* is

increased by interaction with carbonic anhydrase. *J. Biol. Chem.* **280,** 39882–39889
56. Perez-Garcia, O., Escalante, F. M., de-Bashan, L. E., and Bashan, Y. (2011) Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res.* **45,** 11–36
57. Combres, C., Laliberté, G., Sevrin Reyssac, J., and de la Noüe, J. (1994) Effect of acetate on growth and ammonium uptake in the microalga *Scenedesmus obliquus*. *Physiol. Plant.* **91,** 729–734
58. Bedhomme, M., Zaffagnini, M., Marchand, C. H., Gao, X.-H., Moslonka-Lefebvre, M., Michelet, L., Decottignies, P., and Lemaire, S. D. (2009) Regulation by glutathionylation of isocitrate lyase from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **284,** 36282–36291