EXPLORING THE METABOLIC DIVERSITY OF CYANOBACTERIA
FOR GREEN ENERGY PRODUCTION

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by
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ABSTRACT

Cyanobacteria absorb and convert sunlight into chemical potential energy by oxygenic photosynthesis. The energy-rich compounds stored during the day provide the fuel to support growth and maintenance energy production at night. In the presence of oxygen, respiration occurs in most organisms, which allows the oxidation of energy-rich substrates for the production of proton-motive force for ATP synthesis and other biochemical work. This dissertation research focused on elucidating metabolic diversities and capacities in cyanobacteria, some of which have potential applications toward green energy and biomaterial production based on these newly defined metabolic pathways.

Together with glycolysis and the oxidative pentose phosphate pathway, the tricarboxylic acid (TCA) cycle is one of the three most important pathways of central carbohydrate metabolism. It was long believed that cyanobacteria had an incomplete TCA cycle due to the absence of 2-oxoglutarate dehydrogenase (OGDH). Studies from this dissertation demonstrated that the TCA cycle in most cyanobacteria is completed in manner distinct from the classical TCA cycle through the action of two alternative enzymes, 2-oxoglutarate decarboxylase (2-OGDC) and succinic semialdehyde dehydrogenase (SSADH). Some preliminary studies were also performed in metabolic engineering this newly discovered TCA cycle to produce poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate from the newly identified intermediate, succinic semialdehyde.

Though some previous studies of cyanobacteria reported enzyme activities of the glyoxylate cycle (i.e. isocitrate lyase and malate synthase), recent experimental studies found that these two enzymes are not detectable in *Synechocystis* sp. PCC 6803. In this study, genes encoding isocitrate lyase and malate synthase from *Chlorogloeopsis fritschii* PCC 9212 were
identified and characterized. Furthermore, when the two genes encoding isocitrate lyase and malate synthase were expressed in *Synechococcus* sp. PCC 7002, the resulting strain was able to assimilate acetate at a higher rate than the wild type strain. Overall, this study demonstrated that the glyoxylate cycle exists in certain cyanobacterial strains, that it plays an essential role in the assimilation of C₂ carbon sources (i.e., acetate) for growth, and that it may also be involved in balancing carbon and nitrogen metabolism.

The biogenesis of thylakoid membranes in cyanobacteria was also investigated in this study. Previous studies in *Synechocystis* sp. PCC 6803 reported that *vipp1* (sll0617) was essential for viability. By constructing a fully segregated null mutant in *vipp1* (SynPCC7002_A0294) in *Synechococcus* sp. PCC 7002, we show that Vipp1 is not essential. Thylakoid membranes were still observed in *vipp1* mutant cells and resembled those in a *psaAB* mutant that completely lacks photosystem (PS) I. When the *vipp1* mutation was complemented with the orthologous *vipp1* gene from *Synechocystis* sp. PCC 6803 that was expressed from the strong *Pₚción* promoter, PS I content and activities were restored to normal levels, and cells again produced thylakoids that were indistinguishable from those of wild type. This study shows that thylakoids are still produced in the absence of Vipp1 and that normal thylakoid biogenesis in *Synechococcus* sp. PCC 7002 requires expression and biogenesis of PS I, which in turn requires Vipp1.
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LIST OF ABBREVIATIONS

2-OGDC 2-oxoglutarate decarboxylase
2-OGDH 2-oxoglutarate dehydrogenase
3HB 3-hydroxybutyrate
4HB 4-hydroxybutyrate
4HB-CoA 4-hydroxybutyryl-CoA
ATP adenosine triphosphate
CDW cell dry weight
Chl chlorophyll
FBA flux balance analysis
FUM fumarase
GABA gamma-aminobutyric acid
GAD glutamate decarboxylase
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GC-MS gas chromatography–mass spectrometry
GDH glutamate dehydrogenase
HPLC high performance liquid chromatography
ICL isocitrate lyase
IlvB acetolactate synthase
KFOR 2-oxoglutarate:ferredoxin oxidoreductase
MS malate synthase
NAD⁺ nicotinamide adenine dinucleotide
NADH reduced nicotinamide adenine dinucleotide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P3HB</td>
<td>poly-3-hydroxybutyrate</td>
</tr>
<tr>
<td>P3HB-co-4HB</td>
<td>poly-3-hydroxybutyrate-co-4-hydroxybutyrate</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>PHA</td>
<td>polyhydroxyalkanoates</td>
</tr>
<tr>
<td>PHB</td>
<td>polyhydroxybutyrate</td>
</tr>
<tr>
<td>PS I</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>SDH</td>
<td>succinic acid dehydrogenase</td>
</tr>
<tr>
<td>SSA</td>
<td>succinic semialdehyde</td>
</tr>
<tr>
<td>SSADH</td>
<td>succinic semialdehyde dehydrogenase</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
</tr>
<tr>
<td>VIPPI</td>
<td>vesicle-inducing protein in plastids 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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</tbody>
</table>
LIST OF CHEMICAL STRUCTURES

2-Oxoglutarate

Acetyl-CoA

Citrate

Fumarate

gamma-Aminobutyric acid
Succinate

\[
\text{HOOC-CH}_2\text{CH(OH)-COOH}
\]

Succinic semialdehyde

\[
\text{HO-CH}_2\text{CH(OH)-CHO}
\]

Succinyl-CoA

\[
\text{HOOC-CH}_2\text{CH(OH)-COA}
\]
ACKNOWLEDGEMENTS

It was a long journey studying towards this Doctor of Philosophy degree. Many years later, when looking back, the six years in Happy Valley were perhaps the toughest yet the most fascinating time in my life. I have to say, none of the work from this thesis would have been accomplished without Dr. Donald A. Bryant as my advisor in these days, who taught me the essence of thinking critically and challenging the well-established. Thus, first and foremost, I would like to express my deepest appreciation to my Ph.D. advisor, Dr. Donald A. Bryant, for introducing me to this amazing field, sharing his encyclopedic knowledge, providing countless support and allowing me to do independent research. Upon finishing my studies with him, I also feel very fortunate that a Ph.D. is merely the beginning of a relationship and not the end.

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Chapter 1  Introduction

Publication:

Shuyi Zhang and Donald A. Bryant (2014). Learning new tricks from an old cycle: the TCA cycle in cyanobacteria, algae and plants. Perspectives in Phycology, 73-86.
1.1 General background

One of the most daunting challenges for society for the future is finding a sufficient supply of clean energy, which is intimately linked with environmental protection, economic prosperity and the quality of human life. Due to the inherent limitations of fossil energy sources, renewable clean energy will become increasingly important to the future of our society because of the increasing population of humans on Earth, which has grown to more than 7 billion persons (Bloom, 2011). Because the supply and use of petroleum is not sustainable, many people have focused on exploiting new technologies and resources to generate energy (Handke et al., 2011; Logan and Elimelech, 2012; Srirangan et al., 2011; Way et al., 2011). We eventually will need alternatives to coal and crude oil: with the same functions but without the drawbacks.

Biofuels, especially biofuels derived from sunlight, might be one answer. Biofuels can be made from many different starting materials, including waste wood, algae and genetically engineered bacteria (Alper and Stephanopoulos, 2009; Chen, 2009; Fairley, 2011; Grayson, 2011; Savage, 2011; Wells et al., 2011). There are already several biofuels options available. Bioethanol, for example, which is currently produced from sugar and starch crops such as sugar cane and corn, or potentially from lignocellulose, has been the most extensively commercialized option thus far. However, bioethanol has also been criticized due to environmental and ethical issues. For instance, it competes with food crops for arable land, and it aggravates CO$_2$ debt due to land usage changes from clearing natural ecosystems, which are usually more efficient in sequestering CO$_2$ (Fargione et al., 2008; Pimentel et al., 2009; Searchinger et al., 2008). Bioethanol production in Brazil, which is always exemplified as a successful case, has recently been criticized because of competition for arable land and food production in this country, causing more problems than benefits (de Araujo and de Barros Prado Moura, 2011; Loarie et al., 2011; Moraes, 2011). Thus, the applications of bioethanol are limited and may be replaced in future, unless the production techniques change. On
the other hand, biofuel production from microalgae can avoid some of these problems by using land space where no crops grow, such as deserts. However, there are potential problems with microalgae-based biofuels. Open ponds have been found to be the best way for culturing algae, which will greatly increase the cost of producing and extracting the biomass from these ponds, not to mention the huge amounts of water used in such processes (Arumugam et al., 2011; Ghirardi et al., 2007; Pienkos and Darzins, 2009; Radakovits et al., 2010; Rodolfi et al., 2009). Genetically modified algae could also escape to the environment and cause other problems (Pienkos and Darzins, 2009).

Due to these drawbacks, investigations in next-generation biofuels are drawing more and more attention in recent years. The concept of photosynthetic biofuels envisions the application of a single organism, acting both as photo-catalyst and producer of the biofuel (Hellingwerf and Teixeira de Mattos, 2009; Lindberg et al., 2010). These approaches mainly focus on genetically modified bacteria (e.g., cyanobacteria) and produce biofuels in bioreactors, bypassing most of the problems mentioned above.

1.2 Cyanobacteria

Cyanobacteria are regarded as the earliest oxygenic photoautotrophs on earth, which may have appeared prior to 2.5 billion years ago. During photosynthesis, solar energy is captured by cyanobacterial antenna proteins and transformed to chemical energy through the photosynthetic electron transport chain, while water is oxidized to form dioxygen and atmospheric CO₂ is fixed into organic carbon compounds as a long-term method of energy conservation. Compared to eukaryotic microalgae, cyanobacteria have some appealing attributes related to biofuel and biomaterials development, including broad growth conditions (e.g., photoautotrophy, heterotrophy,
mixotrophy); superior growth rates and thus biomass accumulation rates; capabilities of growing from a wide range of light, temperature and salinities; synthesis and excretion of various energy-rich compounds (e.g., glycogen, dihydrogen and hydrocarbons); smaller genomes and advanced genetic tools for mutagenesis and protein expression; versatile metabolisms for metabolic engineering (Dismukes et al., 2008; Dutta et al., 2005; Ladygina et al., 2006). *Synechococcus* sp. PCC 7002, formerly known as *Agmenellum quadruplicatum* strain PR-6 or American Type Culture Collection strain 27167 (Figure 1-1), one of the photoautotrophic cyanobacteria studied in the Bryant laboratory, has been broadly used as a model in studying the direct conversion of sunlight to energy sources (McNeely et al., 2010; Sakamoto and Bryant, 2002; Xu et al., 2006). The cyanobacterium *Synechococcus* sp. PCC 7002 has several advantages in ease of genetic manipulation and efficiency in light energy conversion, and it can thus serve as an ideal candidate for biofuel and biomaterials production. *Synechococcus* sp. PCC 7002 is a euryhaline/marine cyanobacterium, which can tolerate and grow in a wide range of salinities from nearly fresh-water to more than 2 M of NaCl (Batterton and Van Baalen, 1971). In addition to its fast photoautotrophic growth rate (with a doubling time from 2.6 to 4 hours under standard conditions depending on N-source), *Synechococcus* sp. PCC 7002 can alternatively undergo heterotrophic or photomixotrophic growth when the medium is supplemented with glycerol as the carbon and energy source (Ingram et al., 1973). Additionally, it can grow in lowlight conditions and is still able to tolerate extremely high light intensities (~ 5000 µmol photons m⁻² sec⁻¹) (Nomura et al., 2006; Sakamoto and Bryant, 2002; Van Baalen et al., 1971). It produces lactate, hydrogen and acetate as the primary fermentative products (Datta et al., 1995; McNeely et al., 2010). Moreover, this cyanobacterium is naturally transformable and has a very high recombination capacity (Stevens and Porter, 1980), and methods for mutagenesis and overexpression of proteins have been well established (Frigaard et al., 2004; Xu et al., 2011). Furthermore, many transcriptomics, proteomics, metabolomics and functional genomics studies have been performed in this organism, which also provide a solid basis
for further utilization of this strain in biomaterials development (Bennette et al., 2011; Ludwig and Bryant, 2011, 2012a, b; Yang et al., 2014; Yang et al., 2015). Thus, *Synechococcus* sp. PCC 7002 was used throughout this dissertation work as a model system to investigate the metabolic diversities as well as the photosynthesis mechanisms in cyanobacteria, and explore some potential capabilities in producing biomaterials in cyanobacteria.

1.3 The TCA cycle

Oxygenic chlorophototrophic bacteria (i.e., cyanobacteria) as well as algae and plants use sunlight as the energy source to oxidize water and produce ATP and NADPH for CO\textsubscript{2} fixation. Compared to algae and plants, cyanobacteria have somewhat simpler photosystems (Bryant and Frigaard, 2006; Jordan, 2001; Umena et al., 2011), and while all use the reductive pentose phosphate pathway (Calvin-Benson-Bassham cycle) for carbon dioxide fixation, other aspects of their metabolic pathways differ significantly (Tang et al., 2011). The tricarboxylic acid (TCA) cycle, also known as the citric acid or Krebs cycle, was mostly established by Sir Hans Adolf Krebs, who actually established a “trinity of cycles” and received a Nobel Prize for his studies on the TCA cycle (Kornberg, 2000). He first elucidated the reactions of the urea/ornithine cycle in 1932, proposed the TCA cycle in 1937, and then completed his trifecta of cycles by describing the glyoxylate cycle in 1957.

The TCA cycle includes key reactions of central carbohydrate metabolism that support ATP synthesis by oxidative phosphorylation by using reducing equivalents from the oxidation of acetyl-CoA (Fernie et al., 2004; Foyer et al., 2011). However, there is accumulating evidence linking the TCA cycle to other pathways, not only in bacteria (Bott, 2007) but also in algae and plants (Araújo et al., 2012). In heterotrophic bacteria, the TCA cycle has two main functions: (a)
it oxidizes two-carbon, acetyl-CoA units producing NADH that drives oxidative phosphorylation; and (b) it provides essential precursor metabolites (e.g., oxaloacetate, 2-oxoglutarate, and sometimes succinate) that are required for biosynthesis of cellular components, primarily in the biosynthesis of amino acids, porphyrin derivatives (cobalamin, siroheme, heme, chlorophyll, and bilins), and other cellular constituents. Surprisingly, studies have demonstrated that some bacteria have variant TCA cycles (McCammon et al., 2003; Singh et al., 2009; Tian et al., 2005; Zhang and Bryant, 2011), and this also occurs in algae and plants (Sweetlove et al., 2010; Tielens et al., 2002). These variant TCA cycles could be the evolutionary consequences of metabolic adaptations that focus more on the production of biosynthetic precursors than the classic cycle, which favors the generation of energy over the production of metabolic precursors (Figure 1-2).

1.3.1 TCA cycle in chlorophototrophic bacteria

Although chlorophototrophic bacteria (i.e., phototrophic bacteria that synthesize (bacterio)-chlorophylls) have very diverse metabolic lifestyles, their central metabolism is partly dictated by the types of reaction centers they employ. At least three versions of the TCA cycle commonly occur in chlorophototrophic bacteria: the oxidative TCA cycle, the reductive TCA cycle, and the incomplete (branched) TCA cycle. Most chlorophototrophic bacteria are anoxygenic organisms, which use either a homodimeric type-1 (Fe/S-type) or a type-2 (quinone-type) reaction center for the transduction of light into stored chemical potential energy (Bryant and Frigaard, 2006). Bacteria with type-2 reaction centers (e.g., members of the Proteobacteria and filamentous anoxygenic phototrophs from the phylum Chloroflexi) are often facultative phototrophs that can grow under oxic or anoxic conditions; most have an active TCA cycle and many can often perform aerobic respiration (Tang et al., 2011). An interesting recent discovery is
a seventh bacterial phylum containing a member, Gemmatimonas sp. strain AP64, which synthesizes bacteriochlorophyll \( a \) and assembles functional type-2 reaction centers. This bacterium is an aerobic anoxygenic photoheterotroph with a complete TCA cycle (Zeng et al., 2014).

Bacteria that only have homodimeric type-1 reaction centers (e.g., members of the taxa Chlorobi (green sulfur bacteria; (GSB); Firmicutes (heliobacteria), and Acidobacteria) are usually strict anaerobes with specialized, oxygen-sensitive metabolism. An exception is Chloracidobacterium thermophilum, which is a microaerophile with a photosynthetic apparatus typical of that found in green sulfur bacteria (Bryant et al., 2007; Garcia Costas et al., 2012; Tank and Bryant, 2014). In GSB, light-driven oxidation of sulfur compounds produces strongly reducing ferredoxins, which are used in the reductive TCA cycle for fixation of CO\(_2\) and biomass production (Evans et al., 1966; Sirevåg, 1995; Tang et al., 2011). A few GSB have a complete oxidative TCA cycle, but Chlorobaculum tepidum lacks 2-oxoglutarate dehydrogenase (2-OGDH) and has been shown in some studies to utilize only the incomplete oxidative TCA cycle (from citrate to 2-oxoglutarate) during mixotrophic growth with acetate (Feng et al., 2010; Tang and Blankenship, 2010). Heliobacteria can only utilize a limited set of carbon sources, in part due to an incomplete reductive TCA cycle. ATP-dependent citrate lyase and (Si)-citrate synthase activities have not been detected in heliobacteria (Pickett et al., 1994; Tang et al., 2010a). Some more recent studies have indicated that heliobacteria could use the (Re)-citrate synthase to initiate the incomplete oxidative TCA cycle (from citrate to 2-oxoglutarate) and that carbon flux is mostly carried out through the incomplete oxidative TCA cycle (Tang et al., 2010b). Although (Re)-citrate synthase and (Si)-citrate synthase both catalyze the formation of citrate from oxaloacetate and acetyl-CoA, the difference lies in adding the acetyl group to the “pro-R” (by (Re)-citrate synthase) or “pro-S” (by (Si)-citrate synthase) face of citrate (Tang et al., 2011). Interestingly, the recently discovered acidobacterium, C. thermophilum, also seems to lack the
gene for 2-OGDH but has a gene for 2-oxoglutarate:ferredoxin oxidoreductase (KFOR) (Bryant et al., 2007). Because *C. thermophilum* cannot synthesize branched chain amino acids but has the capacity to degrade them (Garcia Costas et al., 2012), this organism may synthesize 2-oxoglutarate primarily by carboxylating succinate (Tank and Bryant, 2015). Finally, it should be noted that two recently described members of the phylum *Chlorobi*, the heterotroph *Ignavibacterium album* (Liu et al., 2012a) and the photoheterotroph “*Candidatus Thermochlorobacter aerophilum*,” (Liu et al., 2012b) are microaerophiles that have complete TCA cycles as well as genes for both KFOR and 2-OGDH.

Nearly 50 years ago, two groups independently concluded that cyanobacteria have an incomplete TCA cycle because they lack 2-OGDH, which converts 2-oxoglutarate to succinyl-CoA (Pearce and Carr, 1967a; Smith et al., 1967). In the intervening years, no gene encoding 2-OGDH has been found in any sequenced cyanobacterial genome (Shih et al., 2013; Wood et al., 2004). Consequently, the observation that most cyanobacteria are obligate photolithoautotrophs was often attributed to their incomplete TCA cycle (Stanier and Bazine, 1977; Wood et al., 2004). However, work from this dissertation demonstrated that two other enzymes, namely 2-oxoglutarate decarboxylase (2-OGDC) and succinic semialdehyde dehydrogenase (SSADH), can functionally replace 2-OGDH and succinyl-CoA synthetase by converting 2-oxoglutarate to succinate (Zhang and Bryant, 2011). More specifically, 2-oxoglutarate is first decarboxylated to succinic semialdehyde by 2-OGDC, and succinic semialdehyde is then oxidized to succinate by SSADH using NADP⁺ as the electron acceptor. Acting together, these two reactions thus complete the TCA cycle in cyanobacteria.
1.3.2 TCA cycle in algae and plants

The main role of mitochondria in algae and plant cells, as in all eukaryotic cells, is the production of ATP, reducing equivalents and metabolic intermediates for use in biosynthesis elsewhere in the cell (Fernie et al., 2004; Nunes-Nesi et al., 2008). The TCA cycle is a central element of mitochondrial respiration linking glycolysis and complete oxidation of acetyl-CoA to the electron transport chain. The TCA cycle is also clearly embedded in a wider metabolic network that allows its activities to contribute to other aspects of metabolism. Although the TCA cycle mainly functions in mitochondria and all enzymes of the TCA cycle apparently occur in mitochondria, many TCA cycle enzymes are also found in other cellular compartments, such as the cytosol (e.g., aconitase, isocitrate dehydrogenase, succinyl-CoA ligase, fumarase, malate dehydrogenase) and peroxisomes (e.g., citrate synthase, isocitrate dehydrogenase, malate dehydrogenase) (Araújo et al., 2012). Whether the TCA cycle is also fully functional in cytosol is still not clear, because 2-OGDH, succinic dehydrogenase and citrate synthase are not located in the cytosol (Araújo et al., 2012; Millar et al., 2011). Interestingly, malate dehydrogenase occurs in chloroplasts (Araújo et al., 2012), in which the TCA cycle is not functional due to the absence of all other TCA cycle enzymes. In C4 plants, malate dehydrogenase is an important, light-activated component of the C4 pathway for photosynthesis and CO₂ concentration (Carr et al., 1999). In C3 plants, the “malate valve” provided by this enzyme is thought to buffer short-term imbalances between light-driven reductant production and consumption (Backhausen et al., 1998).

It has been suggested that the TCA cycle in algae and plants often operates in a modular but not cyclic fashion, especially in plant leaves during daytime when the TCA cycle is mostly open and used to produce 2-oxoglutarate, which implies that different parts of the cycle may have different metabolic functions depending on the physiological context in which that pathway is
operational (Araújo et al., 2012; Sweetlove et al., 2010). In agreement with this idea, studies have shown that the level of accumulation of various organic acids can be extremely variable across species, developmental stages and tissue types in plants (Sweetman et al., 2012). These observations imply that the enzymes involved in the production and interconversion of these metabolic intermediates are tightly controlled. However, even though much progress has been made towards understanding the role of the TCA cycle enzymes in respiration (Noctor et al., 2007; Plaxton and Podestá, 2006), the precise regulation of this cycle, as well as its interactions with photosynthesis and photorespiration remains incomplete (Bauwe et al., 2010; Noctor et al., 2007; Nunes-Nesi et al., 2011). By using isotopic tracing techniques, one study verified that glycolysis and TCA cycle activities are inversely related to the ambient CO$_2$/O$_2$ ratio (Tcherkez et al., 2008). This study also found that high dihydroxyacetone phosphate to glucose-6-phosphate ratios can lead to a reduction of TCA cycle activity, while TCA cycle activity and glutamate synthesis increased under low CO$_2$ conditions (Tcherkez et al., 2008).

Some progress has recently been made using systematic reverse genetics experiments to suppress the activity of each enzyme of the TCA cycle, and these studies have confirmed the importance of TCA cycle metabolism in illuminated leaves (Nunes-Nesi et al., 2008). However, these studies have also revealed a surprising complexity in the response. Suppression of some enzymes led to increased photosynthesis (Carrari et al., 2003; Nunes-Nesi et al., 2005), while suppression of others led to decreased photosynthesis (Nunes-Nesi et al., 2007). While these experiments establish that the enzymatic reactions of the TCA cycle are clearly linked to photosynthetic performance, the mechanisms that underlie the observed responses still need to be fully elucidated.
1.4 The glyoxylate cycle

As described above, the glyoxylate cycle was discovered by Sir Hans Adolf Krebs. (Kornberg, 2000; Kornberg and Krebs, 1957). And due to the sharing of malate dehydrogenase, citrate synthase, and aconitase activities with the TCA cycle, the glyoxylate cycle is usually described as the modified TCA cycle (Figure 1-2). However, the difference lies in the two key enzymes that are used in the glyoxylate cycle but not in the TCA cycle, namely isocitrate lyase and malate synthase, which convert isocitrate and acetyl-CoA into succinate and malate (Figure 1-2). The glyoxylate shunt is usually correlated with the ability of bacteria to metabolize acetate. All chlorophototrophic members of the Chloroflexi (i.e., *Chloroflexus* spp., *Oscillochloris trichoides*, and *Roseiflexus* spp.) have isocitrate lyase and malate synthase. All of these organisms can photoassimilate acetate, and some can grow heterotrophically on acetate using the glyoxylate shunt (Sirevåg, 1995; Zarzycki and Fuchs, 2011). Similarly, most purple sulfur bacteria can photoassimilate acetate and have the enzymes of the glyoxylate shunt, but genes encoding these enzymes appear to be missing in most purple non-sulfur bacteria. Members of the Chlorobi photoassimilate acetate by a different pathway, namely by carboxylation of acetyl-CoA. Finally, heliobacteria and *Chloracidobacterium thermophilum* lack isocitrate lyase and malate synthase.

Until recently, the occurrence of the glyoxylate shunt in cyanobacteria has been controversial, but genome sequencing and improved biochemical analyses seem to provide clarification. Several older studies reported that certain cyanobacteria have isocitrate lyase and/or malate synthase activity (Eley, 1988; Pearce and Carr, 1967b) or could assimilate acetate (Hoare et al., 1967; Miller and Allen, 1972). However, a recent study showed that the enzymes of the glyoxylate shunt are not detectable in *Synechocystis* sp. PCC 6803 (Knoop et al., 2013). Recently, it was reported that two *Cyanothece* spp. (PCC strains 7424 and 7822) have a dicistronic operon encoding isocitrate lyase and malate synthase (Bandyopadhyay et al., 2011). When these genes
are used as database queries of other cyanobacterial genomes, neither gene was present in *Synechococcus* sp. PCC 7492/6301 (*Anacystis nidulans*), *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, *Nostoc* sp. PCC 7120, nor other commonly studied cyanobacteria. Only the genomes of three *Chlorogloeopsis* spp., *Pleurocapsa minor* PCC 7327, *Mastigocoleus testarum*, and *Fischerella* sp. PCC 9605 also contained genes encoding isocitrate lyase and malate synthase. The absence of these two genes in most cyanobacteria implies that they were likely to have been obtained recently by lateral gene transfer. The presence of these two genes in *Chlorogloeopsis* spp. agrees with the demonstrated ability of this organism to assimilate acetate in both the light as well as the dark (Miller and Allen, 1972). Other reports concerning the capacity of cyanobacteria to photoassimilate acetate probably indicate a much more limited capacity for acetate uptake and incorporation and by process(es) that do not employ the glyoxylate shunt.

In algae and plants, the glyoxylate cycle operates in the peroxisome/glyoxasome, providing an effective route to convert C₂-units to C₄-precursors for biosynthesis, which allows growth on fatty acids and C₂-compounds such as acetate and ethanol (Eastmond and Graham, 2001; Kunze et al., 2006). In *Chlamydomonas reinhardtii*, it has been demonstrated that the glyoxylate shunt is required for dark growth on acetate, as well as for efficient growth in the light when acetate is supplied (Plancke et al., 2014). Furthermore, purified isocitrate lyase from *Chlamydomonas reinhardtii* was shown to be inactivated by glutathionylation and reactivated by glutaredoxin, which implies that the glyoxylate shunt may be regulated under specific environmental conditions (Bedhomme et al., 2009). However, the functional significance of these post-translational modifications in response to different growth conditions is not yet well understood. In *Arabidopsis thaliana*, the glyoxylate shunt, together with the β-oxidation of fatty acids, are especially important metabolic pathways in providing carbohydrates and biosynthetic precursors which cannot be generated by photosynthesis during the early stage of seedling
establishment (Eastmond and Graham, 2001; Graham, 2008). The β-oxidation pathway and glyoxylate shunt enzymes are induced for the breakdown of membrane lipids and gluconeogenesis in senescing leaves (Chen et al., 2000). However, the coupling of β-oxidation pathway with the glyoxylate cycle is not essential for seed germination in mutants lacking the key enzyme, isocitrate lyase, of the glyoxylate cycle because photosynthesis can compensate for the absence of the glyoxylate cycle during postgerminative growth (Eastmond et al., 2000; Eastmond and Graham, 2001). However, the glyoxylate cycle plays an important role in seedling survival and recovery during prolonged dark conditions that frequently occur in nature (Eastmond et al., 2000). Furthermore, the linkage between the glyoxylate cycle and β-oxidation was only found to occur in aleurone cells but was absent in embryos of germinating barley (Holtman et al., 1994).

How the glyoxylate cycle and β-oxidation pathways coordinately interact, and the possible regulatory mechanisms that occur during seedling establishment and postgerminative growth, are still important questions to be answered.

1.5 The gamma-aminobutyric acid (GABA) shunt

The GABA shunt pathway is composed of glutamate decarboxylase, GABA aminotransferase and succinic semialdehyde dehydrogenase, and the shunt results in the conversion of glutamate to succinate with GABA and succinic semialdehyde as intermediates. The GABA shunt is conserved in many prokaryotes and eukaryotes. In mammalian systems, it acts as an important channel to synthesize GABA, which is an inhibitory neurotransmitter (Decavel and Van Den Pol, 1990; McCormick, 1989). Severe malfunction of the neural system associated with GABA or 4-hydroxybutyrate accumulation in body fluids was observed when GABA aminotransferase or SSADH in humans was disrupted, respectively (Chambliss et al.,
In addition, the GABA shunt has been found to be involved in many physiological responses in plants, including stress sensing and plant development (Bouché et al., 2003; Coleman et al., 2001; Palanivelu et al., 2003), plant-bacteria signaling and communication and carbon/nitrogen balancing (Chevrot et al., 2006; Fait et al., 2008; Fait et al., 2011). It was also reported in *Bradyrhizobium japonicum* that the GABA shunt was used in a mutant lacking the 2-oxoglutarate dehydrogenase (Green et al., 2000).

Some other interesting research in plants recently demonstrated that reducing the activity of enzymes of the TCA cycle, such as citrate synthase or succinyl-CoA synthetase, produced relatively small changes in the rate of respiration within photosynthetic tissues (Sienkiewicz-Porzucek et al., 2008; Studart-Guimarães et al., 2007). It was suggested that this modest phenotype may due to compensatory up-regulation of the GABA shunt (Figure 1-2), which is known to be an important but only partly characterized plant pathway, and which is associated with numerous physiological responses conditions (Bouché and Fromm, 2004; Fait et al., 2008). Microarray profiling of transcript levels for genes encoding components of the TCA cycle and GABA shunt have revealed that they are differentially regulated (Fait et al., 2008). It seems reasonable to assume that flux through the GABA shunt increases in response to changing environmental conditions, and the GABA shunt could serve as an adaptive mechanism to maintain the rate of respiration under certain stress situations (Fait et al., 2008).

### 1.6 Photosystems and thylakoid membrane assembly in cyanobacteria

In cyanobacteria, the conversion of light to chemical energy by the process of photosynthesis is localized on the thylakoid membrane network. Two types of photosystems (PS) cooperate with each other in the utilization of light energy to produce the reducing power (e.g.,
NADPH) and chemical energy (e.g., ATP), which are required for carbon dioxide fixation and many other cellular metabolisms. PS II catalyzes the light-dependent oxidation of water and PS I carries out the light-dependent oxidation of plastocyanin (or cytochrome c6). PS I is a large, multisubunit complex that can be purified in the forms of both monomer and trimers from cyanobacteria. X-ray crystallographic structures of PS I and PS II complexes have been obtained from the thermophilic cyanobacteria species Thermozynechococcus (Jordan, 2001; Umena et al., 2011). The 2.5-Å crystal structure shows that each PS I monomer comprises 12 polypeptides, 96 chlorophyll (Chl) a molecules, 22 beta-carotene molecules, 3 [4Fe-4S] clusters, 2 phylloquinones, 4 lipids, and a putative Ca\(^{2+}\) ion (Jordan, 2001). Six redox centers are involved in light-induced electron transfer in PS I, including the primary electron donor P700 as well as A\(_0\), A\(_1\), F\(_X\), which are harbored by PsaA/PsaB heterodimer, and two terminal electron acceptors, F\(_A\) and F\(_B\), which are located on the extrinsic PsaC protein (Vassiliev et al., 2001). The 1.9-Å structure of PS II show that each PS II monomer additionally binds 35 Chl a molecules, two pheophytin (Phe) a molecules, two plastoquinones, 11 β-carotenes, at least 20 lipids, and many inorganic ions or atoms including two heme irons, one non-heme iron, four manganese atoms, 1 Ca\(^{2+}\) ion, ~3 to 4 Cl\(^{-}\) ions, and one bicarbonate ion (Umena et al., 2011).

The complicated structure of photosystems indicates that biogenesis of photosynthetic complexes in cyanobacteria is a complex multistep process that includes apo-protein translation and folding, cofactor binding and iron-sulfur cluster assembly, ordered association of each individual subunits, and insertion of multisubunit complexes into the thylakoid membrane. Despite the fact that several cofactors have been found to be involved in the assembly or stability of PS I, the underlying molecular mechanisms are still not clear (Bartsevich and Pakrasi, 1997; Boudreau et al., 1997; Naver et al., 2001). Besides, targeting proteins into and across the thylakoids have also been described in several pathways, but little is known about the origin of this membrane system or how the lipid backbone of the thylakoids is transported and fused with the target membrane (Fuhrmann
et al., 2009; Kroll et al., 2001). Moreover, the relationship between the cytoplasmic and thylakoid membranes in cyanobacteria is still very poorly understood. There is general agreement that the biogenesis of thylakoid membranes is a complex, multidimensional process. During this process, lipids, proteins, and pigments, as well as other cofactors must be synthesized, transported, assembled and inserted into these membranes, but few mechanistic details are available.

Recent studies have revealed that the product of the *vipp1* gene (*Vesicle-Inducing Protein in Plastids 1*) is involved in this process of thylakoid biogenesis (Aseeva et al., 2007; Gao and Xu, 2009; Kroll et al., 2001; Li et al., 1994; Westphal et al., 2001). An *Arabidopsis thaliana* mutant strain, in which the Vipp1 was expressed to only about 20% of the Vipp1 protein levels that occur in wild type under normal growth conditions, lost most of the thylakoid membranes and the mutant was incapable of photoautotrophic growth on soil (Kroll et al., 2001). However, a fully segregated null mutant for *vipp1* could not be produced, and thus the product of *vipp1* was believed to be essential for viability (Kroll et al., 2001). Genes similar to *vipp1* are also found in most cyanobacteria (Huang et al., 2002; Srivastava et al., 2006; Westphal et al., 2001). Recently, attempts were made to construct *vipp1* mutants of *Synechocystis* sp. PCC 6803, but in none of these studies could null mutations of the *vipp1* gene be fully segregated. These *Synechocystis* sp. PCC 6803 merodiploids had a similar phenotype to the knock-down strains of *A. thaliana*, and they exhibited a comparable loss of thylakoid membrane content and structure and also had reduced photosynthetic activity (Gao and Xu, 2009; Westphal et al., 2001). Thus, it was suggested that Vipp1 is also essential in cyanobacteria, because it apparently plays an essential role in thylakoid membrane biogenesis. However, because null alleles of *vipp1* never fully segregated in these *Synechocystis* sp. PCC 6803 strains, the results obtained from the characterization of the merodiploid strains were inconclusive and must be interpreted cautiously until fully segregated deletion mutant of *vipp1* was generated. Characterization of cyanobacteria strains with *vipp1* fully deleted will not only help us to understand the function of *vipp1* gene in
thylakoid membrane biosynthesis and photosynthetic protein assembly, but may also help us to explore new approaches in metabolic engineering new cyanobacterial strains for biofuel applications with high efficiency in conversion of sunlight into chemical energy through photosynthesis.

1.7 Aims and scopes of dissertation research

As mentioned, cyanobacteria are one group of essential oxygenic photosynthetic organisms and are projected to play a pivotal role in supplying future renewable energy needs. Thus, our abilities to understand and utilize these organisms efficiently are extremely important. This capability is directly dependent on fundamental studies to understand key metabolic interactions and regulatory processes, as well as the studies of photosystem assembly and photosynthetic processes in these organisms. The combination of these two approaches will enrich the understanding and thus facilitate potential applications in maximizing both the light absorbance efficiency and the metabolic engineering capacity in cyanobacteria.

This dissertation will focus on the discovery of key metabolic pathways and illustration of the photosynthetic processes mechanisms in cyanobacteria, in order to provide novel insights into metabolic and pathway evolution and also to provide new opportunities for biotechnological applications through metabolic engineering. These discoveries should also help to understand carbon–nitrogen balance better in algae and plants. Additionally, the vast amounts of rapidly accumulating genome sequence data from prokaryotic and eukaryotic sources also provide opportunities to study the molecular evolution of individual enzymes as well as of complete pathways with multiple enzymatic reactions. Thus, the metabolic rationale for the acquisition and incorporation of new genes/enzymes into metabolic pathways during evolution will also be
investigated. Furthermore, by utilizing the diverse metabolic capacities in cyanobacteria and these newly demonstrated metabolic pathways, the possible metabolic engineering strategies to produce green and sustainable biofuels and biomaterials were also explored in cyanobacteria. Finally, proteins (*vipp1*) related to the transport and assembly processes of photosystems and thylakoid membrane were also studied, because this knowledge could help those attempting to maximize photosynthetic efficiency in engineered cyanobacteria and photosynthetic eukaryotes.

### 1.8 References


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Figure 1-1 Bright field microscopy image of *Synechococcus* sp. PCC 7002

*Synechococcus* sp. PCC 7002 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% (vol/vol) CO$_2$ in air.
Figure 1-2 Scheme showing the TCA cycle and related variants

Abbreviations: 2-oxoglutarate, 2-oxoglutarate; 2-OGDC, 2-oxoglutarate decarboxylase; 2-OGDH, 2-oxoglutarate dehydrogenase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; ACL, ATP-dependent citrate lyase; ACO, Aconitase; CS: Citrate synthase; FUM, Fumarase; FRD, Fumarate reductase; GABA, gamma-Aminobutyric acid; GABA-TK, GABA aminotransferase (2-oxoglutarate depended); GABA-TP, GABA aminotransferase (pyruvate depended); GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GOGAT; glutamine:2-oxoglutarate amidotransferase; GS, glutamate synthetase; ICL, Isocitrate lyase; IDH, Isocitrate dehydrogenase; KFOR, 2-oxoglutarate:ferredoxin oxidoreductase; MDH, Malate dehydrogenase; MS: malate synthase; PDH, Pyruvate dehydrogenase; SCS, Succinyl-CoA synthetase; SDH, Succinic acid dehydrogenase; SSA: succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase. The blue arrows indicate enzymatic reactions (ACL, FRD, KFOR) that are specific to the reverse TCA cycle. The TCA cycle can also operate as a branched pathway.
depending on the physiological conditions and consumption of 2-oxoglutarate and oxaloacetate (e.g., modular operation in illuminated plant leaves or in cyanobacteria and other bacteria under many conditions; see main text for details). Although the GABA shut accomplishes the same overall reaction as the 2-OGDC and SSADH, the genes encoding this pathway are only found in a few cyanobacteria. Nitrogen assimilation can include ammonium uptake, nitrate/nitrite reduction, N₂ fixation, and/or cleavage of urea by urease. (Figure reproduced from Zhang and Bryant, 2014)
Chapter 2  The TCA cycle in cyanobacteria

Publications:


Xiao Qian, Shuyi Zhang, Donald A. Bryant and Charles G. Dismukes. Functional involvements of the 2-OGDH/SSADH bypass of the TCA cycle in Synechococcus sp. PCC 7002. Manuscript in preparation.
2.1 Abstract

It is generally accepted that cyanobacteria have an incomplete TCA cycle because they lack 2-oxoglutarate dehydrogenase and thus cannot convert 2-oxoglutarate to succinyl-CoA. Genes encoding a novel 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase were identified in the cyanobacterium *Synechococcus* sp. PCC 7002. Together, these two enzymes convert 2-oxoglutarate to succinate and thus functionally replace 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. These genes are present in all cyanobacterial genomes except those of *Prochlorococcus* and marine *Synechococcus* species. Closely related genes occur in the genomes of some methanogens and other anaerobic bacteria, which are also thought to have incomplete tricarboxylic acid (TCA) cycles. Besides, genes encoding fumarase were also identified in the cyanobacterium *Synechococcus* sp. PCC 7002. These results established that the complete TCA cycle exists in cyanobacteria, though different compared to the classical TCA cycle.
2.2 Introduction

Cyanobacteria are a highly diverse group of oxygenic chlorophototrophic bacteria, which may have evolved as much as 2.5 billion years ago. Once they evolved the capacity for oxygenic photosynthesis, cyanobacteria played a crucial role in creating an oxidizing atmosphere and providing organic carbon by modifying the evolution of other organisms as well (Des Marais, 2000). In the dark under oxic conditions, cyanobacteria perform aerobic respiration, but anaerobic respiration is extremely uncommon in cyanobacteria. Although common among (facultative) anaerobes, no cyanobacterium has been reported to use nitrate as an electron acceptor for anaerobic respiration. However, *Oscillatoria limnetica* has been reported to perform anaerobic respiration using elemental sulfur as the electron acceptor (Oren and Shilo, 1979). Cyanobacteria typically perform fermentative metabolism under anoxic conditions by consuming endogenous carbohydrate reserves such as glycogen (Stal and Moezelaar, 1997). It has long been accepted that a complete tricarboxylic acid (TCA) cycle is important if not essential for respiratory energy production in the dark for eukaryotes. However, because biomass production is a more central concern for cyanobacteria, the TCA mostly operates as a branched pathway for the production of two essential precursor metabolites, 2-oxoglutarate and oxaloacetate. Interestingly, one group of nitrogen-fixing marine cyanobacteria are proposed to be obligate photoheterotrophs due to the absence of Photosystem II, autotrophic carbon assimilation pathways, and the TCA cycle (Tripp et al., 2010; Zehr et al., 2008). The absence of TCA cycle enzymes for the synthesis of 2-oxoglutarate and oxaloacetate implies that this organism must take up exogenous dicarboxylic acids, or amino acids such as aspartate and glutamate, to provide essential metabolic precursors.

The TCA cycle has two functions in bacteria: it oxidizes two-carbon units derived from acetyl-CoA producing NADH to drive oxidative phosphorylation, and it provides essential precursor metabolites (e.g., oxaloacetate, 2-oxoglutarate, and in some species succinate) that are
required for biosynthesis of cellular components. In 1967 two groups reported the failure to detect 2-oxoglutarate dehydrogenase (2-OGDH), which converts 2-oxoglutarate to succinyl-CoA, in various cyanobacteria (Pearce and Carr, 1967; Smith et al., 1967) (Figure 2-1). For the ensuing 44 years, it has been generally accepted that these organisms have an incomplete TCA cycle, and that this is at least a major contributing factor to explain why most of these organisms are obligate photolithoautotrophs (Ohashi et al., 2011; Pearce and Carr, 1967; Schwarz et al., 2011; Smith et al., 1967; Stanier and Bazine, 1977; Wood et al., 2004). In support of these observations, no fully sequenced cyanobacterial genome encodes the genes for 2-OGDH. The incomplete TCA cycle has been incorporated into various metabolic models for the cyanobacterium Synechocystis sp. PCC 6803 (Fu, 2009; Shastri and Morgan, 2005).

Alternatives to 2-oxoglutarate dehydrogenase are known to participate in the TCA cycles of a few organisms, including those of Euglena gracilis mitochondria (Shigeoka, 1986; Shigeoka and Nakano, 1991) and Mycobacterium spp. (Tian et al., 2005a; Tian et al., 2005b). In the latter organisms, a thiamine-pyrophosphate (TPP)-dependent enzyme, which is structurally related to the large subunit of 2-oxoglutarate dehydrogenase, 2-oxoglutarate decarboxylase (2-OGDC) produces succinic semialdehyde (Tian et al., 2005a). Succinic semialdehyde is subsequently oxidized by succinic semialdehyde dehydrogenase (SSADH) to produce succinate. Although cyanobacteria lack homologs of this type of 2-OGDC, many cyanobacteria, including Synechococcus sp. PCC 7002, encode homologs of SSADH. This observation, as well as the fact that mutants of Synechocystis sp. PCC 6803 lacking succinate dehydrogenase still synthesize succinate (Cooley et al., 2000), strongly suggested that cyanobacterial genomes encode a previously unrecognized 2-OGDC. A gene neighborhood analysis revealed that the gene encoding SSADH (SynPCC7002_A2771) occurred as part of an apparent operon comprising two genes in most cyanobacteria, in which one of the genes (SynPCC7002_A2770) encodes an enzyme similar to, but phylogenetically distinct from, acetolactate synthase (IlvB) (Figure 2-2).
Because acetolactate synthase is a TPP-dependent enzyme, like 2-OGDH and 2-OGDC (Chipman et al., 2005), it seemed possible that these two open reading frames might encode the enzymes replacing 2-OGDH. An examination of transcription data for these genes under many different growth conditions strengthened this hypothesis, because the transcript levels of these two genes varied coordinately with those for other genes encoding enzymes of the TCA cycle (Ludwig and Bryant, 2011, 2012a, b).

Further confusing the properties of the TCA 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 open reading frame of SYNCC7002_A2041 had the highest sequence identity (43%) to the fumarase (slr0018) from Synechocystis sp. PCC 6803 (Figure 2-3). Although it had initially been misannotated as aspartate ammonia-lyase, it thus seemed likely that this gene encodes fumarase.

In this study, proteins encoded by SynPCC7002_A2770 and SynPCC7002_A2771 were separately produced in Escherichia coli and purified, and enzyme assays were then performed. When the product of SynPCC7002_A2771 was incubated with succinic semialdehyde, succinate was produced and NADP⁺ was reduced to NADPH. When the products from SynPCC7002_A2770 and SynPCC7002_A2771 were incubated with 2-oxoglutarate, 2-oxoglutarate was converted to succinate and NADP⁺ was reduced; however, 2-oxoglutarate was consumed but no succinate was produced when NADP⁺ was omitted. When SynPCC7002_A2770 was incubated with 2-oxoglutarate alone, an aldehyde was produced that could be detected using Schiff’s reagent. These observations establish that SynPCC7002_A2770 encodes a novel 2-OGDC that produces succinic semialdehyde and SynPCC7002_A2771 encodes SSADH, which jointly complete the TCA cycle. Together, these two enzymes convert 2-oxoglutarate to succinate...
and thus functionally replace 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. Besides, enzyme assays also demonstrated that the protein product of SynPCC7002_A2041 catalyzed the reversible reaction between malate and fumarate, and thus established that SynPCC7002_A2041 is the gene for fumarase. In together, these results validated that the TCA cycle is complete in cyanobacteria.

2.3 Materials and Methods

2.3.1 Protein purification

Open reading frames SynPCC7002_A2770, SynPCC7002_A2771 SynPCC7002_A2041 of *Synechococcus* sp. PCC 7002 were amplified by polymerase chain reaction (PCR) with Phusion DNA polymerase (New England Biolabs) and cloned into plasmid pAQ1Ex-P_{cpcBA} (Xu et al., 2011). SynPCC7002_A2770, SynPCC7002_A2771 and SynPCC7002_A2041 were amplified using primer sets A2770ExF-A2770ExF, A2771ExF-A2771ExF, A2041ExF-A2041ExF, respectively (Table 2-1). *Escherichia coli* DH5-alpha cells were transformed with pAQ1Ex-P_{cpcBA} encoding SynPCC7002_A2770, SynPCC7002_A2771 or SynPCC7002_A2041. Cells were grown overnight in Luria-Bertani medium (2 L) amended with gentamycin (50 µg ml⁻¹), harvested by centrifugation at 4 °C at 5,000 × g, and washed once with 50 mM Tris-HCl buffer, pH = 8.0. Cell pellets were collected and resuspended in BugBuster™ protein extraction reagent (Novagen), with a volume equal to 5× the weight of the pellet. Cells were disrupted after 30 min of incubation at room temperature (RT) with gentle vortexing. Soluble lysates were obtained by centrifugation at 20,000 × g and loaded on Ni²⁺-NTA affinity resin (Goldbio), which was pre-equilibrated with 10 mM imidazole in 25 mM Tris-HCl, pH 8.0, and washed with 30 mM
imidazole in 25 mM Tris-HCl, pH 8.0, 300 mM NaCl. Proteins were eluted stepwise with 50, 100, 150, 200, and 250 mM imidazole in 25 mM Tris-HCl, pH 8.0, 300 mM NaCl. Fractions containing recombinant proteins were monitored by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), concentrated by ultrafiltration using Centriprep columns (Millipore), and dialyzed (SynPCC7002_A2770 was dialyzed in 50 mM K-phosphate buffer, pH 7.0, 0.3 mM thiamine pyrophosphate (TPP), 1 mM MgCl₂, while SynPCC7002_A2771 and SynPCC7002_A2041 were dialyzed in 50 mM K-phosphate buffer, pH = 7.0). Purified proteins were analyzed by SDS-PAGE and immunoblotting as previously described (Shen et al., 2002) with commercial antibodies (Rockland) to the [His]₆-tag (Figure 2-4). Proteins were also identified by tryptic peptide mass fingerprinting.

2.3.2 Enzyme assays

2.3.2.1 Enzyme assay for Fumarase

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 (RT) 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) containing 2.5 mM fumarate, 50 mM K-phosphate, pH 7.0 and 50 µg of purified SynPCC7002_A2041. The assay mixture was incubated at RT 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.

2.3.2.2 Enzyme assay for SSADH

The reaction mixture (0.2 ml) containing 1.5 mM succinic semialdehyde, 50 mM K-phosphate, pH 7.0, 1 mM DTT, 2 mM NADP\(^+\), and 50 µg purified SynPCC7002_A2771 was pre-incubated at room temperature (RT) for 2 h, then a 20 µl aliquot of the reaction mixture was injected into the HPLC for analysis. Control experiments were performed the same way, without enzyme or NADP\(^+\). Reaction mixtures (0.5 ml) containing 50 mM KPi, pH 7.0, 200 µM DTT, 400 µM NADP\(^+\) and different amounts of purified SynPCC7002_A2771 were pre-incubated at RT for 15 min. The substrate, 20 µM succinic semialdehyde, was then added to the reaction mixture, and the formation of NADPH was monitored spectrophotometrically at 340 nm (Figure 2-10).

2.3.2.3 2-OGDC and Schiff's test

The reaction mixture (1.0 ml) containing 10 mM 2-oxoglutarate, 50 mM K-phosphate, pH 7.0, 0.3 mM TPP, 1 mM MgCl\(_2\), and 100 µg purified SynPCC7002_A2770 was pre-incubated at RT for 2 h. Schiff's reagent (1.0 ml; Sigma-Aldrich) was added to the reaction mixture. The color was recorded after 5 min. Control experiments were performed in the same way but without added enzyme or 2-oxoglutarate.
2.3.2.4 Coupled reaction

Reaction mixture A (0.2 ml) containing 4 mM 2-oxoglutarate, 50 mM K-phosphate, pH 7.0, 0.3 mM TPP, 1 mM MgCl₂, and 50 µg purified SynPCC7002_A2770 was pre-incubated at RT for 1 h. Reaction mixture B (0.2 ml) containing 50 mM K-phosphate, pH 7.0, 2 mM DTT, 4 mM NADP⁺, and 50 µg of purified SynPCC7002_A2771 was then combined with mixture A and incubated at RT for 3 h, and aliquots (20 µl) were injected into the HPLC for analysis. Control experiments were performed the same way but without enzymes or NADP⁺ in mixture B.

2.3.3 HPLC parameters

Samples were analyzed on a Shimadzu LC-20AB HPLC equipped with a 210-nm UV detector SPD-20A. The substrates and products were separated on a Supelcogel C610H column (Supelco, Bellfonte, PA), with 4 mM H₂SO₄ as the mobile phase. The flow rate was 0.5 ml min⁻¹ and the chromatography was performed at 30 °C. The HPLC elution profiles for equal amounts (100 nmol) of 2-oxoglutarate and succinate were also analyzed. The molar extinction coefficient of 2-oxoglutarate is about 20× that for succinate at 210 nm (Figure 2-8).

2.3.4 Preparation of whole-cell lysates

Cells of the wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were harvested by centrifugation at 4000 × g for 10 min at 4 °C. The pellets were resuspended in 0.3 mL of BugBuster™ Master Mix protein extraction reagent (Novagen). Cells were disrupted after a 20-min incubation at RT with gentle shaking. Cell debris was removed by centrifugation at 18,000 ×
g for 30 min at 4 °C. The protein concentration of the cell free extract was determined by using the Quant-iT™ Protein Assay Kit (Invitrogen).

2.3.5 Enzyme assays with whole-cell lysates

Enzyme activities in whole-cell lysates were determined by monitoring the formation of NADPH at 340 nm. To assay the SSADH activity of the whole cell lysate, the reaction mixture (0.5 ml) contained 1.5 mM succinic semialdehyde, 50 mM K-phosphate, pH 7.0, 1 mM DTT, 2 mM NADP⁺, and an appropriate amount of the specified whole-cell lysate. To determine the coupled activity (conversion of 2-oxoglutarate to succinate), the reaction mixture (0.5 ml) contained 2 mM 2-oxoglutarate, 0.3 mM TPP, 1 mM MgCl₂, 50 mM K-phosphate, pH 7.0, 2 mM NADP⁺, 1 mM DTT, and an appropriate amount of the specified whole-cell lysate. No difference was observed if the DTT was omitted from the assay buffer.

2.3.6 Construction of deletion mutants

The coding sequences of SynPCC7002_A2770, SynPCC7002_A2771, or sdhB (SynPCC7002_A1094; succinate dehydrogenase Fe/S subunit, SdhB) were deleted and replaced by DNA fragments encoding an antibiotic resistance gene to produce the corresponding mutant strains of *Synechococcus* sp. PCC 7002. The *erm* gene (erythromycin resistance) was used for SynPCC7002_A2770, and the *aphII* (aminoglycoside phosphotransferase II; kanamycin resistance) gene was used for SynPCC7002_A2771 and *sdhB*. Transformation and selection were performed as previously described (Frigaard et al., 2004). The complete segregation of alleles
was verified by PCR with template DNAs derived from the wild type and mutant strains (Figure 2-15). The primers used are listed in Table 2-1.

### 2.3.7 Construction of overexpression strains

Strains overexpressing open reading frame SynPCC7002_A2770 or SynPCC7002_A2771 were generated using the pAQ1-based expression system previously described (Xu et al., 2011). Plasmids pAQ1Ex-\(P_{\text{pcpBA}}::\text{A2770}\) or pAQ1Ex-\(P_{\text{pcpBA}}::\text{A2771}\) were separately transformed into the \(\Delta\text{sdhB}\) mutant to produce strains overproducing 2-OGDC and SSADH. Growth of the mutant and wild-type strains was followed by measuring OD\(_{730\text{ nm}}\) as a function of time (Figure 2-17 and Figure 2-18). Growth rates were calculated from the increase in optical density prior to the onset of light limitation at OD\(_{730\text{ nm}}\) ~0.7. Cells were grown in medium A\(^+\) (containing 10 mM nitrate as N-source) under constant irradiance (~250 \(\mu\text{mol photons m}^{-2}\text{ s}^{-1}\)) at 38°C with continual sparging with 1% (v/v) CO\(_2\) in air (standard photoautotrophic growth conditions for this cyanobacterium).

### 2.3.8 Intracellular metabolite pool analysis (LC/MS)

Metabolite extraction and analysis was done as previously described (Bennette et al., 2011). Briefly, the cells (1ml) from the cell culture were collected and centrifuged, and the supernatant was discarded. After vacuum dry of remnant media, 1.8 ml of 80:20 MeOH/H\(_2\)O (precooled to -20 °C) was added to each tube. After 20 min of incubation in -20 °C, the tubes were centrifuged at 14,000 g at 4 °C for 5 min, and 120\(\mu\text{l}\) of the solvent was transferred to another tube and vacuum-centrifuged (Conco Centri-Vap Concentrator), after which the pellet
was resuspended in 40 µl of LC/MS grade H₂O and transferred to LC/MS vials for analysis. The metabolites were analysed by injecting 10µl of the sample into Agilent’s 1200 series HPLC coupled to 6490 QQQ mass analyzer equipped with an ion spray source (Agilent Technologies, Waldbronn, Germany). The samples were column separated using XRs 3 C18 50 X 2.0 mm column with gradients of MeOH and 11mM acetic acid + 10 mM TBA in water as mobile phases with the flow rate of 0.33 ml/ min. The MS was operated in negative ionization mode and the data acquired was analyzed using Agilent Mass Hunter software (Build 1.04).

2.4 Results

2.4.1 Enzymatic assays

Open reading frames SynPCC7002_A2770, SynPCC7002_A2771 and SynPCC7002_A2041 were separately expressed in *Escherichia coli*, and the resulting soluble proteins were purified by metal chelation chromatography as described in the Materials and Methods part (section 2.3.1). The enzyme activity for fumarase was firstly assayed. When the product of SynPCC7002_A2041 was incubated with malate at RT, fumarate was formed and malate was consumed (Figure 2-5). When the product of SynPCC7002_A2041 was incubated with fumarate at RT, malate was formed and fumarate was consumed (Figure 2-5). These results validate that SynPCC7002_A2041 encodes for fumarase and catalyzes the reversible reaction between malate and fumarate.

When the product of SynPCC7002_A2771 was incubated with succinic semialdehyde, succinate was produced and NADP⁺ was reduced (Figure 2-6). When the products from SynPCC7002_A2770 and SynPCC7002_A2771 were incubated with 2-oxoglutarate, succinate
was produced and NADP⁺ was reduced; however, 2-oxoglutarate was consumed but no succinate was produced when NADP⁺ was omitted (Figure 2-7). When SynPCC7002_A2770 was incubated with 2-oxoglutarate alone, an aldehyde was produced that could be detected using Schiff’s reagent (Figure 2-9). These observations establish that SynPCC7002_A2770 encodes a novel 2-OGDC that produces succinic semialdehyde and SynPCC7002_A2771 encodes SSADH.

2.4.2 Substrate inhibition study of SSADH

Due to the easy detection of the formation of NADPH, the kinetic mechanism for SSADH was further investigated. Surprisingly, decreased initial reaction rate was observed with increased substrate concentrations of succinic semialdehyde when succinic semialdehyde concentration was above 40 µM (Figure 2-11), suggesting that SSADH was not following the typical Michaelis–Menten kinetics, but was more likely a substrate inhibited enzyme (Reed et al., 2010). One mechanism for the explanation of substrate inhibition is that the enzyme has two binding sites for its substrate, a catalytic site for binding a substrate that can produce the product, and a non-catalytic site that could also be binding another substrate. Based on this mechanism, a kinetic model could be derived as shown in Figure 2-12. The experimental data fit very well when the substrate inhibition model was applied, and the calculated parameters were: the $V_{\text{max}}$ was 1.50 µM/sec/µM Enzyme, the $K_{m}$ was 3.36 µM and the $K_{i}$ was 204.35 µM. In agreement with this proposed mechanism, the recently solved crystal structure (Figure 2-12) also demonstrated that SSADH is a homodimeric enzyme and thus has two potential substrate binding sites (Park and Rhee, 2013).
2.4.3 *In vivo* enzymatic assay

Peptides from SynPCC7002_A2770 and SynPCC7002_A2771 were present in the soluble proteome of *Synechococcus* sp. PCC 7002 (Cannon et al., 2011). To investigate the function of these two enzymes in *Synechococcus* sp. PCC 7002, deletion mutants lacking either SynPCC7002_A2770 or SynPCC7002_A2771 were constructed. Additionally, the two deletion strains were complemented with plasmids to overproduce 2-OGDC and SSADH. Figure 2-13 shows the results of enzymatic assays for SSADH activity. Succinic semialdehyde oxidation activity was present in whole-cell extracts of wild-type cells, but no activity was detected in the SynPCC7002_A2771 deletion mutant. To demonstrate 2-OGDC activity, a coupled assay for the conversion of 2-oxoglutarate to succinate in the presence of NADP⁺ was used. As shown in Figure 2-14, wild-type whole-cell extracts exhibited this activity, but no activity was detected in whole-cell extracts prepared from either of the two deletion mutants. These data clearly establish that both 2-OGDC and SSADH are present and active in wild-type *Synechococcus* sp. PCC 7002 cells grown under standard photoautotrophic growth conditions.

2.4.4 Intracellular metabolites concentration detection

To investigate the enzyme activities *in vivo* further, the intracellular metabolites concentrations in the wild type and mutants were also measured as described in the Material and Methods (section 2.3.8). Our results show that succinate accumulated to a high level in the succinate dehydrogenase mutant compared to wild type strain (~9-fold higher), while 2-oxoglutarate also accumulated (~1.5-fold higher). On the other hand, 2-oxoglutarate accumulated (~2-fold higher) in the 2-OGDC and SSADH single mutants as well as the double mutant, while
succinate was nearly depleted (~10% of the amount in the wild-type strain). These data validate that 2-OGDC and SSADH are functional in cells, and serve as a major contributing pathway for the biosynthesis of succinate from 2-oxoglutarate.

2.4.5 Mutants growth phenotype

To assess the impact of deletion mutations on growth, cells were grown under constant light or under a 12 h light:12 h dark regime. During constant light, both mutants grew slower than the wild type. The mutant lacking SSADH grew about 12% slower and had a doubling time of 4.6 h while the mutant lacking 2-OGDC grew about 30% slower and had a doubling time of 5.3 h (4.1 h for the wild type). A deletion mutant for a subunit of succinate dehydrogenase (SynPCC7002_A1094) was also constructed and had a doubling time of 4.2 h. These data are generally consistent with studies showing that *Synechocystis* sp. PCC 6803 mutants lacking succinate dehydrogenase still had a comparable photoautotrophic growth rate as that of wild type strain (Cooley et al., 2000).

Strains overproducing SynPCC7002_A2770 and SynPCC7002_A2771 were also studied under constant light and light:dark growth regimes. The largest effect was observed for the mutant overproducing 2-OGDC, which grew much slower than the wild-type strain (doubling time of 9 h). 2-oxoglutarate is one of the most important metabolites in cyanobacterial cells and is important for ammonia assimilation, regulation of nitrogen and carbon metabolism, and as a precursor metabolite for heme and chlorophyll biosynthesis (Fokina et al., 2010; Forchhammer, 2004). Thus, it is not surprising that overproduction of an enzyme that should lower the intracellular levels of this metabolite could have a negative impact on cell growth rate. It is also possible that succinic semialdehyde could accumulate and cause some toxicity effects in this
mutant. Conversely, overproduction of SSADH had a smaller effect on cell growth. succinic semialdehyde production would presumably still be restricted by the amount of 2-OGDC activity in cells.

2.5 Discussion

Collectively, these data demonstrate that *Synechococcus* sp. PCC 7002 has two enzymes that replace the activities of 2-OGDH and succinyl-CoA synthetase, although the replacement enzymes produce NADPH rather than NADH and are unable to synthesize GTP by substrate-level phosphorylation. The genes encoding these two enzymes are organized in an apparent operon in *Synechococcus* sp. PCC 7002 and numerous other cyanobacteria (but not in *Synechocystis* sp. PCC 6803). Furthermore, these two genes are highly conserved and, with the exception of *Prochlorococcus* spp. and some marine *Synechococcus* spp. (the alpha cyanobacteria), are found in the majority of cyanobacteria with completely sequenced genomes (Zhang and Bryant, 2011), and these two genes are also found in some anaerobes (e.g., *Methanosarcina* and *Clostridium* spp.) that have been reported to have incomplete TCA cycles due to the absence of 2-OGDH.

Because at least some cyanobacteria (e.g., *Prochlorococcus* and marine *Synechococcus* spp.) apparently have incomplete TCA cycles (Zhang and Bryant, 2011), the discovery of the TCA cycle variant in other cyanobacteria raises the question, “is there an advantage to the acquisition of these genes in most cyanobacteria?” Given the broad distribution of these two genes, and given the fact that only some cyanobacteria are able to grow heterotrophically in the dark, it is clear that neither an incomplete TCA cycle nor the recently discovered TCA cycle shunt are solely responsible for the obligate photoautotrophic growth exhibited by some
cyanobacteria (Meeks, 2011). Instead, the absence of transporters for potential carbon sources or some other limitation resulting from a metabolic control process probably restricts the growth of cyanobacteria in the dark. This also causes one to question whether TCA cycle variants utilize different regulatory molecules that might efficiently coordinate flux towards specific reactions and thus metabolic intermediates, which might be needed to respond to changes in environmental conditions.

2-oxoglutarate has important regulatory functions, and its levels are monitored by signaling proteins such as GlnB (P\text{II}) or CcmR (Jiang and Ninfa, 2009; Moorhead and Smith, 2003). The phosphorylation state of GlnB regulates central nitrogen assimilatory processes and is believed to be important in establishing control over carbon/nitrogen metabolism at various systems levels, it may be a good candidate for such control (Forchhammer, 2004; Steinhauser et al., 2012). It is demonstrated that GlnB proteins can act as sensors of the cellular adenylate energy charge (ATP/ADP) and the 2-oxoglutarate level. In response to these metabolic signals, GlnB can regulate many cellular processes, from nutrient transport to gene expression through several protein–protein interactions with GlnB receptor proteins (Forchhammer, 2004; Steinhauser et al., 2012). However, the regulatory mechanisms controlling the TCA cycle are still unclear and need more investigation. The LysR-type transcription factor, CcmR, controls the expression of genes encoding the carbon concentration mechanism of cyanobacteria (Daley et al., 2012). Surface plasmon resonance studies recently showed that 2-oxoglutarate and NADP\textsuperscript{+} function as co-repressors of CcmR, which represses its own transcription along with structural genes encoding high affinity C\textsubscript{i} transporters in \textit{Synechocystis} sp. PCC 6803 (Daley et al., 2012). This indicates that CcmR is also likely to play an extremely important role in connecting central metabolism to inorganic carbon acquisition for photosynthesis.

Traditional biochemistry and related experimental approaches have provided much valuable knowledge about metabolism and physiology of cyanobacteria with regards to the
regulatory mechanisms of the TCA cycle. However, flux balance analysis (FBA) and related
methods (e.g., $^{13}$C-metabolic flux analysis; $^{13}$C-MFA) are comprehensive and powerful tools to
investigate the organization of large-scale metabolic networks, can help to decipher functions
predicted from genome analysis, and can unravel cell phenotype in cyanobacteria under different
metabolic conditions (Vu et al., 2013; Young et al., 2011). Flux balance analysis of
photoautotrophic metabolism in cyanobacteria has shown that the main flux occurs within the
Calvin–Benson cycle and that the TCA cycle mostly acts as an incomplete, branched pathway
(Knoop et al., 2013; Steuer et al., 2012). This is consistent with the absence of a complete TCA
cycle in some cyanobacteria and with the ability to construct single or double mutants lacking 2-
OGDH and/or SSADH (Zhang and Bryant, 2011). On the other hand, FBA implies that dark
consumption of glycogen in cyanobacteria should include cyclic flux through the TCA cycle,
mediated by 2-OGDC and SSADH (Knoop et al., 2013; Steuer et al., 2012).

Recent $^{13}$C-MFA studies in *Synechocystis* sp. PCC 6803 showed that the 2-oxoglutarate
was converted to succinate when glutamate was added (You et al., 2014), in agreement with a
previous study showing that mutants of *Synechocystis* sp. PCC 6803 lacking succinate
dehydrogenase could still synthesize succinate when 2-oxoglutarate was supplied to the cells
(Cooley et al., 2000). However, significant fractions of unlabeled 2-oxoglutarate, succinate, and
malate were observed after unlabeled glutamate was added to $^{13}$C-labeled cultures, which may be
accomplished by the newly identified TCA bypass through succinic semialdehyde (You et al.,
2014). Meanwhile, this study also indicated that the flux from 2-oxoglutarate through the
complete TCA cycle was very small compared to other metabolite fluxes (e.g. fluxes through
glycolysis or the Calvin-Benson-Bassham cycle). A low rate of conversion of 2-oxoglutarate to
its downstream metabolites in the TCA cycle was also observed in *Synechococcus elongatus* PCC
7942 expressing 2-oxoglutarate permease, in which 2-oxoglutarate was mainly converted into
glutamate and glutamine instead of downstream metabolites of the TCA cycle (Vázquez-
Bermúdez et al., 2000). In summary, current evidence suggests that, although a complete TCA cycle exists in many cyanobacteria, there may only be a large flux through this cycle under specific growth conditions. The 2-OGDC/SSADH bypass may mostly serve to regenerate intermediates of the cycle (e.g. succinic semialdehyde under certain conditions may be a useful substrate) or to fine-tune the metabolic balance under certain photomixotrophic conditions. Future research applying systems biology-oriented approaches that integrate data for the transcriptome, proteome, and metabolome may ultimately provide details of how the TCA cycle is regulated in cyanobacteria, as well as show how global metabolic fluxes are controlled under different environmental conditions in organisms that have variant TCA pathways (i.e., cycles vs. branched pathways).

It is generally accepted that eukaryotes originated from an ancestral, nucleated heterotroph by the engulfment of free-living bacteria through a process termed endosymbiosis or endocytobiosis (Gray et al., 2001; Martin et al., 2002). This led to organelles with new functionalities for these cells (i.e., mitochondria for respiration and chloroplasts for oxygenic photosynthesis). Mitochondria are generally thought to have arisen from engulfment of an alpha-proteobacterium (Gray et al., 2001), and eukaryotes gained chloroplasts and the capacity for oxygenic photosynthesis through primary endosymbiosis of a cyanobacterium-like cell (Hohmann-Marriott and Blankenship, 2011). Many genes from the cyanobacterial endosymbiont were transferred to the nucleus of the eukaryotic host, leading to the evolution of algae and subsequently plants (Deusch et al., 2008; Reyes-Prieto et al., 2007; Rujan and Martin, 2001). The transferred genes encoded proteins involved in photosynthesis, respiration, and many other metabolic processes, including regulatory functions (Kern et al., 2011; Martin et al., 2002). Although the TCA cycle is typically a mitochondrial pathway in eukaryotes, all of the enzymes of the TCA cycle are encoded in the nucleus in algae and plants (Schnarrenberger and Martin, 2002). A similar situation exists for genes of the Calvin-Benson-Bassham cycle and chloroplasts;
nearly all of the genes of this plastid-localized pathway are encoded in the nucleus (Martin and Schnarrenberger, 1997). It has been suggested that prior to the evolution of a molecular machine to import proteins from the host cell cytoplasm into the symbiont, many proteins involved in central metabolic pathways that once belonged to the symbiont could have been transferred to the cytosol after the genes encoding these proteins had been incorporated into the host chromosome (Martin and Miklós, 1998). However, because some enzymes (e.g. succinate dehydrogenase) of the TCA cycle would have been located on the cytoplasmic membrane of the mitochondrial ancestor, a complete transfer of the enzymes in this pathway to the cytosol initially could not occur, and this limitation might have restricted the TCA cycle to mitochondria. In order for these nuclear-encoded proteins to be transported and be functional in the symbiont, the genes that were incorporated into the host chromosome must have eventually obtained signal sequences in order to be translocated back to the symbiont. In accordance with this idea, it has been shown that the psaE gene, which is located on nuclear genome of *Paulinella chromatophora* (Nakayama and Ishida, 2009), has gained four potential non-AUG translation initiation codons upstream of the previously proposed start codon. One of these non-AUG start codons appears to initiate translation of the associated signal peptide that allows translocation of PsaE into the chromatophores (Mackiewicz and Bodyl, 2010; Nakayama and Ishida, 2009).

Detailed phylogenetic analyses indicate that all plant TCA enzymes, except cytosolic aconitase and mitochondrial isocitrate dehydrogenase, are clearly more similar to eubacterial homologs than they are to archaeal homologs (Schnarrenberger and Martin, 2002), and the reductive TCA cycle is a major pathway in many archaea (Schäfer et al., 1989). However, phylogenetic analyses also show that only about half of the enzymes of the TCA cycle in plants can specifically be traced back to an alpha-proteobacterial donor. Origins of the rest of the enzymes in this pathway in plants either have nearly equal similarity to homologs in alpha-proteobacteria and archaea (isocitrate dehydrogenase), or they are more similar to enzymes from
eubacterial donors but not specifically to those from an alpha-proteobacterial source (e.g. malate dehydrogenase) (Schnarrenberger and Martin, 2002). Adding to this complexity is the fact that algae and plants contain both mitochondria and chloroplasts. Considering that variants of TCA cycles exist in both cyanobacteria, algae and plants, a question arises as to whether the genes for enzymes of the TCA cycle in cyanobacteria after primary endosymbiosis were transferred to the chromosome of algae and plants and how those would have interacted with the genes that were already present on the chromosome. Alternatively, the TCA cycle genes could simply have been completely lost from the endosymbiotic cyanobacterium during evolution. Some studies imply that the non-alpha-proteobacterial components of the TCA cycle are encoded by genes that were originally present in the ancestral eukaryotic host before the arrival of the mitochondrial, and that the products of those genes eventually replaced the products of the genes in the evolving mitochondrion (Gray, 2014). Serving as an example of a very recent primary endosymbiotic event, the recently sequenced genome of the chromatophores in *Paulinella chromatophora* provides tantalizing information. The genes for the TCA cycle are already missing in these chromatophores and presumably have been lost or transferred to the nucleus (Nowack et al., 2008). In the recently sequenced genome of the evolutionarily important Glaucohyte, *Cyanophora paradoxa*, genes encoding 2-OGDH, KFOR, 2-OGDC and SSADH are all missing, although it contains genes for the remaining enzymes in the TCA cycle (Price et al., 2012). This result strongly implies that, like some cyanobacteria, *C. paradoxa* has a branched TCA cycle or that it has some other undescribed TCA cycle variant for the conversion of 2-oxoglutarate into succinate. Future genome sequencing, phylogenetic analyses of additional cyanobacteria and selected algae, and further biochemical studies, may provide answers to these unresolved questions.

Although SSADH was correctly identified in most genome annotations, 2-OGDC is a novel thiamine pyrophosphate-dependent decarboxylase that is a member of the acetolactate
synthase family and thus is distantly related to IlvB. 2-OGDH is also a TPP-dependent decarboxylase, and both decarboxylases are distantly related to one of the subunits of pyruvate dehydrogenase (Zhang and Bryant, 2011). It was possible to produce null mutants lacking either or both genes, and a significant growth defect was observed when 2-OGDC was overexpressed. Because 2-oxoglutarate is an essential precursor metabolite for heme, bilin, and chlorophyll biosynthesis, in addition to its role in amino acid and protein biosynthesis (Lancien et al., 2000), it is perhaps understandable that overexpression of 2-OGDC might dramatically lower the intracellular levels of 2-oxoglutarate and cause a severe, negative impact on cell growth rate. This slower growth rate could also partly be due to the production of succinic semialdehyde, which is likely to be toxic and inhibit cell growth. A surprising and still unexplained result is that mutants lacking either the flavin- or Fe/S-cluster carrying subunits of succinate dehydrogenase exhibited no detectable growth defect, but mutations in the genes encoding either 2-OGDC or SSADH consistently grew slower than wild-type *Synechococcus* sp. PCC 7002 (Zhang and Bryant, 2011). One possible interpretation of this result is that succinic semialdehyde is required to produce a compound that is not essential but has a positive effect on cell growth. An important consequence of these findings is that there is new biochemical intermediate, succinic semialdehyde, in a major pathway in cyanobacteria. For example, succinic semialdehyde can be converted in four steps into 1,4-butanediol (Yim et al., 2011), and potentially other useful compounds could use succinic semialdehyde as a building block. Overall, this study corrects a long-standing misconception about cyanobacterial metabolism and illustrates why important biochemical conclusions should not be inferred from exclusively negative data.
2.6 References


Jiang, P., and Ninfa, A.J. (2009). Sensation and signaling of α-ketoglutarate and adenylylate energy charge by the *Escherichia coli* PII signal transduction protein require cooperation of the three ligand-binding sites within the PII trimer. Biochemistry 48, 11522-11531.


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. Frontiers in Microbiology 2, 1-23.


Figure 2-1 The TCA cycle in cyanobacteria and the missing steps

Two steps (2-oxoglutarate dehydrogenase and fumarase) are missing from the classical TCA cycle in cyanobacteria, as indicated by red boxes. Figure was adapted from KEGG. http://www.genome.jp/kegg-bin/show_pathway?syp00020.
Figure 2-2 Neighbor-joining phylogenetic tree

The tree shows the evolution relationship of 2-OGDC (2-oxoglutarate decarboxylase) and IlvB (acetolactate synthase) homologs in different cyanobacterial species. The tree was generated using 1000 bootstrap resamplings, and bootstrap support values per 100 resamplings are shown for each node when the value was greater than 50%. IlvB from *Escherichia coli* was used as the out-group, and the tree shows that that 2-OGDC is distantly related to the paralogous IlvB family. The arrows to the right of the 2-OGDC portion of the tree show the organization of the genes encoding SSADH (succinic semialdehyde dehydrogenase) and 2-OGDC, which are apparently encoded as an operon in many cyanobacteria as shown in the figure but are not colocalized in *Synechocystis* sp. PCC 6803.

Abbreviations:
- SYN: *Synechocystis* sp. PCC 6803
- Npun: *Nostoc punctiforme* PCC 73102
- ANA: *Anabaena* sp. 90
- SYNPCC7002: *Synechococcus* sp. PCC 7002
- Lepto7376: *Leptolyngbya* sp. PCC 7376
- PCC8801: *Cyanothece* sp. PCC 8801
- NIES39: *Arthrospira platensis* NIES-39
- GVI: *Gloeobacter violaceus* PCC 7421
- Mic7113: *Microcoleus* sp. PCC 7113
- Oscill6304: *Oscillatoria acuminata* PCC 6304
- ECDH1: *Escherichia coli* DH1
Figure 2-3 BLASTP result

BLASTP search result shows that the missing fumarase in *Synechococcus* sp. PCC 7002 was mis-annotated as aspartate ammonia-lyase. BLASTP result was generated using the protein sequence of fumarase from *Synechocystis* sp. PCC 6803 as the query against the whole protein library of *Synechococcus* sp. PCC 7002.
Figure 2-4 SDS-PAGE and immunoblotting analysis

Purified proteins of 2-OGDC (SynPCC7002_A2770; lanes 1 and 2), SSADH (SynPCC7002_A2771; lanes 3 and 4) and the putative fumarase (SynPCC7002_A2041; lanes 5 and 6) were analyzed. Lanes 1, 3 and 5 were stained with Coomassie blue and lanes 2, 4 and 6 were detected by immunoblotting with antibodies to the poly-[His]₆ tag.
Figure 2-5 HPLC analysis of fumarase assay components
A, Reaction was catalyzed by fumarase (SynPCC7002_A2041), showing the formation of malate from fumarate. The control reaction was performed lacking enzyme. B, Reaction was catalyzed by fumarase (SynPCC7002_A2041), showing the formation of fumarate from malate. The control reaction was performed lacking enzyme.
Figure 2-6 HPLC analysis of SSADH assay components

Reactions catalyzed by SSADH showing the formation of succinate and NADPH from succinic semialdehyde and NADP$^+$ catalyzed by SSADH (SynPCC7002_A2771). Purified SSADH was incubated with 1.5 mM succinic semialdehyde and 2 mM NADP$^+$. HPLC analysis of 2 mM NADPH in distilled water was also included as standard. The control assays were performed lacking enzyme and NADP$^+$, respectively.
Reactions were catalyzed by the purified 2-OGDC and SSADH, showing the conversion of 2-oxoglutarate into succinate and the conversion of NADP$^+$ into NADPH. The assays were performed as described in the Materials and Methods (2.3.2.4). Control experiments were performed lacking enzymes and NADP$^+$, respectively.
Figure 2-8 HPLC analysis of 2-oxoglutarate and succinate standards

HPLC analysis of equal amounts (100 nmol) of 2-oxoglutarate and succinate with detection at 210 nm. Note that the absorption scales for the two analyses differ by a factor of 10. Thus, the molar extinction coefficient for 2-oxoglutarate at 210 nm is ~20× larger than that for succinate.
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</table>

**Figure 2-9 Detection of succinic semialdehyde by Schiff’s reagent.**

The reaction contains the complete assay mixture for 2-OGDC. The reaction mixture (1.0 ml) containing 10 mM 2-oxoglutarate (2-OG), 50 mM K-phosphate, pH 7.0, 0.3 mM TPP, 1 mM MgCl₂, and 100 µg purified SynPCC7002_A2770. Schiff’s reagent (1.0 ml; Sigma-Aldrich) was added to the reaction mixture. The color was recorded after 5 min. Control experiments were performed without the enzyme or the 2-oxoglutarate added, respectively.
Figure 2-10 Assays for SSADH activity

SSADH activities were assayed by monitoring NADPH production by the absorbance increase at 340 nm. The assays contained 20 µM succinic semialdehyde, 400 µM NADP⁺ and 0, 14, 21, and 28 µg SSADH per ml as indicated. The data shown are for a single set of assays, but similar results were obtained in multiple trials.
Initial reaction rates were calculated based on different amounts of the substrate succinic semialdehyde (SSA), showing that decrease initial reaction rates were observed with increased substrate concentrations. The assays mixture contained 400 µM NADP⁺ and 5 µM SSADH were premixed and incubated for 5 min at room temperature, after which different concentrations of succinic semialdehyde were added as indicated. The blue line shows typical Michaelis–Menten kinetics and the green line shows the fitting result using the substrate inhibition model.
Figure 2-12 Substrate inhibition mechanism and crystal structure of SSADH

A. The scheme shows the possible substrate inhibition mechanism, indicating the binding of another substrate (SES) to the enzyme substrate intermediate (ES). Bottom part shows the derived kinetic formula between substrate concentrations and initial reaction rate. $K_m$ the Michaelis–Menten constant, and $K_i$ is the dissociation equilibrium between SES and ES. B. Crystal structure shows that SSADH is a dimer and has two potential substrate binding sites. Figure was adapted from Park and Rhee, 2013.
Figure 2-13 Whole cell lysate enzyme assay for SSADH

Activity assay for SSADH in whole-cell extracts of wild-type Synechococcus sp. PCC 7002 cells at two extract concentrations (30 µg and 60 µg of protein) and for a deletion mutant D71 lacking SynPCC7002_A2771 (30 µg protein). Activity was monitored as NADPH production by absorption increase at 340 nm.
Coupled activity assay for 2-OGDC and SSADH, which convert 2-oxoglutarate to succinate in the presence of NADP⁺. Whole-cell extracts of wild-type *Synechococcus* sp. PCC 7002 cells were assayed at two extract concentrations (20 µg and 60 µg of protein). Extracts for deletion mutants D70 and D71 were also assayed for 30 µg of protein. Activity was monitored as NADPH production by absorption increase at 340 nm.
Figure 2-15 Construction of mutants and verification

A. General scheme showing how the target genes were replaced with antibiotic resistance cassettes. Polymerase chain reaction was used to amplify the upstream and downstream flanking regions for a gene of interest using primer pairs p1-p2 and p3-p4, and these fragments were ligated to the antibiotic cassette. The resulting construction was transformed into *Synechococcus* sp. PCC 7002, and after segregation of alleles occurred during selection and repeated streaking, transformants were verified by PCR amplification using primers p1 and p4 and template DNA derived from wild-type or transformed cells. B. Agarose gel electrophoretic analysis of amplicons from PCR reactions to verify complete segregation of alleles. Lanes 2, 4 and 6 contained template DNA from the wild-type strain and primers p1 and p4 for SynPCC7002_A2770 (lane 2), SynPCC7002_A2771 (lane 4), and *sdhB* (SynPCC7002_A1094) (lane 6), respectively. Lanes 1, 3 and 5 contained templates DNA from antibiotic-resistant transformants in which
SynPCC7002_A2770 (lane 1), SynPCC7002_A2771 (lane 3), and SynPCC7002_A1094 (lane 5) had been replaced by *erm* or *aphII* as described above.
Relative intracellular concentrations for 2-oxoglutarate and succinate in wild type strain (WT) as well as mutant strains were determined as described in the Materials and Methods (section 2.3.8). ΔSDH: *sdhB* deletion mutant; Δ2-OGDC: SynPCC7002_A2770 deletion mutant; ΔSSASH: SynPCC7002_A2771 deletion mutant; Δ2-OGDCΔSSADH: double mutant of SynPCC7002_A2770 and SynPCC7002_A2771.
Cells were grown in medium A+ (containing 10 mM nitrate as N-source) under a constant irradiance of ~250 μmol photons m$^{-2}$ s$^{-1}$ at 38 °C with sparging with 1% CO$_2$ in air. WT, wild type; sdhB, sdhB deletion mutant; 70D, SynPCC7002_A2770 deletion mutant; 71D, SynPCC7002_A2771 deletion mutant; 70OVER, sdhB mutant harboring pAQ1Ex-$P_{cpcB4}$::SynPCC7002_A2770 to overproduce 2-OGDC; 71OVER, sdhB mutant harboring pAQ1Ex-$P_{cpcB4}$::SynPCC7002_A2771 to overproduce SSADH. The plotted data are the average values obtained from three independent experiments and bars indicate the standard deviations.
Cells were grown in medium A+ (containing 10 mM nitrate as N-source) under a constant irradiance of ~250 µmol photons m⁻² s⁻¹ at 38 °C with sparging with 1% CO₂ in air. WT, wild type; sdhB, sdhB deletion mutant; 70D, SynPCC7002_A2770 deletion mutant; 71D, SynPCC7002_A2771 deletion mutant; 70OVER, sdhB mutant harboring pAQ1Ex-\textit{P}_{\text{cpcB}}::\textit{SynPCC7002}_A2770 to overproduce 2-OGDC; 71OVER, sdhB mutant harboring harboring pAQ1Ex-\textit{P}_{\text{cpcB}}::\textit{SynPCC7002}_A2771 to overproduce SSADH. The plotted data are the average values obtained from three independent experiments and bars indicate standard deviations.

\textbf{Figure 2-18 Growth comparison under dark-light cycle}
Table 2-1 Primers used in this chapter

The open reading frame is designated by A2770 (SynPCC7002_A2770), A2771 (SynPCC7002_A2771) or A1094 (SynPCC7002_A1094) and the primer number (e.g., p1) corresponds to the numbering scheme used in Figure 2-15.

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Chapter 3  The glyoxylate cycle and GABA shunt in cyanobacteria

Publications:


3.1 Abstract

Cyanobacteria are important photoautotrophic bacteria with extensive but variable metabolic capacities. The existence of the glyoxylate cycle, an important 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 two enzymes are missing. The GABA shunt is another controversial variant of the TCA cycle that may occur in some cyanobacteria. In this study the genes encoding glutamate decarboxylase and GABA-aminotransferase from cyanobacteria were biochemically characterized, and metabolite analysis was also performed to study the possible relation between GABA shunt and the TCA cycle. Additionally, in this study the genes encoding isocitrate lyase and malate synthase from Chlorogloeopsis fritschii PCC 9212 were identified, and the enzymes were biochemically characterized. Consistent with the presence of the enzymes of the glyoxylate cycle, C. fritschii PCC 9212 could assimilate acetate under both light and dark growth conditions. Transcript abundances for isocitrate lyase and malate synthase increased, and C. fritschii PCC 9212 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 the wild type. Database searches showed that the genes for the glyoxylate cycle occur in only a few other cyanobacteria, all of which are able to fix nitrogen. This study demonstrates that the glyoxylate cycle exists in some 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. Similarly, the enzymes of the GABA shunt also are found in only a few cyanobacteria, including the common model organism *Synechocystis* sp. PCC 6803.
3.2 Introduction

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 (Brown et al., 1977; Kreth et al., 2013; Pinchuk et al., 2011). 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$_2$ (Ihlenfeldt and Gibson, 1977). The assimilation of dissolved carboxylic acids by heterotrophic bacteria has been known and studied for decades (Gutowski and Rosenberg, 1975; Kay and Kornberg, 1971). 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 (Gibbs et al., 1986; Wright and Hobbie, 1966). Once acetate is transported into the cytosol, it is first converted by acetyl-CoA synthetase to acetyl-coenzyme A (acetyl-CoA), 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 (Boyle and Morgan, 2009; Kornberg, 1966).

Sir Hans Adolf Krebs, who also established the urea/ornithine cycle as well as the TCA cycle, discovered the glyoxylate cycle, (Kornberg, 2000; Kornberg and Krebs, 1957). 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 (Figure 3-1). 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 (Figure 3-1). In more detail, isocitrate
is split into succinate and glyoxylate by isocitrate lyase, after which malate synthase catalyzes the condensation of the glyoxylate and acetyl-CoA to form malate with the release of CoA. 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 while avoiding the CO₂-releasing, oxidative steps of the TCA cycle. Thus, the glyoxylate cycle enables cells to utilize C₂-units (i.e. acetyl-CoA) more efficiently. These C₂-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 (Dunn et al., 2009).

The glyoxylate cycle has been found in many chlorophototrophic bacteria (Tang et al., 2011; Zhang and Bryant, 2014). 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 (Sirevåg, 1995; Zarzycki and Fuchs, 2011). 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 (Tang et al., 2011). Heliobacteria, green sulfur bacteria and Chloracidobacterium thermophilum lack isocitrate lyase and malate synthase, and thus the glyoxylate cycle is absent in these bacteria. Interestingly, 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 CO₂ at the same time (Feng et al., 2010; Madigan, 2006).

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 (Zhang and Bryant, 2011). Although it has been reported that isocitrate lyase and/or malate synthase activities were detected in some cyanobacteria (Eley, 1988; Pearce and Carr, 1967), and that some cyanobacteria were able to assimilate acetate (Hoare et al., 1967; Miller and Allen, 1972), a recent study in *Synechocystis* sp. PCC 6803 failed to detect the enzymes of the glyoxylate cycle (Knoop et al., 2013). However, a recent genome sequencing study reported that two *Cyanothecaceae* spp. (strains PCC 7424 and PCC 7822) have an operon encoding the isocitrate lyase and malate synthase (Bandyopadhyay et al., 2011). 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 (Miller and Allen, 1972).

In addition to the controversy of the glyoxylate cycle, the existence of GABA shunt in cyanobacteria is also poorly understood. Glutamate decarboxylase, GABA aminotransferase and succinic semialdehyde dehydrogenase, form the GABA shunt pathway, which results in the conversion of glutamate to succinate with GABA and succinic semialdehyde as intermediates. A recent study reported that N-acetylornithine aminotransferase from *Synechocystis* sp. PCC 6803 could also function as GABA aminotransferase, which together with glutamate decarboxylase forms the GABA shunt and thus closes the TCA cycle (Xiong et al., 2014). Genes encoding enzymes for the GABA shunt can be found in the genomes of some cyanobacteria (e.g., *Synechocystis* sp. PCC 6803, *Nostoc* sp. PCC 7107, *Prochlorococcus marinus* str. MIT 9303 and *Synechococcus* sp. RCC307). However, *Prochlorococcus marinus* str. MIT 9303 and *Synechococcus* sp. RCC307 lack the 2-OGDC/SSADH bypass, and many other *Prochlorococcus* and marine *Synechococcus* species seemingly lack the GABA shunt as well as the 2-OGDC/SSADH bypass. Such organisms either have yet another alternative bypass or must have a
branched TCA cycle. Similarly, many other cyanobacteria (e.g., *Synechococcus* sp. PCC 7002) apparently lack the glutamate decarboxylase needed to complete the GABA shunt. Thus, the GABA shunt seems to be only present in a few cyanobacteria and does not appear to be universal (Xiong et al., 2014). However, the biochemical validation of the putative bi-functional N-acetylornithine aminotransferase was not performed yet. In this study, we biochemically validated that N-acetylornithine aminotransferase could also function as GABA aminotransferase. Furthermore, the glutamate decarboxylase from *Synechocystis* sp. PCC 6803 was also transformed to *Synechococcus* sp. PCC 7002 which normally lacks this enzyme. Intracellular metabolite concentrations were measured in this overexpression strain and the potential interaction between the GABA shunt and the TCA cycle was investigated.

Additionally, in this study, we describe the biochemical validation of the two genes in *Chlorogloeopsis fritschii* PCC 9212 that encode the key enzymes, isocitrate lyase and malate synthase, of the glyoxylate cycle. We showed that *C. fritschii* PCC 9212 can take up acetate under both light and dark conditions, and that the organism grew faster when acetate was supplied in the medium. Whole-cell transcription profiling showed that the mRNA levels of these two genes were increased somewhat 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 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 glyoxylate cycle in cyanobacteria but shows 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.

3.3 Materials and Methods

3.3.1 Strains and growth conditions

*C. fritschii* PCC 9212 was obtained from the Pasteur Culture Collection (PCC) and was routinely grown in medium BG-11 at 26°C (Rippka et al., 1979). To emphasize the effects of acetate supplementation, cells were grown under constant irradiance of 50 µmol photons m\(^{-2}\) s\(^{-1}\), that was provided by cool white fluorescent tubes, and cultures were sparged with 1% (vol/vol) 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 of the glyoxylate cycle (strain *Synechococcus* 7002-glyox) were grown in liquid A\(^{+}\) medium under standard conditions for this organism (Ludwig and Bryant, 2011): 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% (vol/vol) 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.
3.3.2 **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 a 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 H2SO4 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.

3.3.3 **Cloning, protein purification, and protein identification**

Open reading frames *sll1641*, encoding the glutamate decarboxylase and *slr1022*, encoding the N-acetylornithine aminotransferase of *Synechocystis* sp. PCC 6803; open reading frames *SYNPCC7002_A0326*, encoding the N-acetylornithine aminotransferase of *Synechococcus* sp. PCC 7002 were amplified by polymerase chain reaction (PCR) with Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and cloned into plasmid pAQ1Ex-*P_{cpcBA}* (Xu et al., 2011). Open reading frames *UYEDRAFT_02681*, encoding the putative isocitrate lyase and *UYEDRAFT_02682*, encoding the putative malate synthase of *C. fritschii* PCC 9212 were also amplified and separately cloned into pAQ1Ex-*P_{cpcBA}* using the same procedures (Xu et al., 2011). Primer set ICLF-ICLR was used to amplify *UYEDRAFT_02681*; primer set MSF-
MSR was used to amplify UYEDRAFT_02682; primer set GADF-GADR was used to amplify sll1641; primer set 6803ArgDF-6803ArgDR was used to amplify slr1022; primer set 7002ArgDF-7002ArgDR was used to amplify SYNPCC7002_A0326 (Table 3-1). An N-terminal [His]$_{10}$-tag was introduced into all the putative enzymes except malate synthase 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 of malate synthase.

The resulting plasmids pAQ1Ex-$P_{pcpBA}$:: sll1641, pAQ1Ex-$P_{pcpBA}$:: slr1022, pAQ1Ex-$P_{pcpBA}$:: A0326, pAQ1Ex-$P_{pcpBA}$::U02681 and pAQ1Ex-$P_{pcpBA}$::U02682 were verified by DNA sequencing and were transformed into E. coli strain DH5-alpha. Cells were grown overnight in 1 liter 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 were 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 dodecylsulfate (SDS-PAGE) and were 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 (Zhang and Bryant, 2011).
3.3.4 Expression of glyoxylate cycle genes and glutamate decarboxylase

Open reading frames UYEDRAFT_02681 (isocitrate lyase) and UYEDRAFT_02682 (malate synthase) 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 (Xu et al., 2011) using primer set MSF and ICLR (Table 3-1). The resulting plasmid was verified by DNA sequencing and transformed into wild-type *Synechococcus* sp. PCC 7002 as previously described to generated the glyoxylate cycle expression strain (*Synechococcus* 7002-glyox) (Frigaard et al., 2004). In order to construct an expression strain of glutamate decarboxylase (*Synechococcus* 7002-GAD), pAQ1Ex-*pccBA::sll1641* was also transformed into wild-type *Synechococcus* sp. PCC 7002 in a similar manner. The presence of the desired glyoxylate genes in strain *Synechococcus* 7002-glyox and the presence of glutamate decarboxylase gene in *Synechococcus* 7002-GAD, were confirmed by PCR using primer set MSF-ICLR and GADF-GADR, respectively (Table 3-1).

3.3.5 Enzymatic assays

3.3.5.1 Isocitrate lyase

For enzyme assays with isocitrate lyase, the reaction mixture (0.2 ml) contained 2 mM isocitrate, 50 mM K-phosphate, pH 7.8, 1 mM MgCl$_2$, and 50 µg of purified UYEDRAFT_02681. The mixture was incubated at RT 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 MgCl₂, and 50 µg of purified UYEDRAFT_02681. The mixture was incubated at RT 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.

3.3.5.2 Malate synthase

For enzyme assays with malate synthase, the reaction mixture (0.2 ml) contained 2 mM acetyl-CoA, 2 mM glyoxylate, 2 mM MgCl₂, 50 mM K-phosphate, pH 7.8 and 50 µg purified UYEDRAFT_02682. The mixture was incubated at RT 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.

3.3.5.3 Glutamate decarboxylase

For enzyme assays with glutamate decarboxylase, the reaction mixture (0.2 ml) contained 1 mM glutamate, 50 mM K-phosphate, pH 4.5 and 50 µg purified sll1641, and control experiments were performed similarly but without the addition of the purified enzyme. The mixture was incubated at RT for 1 h, and the produced GABA was detected by using Edman’s reagent as previously described with modifications (Tank and Bryant, 2015). In details, an aliquot (30 µl) of the reaction mixture was transferred to a glass test tube and dried by flushing with nitrogen. The dried sample was then dissolved in 100 µL coupling solution (mixture containing
50% acetonitrile, 25% pyridine, 10% triethylamine and 15% water) and the resulted solution was dried again. Then the dried sample was dissolved in 100 µL coupling solution, after which 5 µL of PITC was added to the solution and reaction was allowed to proceed for 5 minutes at RT. After reaction, the liquid sample was dried and the resulting pellet was dissolved in 250 µL analysis solvent. An aliquot (20 µL) of the solution was injected on Shimadzu LC-20AB HPLC system equipped with 254-nm UV detector SPD-20A. Different components in the solution were separated on a Kinetex 5-µm C18 100Å column (15 cm × 4.6 174 mm ID) protected by a SecurityGuard ULTRA cartridge UHPLC C18 for 4.6-mm ID columns 175 (Phenomenex, Torrance, CA, USA). The HPLC analysis method consisted of a 2-solvent gradient (solvent A and B) developed over a 20-min period with a flow rate of 0.5 ml/min at 30 °C. The initial condition was 100% solvent A (0.14 M sodium acetate, pH 6.2 containing 0.5 mM triethanolamine), which decreased over 10 min to 82.5% and from 10-15 min to 0%. Solvent B was a 40:60 (v/v) mixture of HPLC-grade water and acetonitrile. The elution times and concentrations of substrates and products were determined by comparison of results obtained from analyses of individual standard compounds with the same procedure.

3.3.5.4 *N*-acetylornithine aminotransferase

For enzyme assays with *N*-acetylornithine aminotransferase, the reaction mixture (0.2 ml) contained 1 mM L-ornithine, 1 mM 2-oxoglutarate, 50 mM K-phosphate, pH 9 and 50 µg purified slr1022 or SYNPCC7002_A0326. The mixture was incubated at RT for 1 h, and an aliquot (30 µl) of the reaction mixture was further analyzed using the same procedure as described in the enzyme characterization of glutamate decarboxylase to detect product formation. 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.

### 3.3.5.5 GABA aminotransferase

For enzyme assays with GABA aminotransferase, the reaction mixture (0.2 ml) contained 1 mM GABA, 1 mM 2-oxoglutarate, 50 mM K-phosphate, pH 9 and 50 µg purified slr1022 or SYNPPCC7002_A0326. The mixture was incubated at RT for 1 h, and an aliquot (30 µl) of the reaction mixture was further analyzed using the same procedure as described in the enzyme characterization of glutamate decarboxylase to detect product formation. 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.

### 3.3.6 PHB extraction and quantification

Quantification of PHB was performed as previously described (Braunegg et al., 1978; Tsang et al., 2013). 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-Aldrich, St. Louis, MO) were placed into glass tubes with sealed rubber caps. Chloroform (1 ml) and acidified methanol (15% v/v H₂SO₄) (1 ml) were added to each sample. The samples were heated in a 97°C water bath for 3 hours 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 (Tsang et al., 2013). The concentrations and inferred cellular contents of PHB were calculated on the basis of a standard curve generated with known concentrations of PHB (Sigma-Aldrich, St. Louis, MO).

3.3.7 Transcription profiling

Global transcriptome profiling was performed by RNA-seq as previously described (Gan et al., 2014b). 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 OD$_{750\text{ nm}}$ = 1. Total RNA was then extracted from these two strains, and rRNA depletion was performed as described (Gan et al., 2014b). The construction of cDNA libraries and Illumina sequencing (50-nt, 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 maximum of four mismatches per read. The resulting alignment files were further analyzed with self-developed scripts to extract relative expression levels for each gene (Ludwig and Bryant, 2011). In order to compare the relative expression levels of the same gene from different samples that were grown under different growth conditions, the number of aligned sequences was normalized relative to the total number of mRNA counts in each sample. The RNA sequencing data have been deposited in the NCBI Sequence Read Archive under accession number SRP052045.
3.4 Results

3.4.1 Enzyme characterizations of glyoxylate cycle gene products

The isocitrate lyase (ORF UYEDRAFT_02681) of *C. fritschii* strain PCC 9212 was successfully expressed and purified from *E. coli* as an N-terminally poly-[His]₆-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]₆ tag (Figure 3-2). The purified protein was conclusively identified by tryptic peptide mass fingerprinting (data not shown). As mentioned in the Materials and Methods, 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]₆-tag, possibly due to protein misfolding. However, moving the poly-[His]₆ 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]₆ tag (Figure 3-2). 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 isocitrate, isocitrate was consumed and succinate and glyoxylate were produced (Figure 3-3). Isocitrate was produced when succinate and glyoxylate were incubated with the product of UYEDRAFT_02681, demonstrating that this reaction is reversible (Figure 3-3). When the protein product from ORF UYEDRAFT_02682 was incubated with glyoxylate and acetyl-CoA, malate was produced (Figure 3-4). 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 central metabolism. These biochemical assays also confirm that the glyoxylate cycle is present and probably active in \(C.\ fritschii\) PCC 9212.

3.4.2 Enzyme characterizations of GABA shunt gene products

The glutamate decarboxylase (sll1641) and N-acetylmornithine aminotransferase (slr1022) of \(Synechocystis\) sp. PCC 6803, as well as the N-acetylmornithine aminotransferase (SYNPCC7002_A0326) of \(Synechococcus\) sp. PCC 7002 were successfully expressed and purified from \(E.\ coli\) as an N-terminally poly-[His]\(_6\)-tagged protein. The purified proteins were positively immunoreactive with commercial antibodies to the poly-[His]\(_6\) tag (Figure 3-5), and the purified proteins were further confirmed by tryptic peptide mass fingerprinting (data not shown).

The enzyme activity of glutamate decarboxylase was firstly characterized. When the protein product from ORF sll1641 was incubated with glutamate, GABA was produced and glutamate was consumed (Figure 3-6). This biochemical result established that sll1641 encodes glutamate decarboxylase and catalyzes the conversion of glutamate to GABA. The enzyme activity of N-acetylmornithine aminotransferase was also assayed. When the protein product from ORF slr1022 or ORF SYNPCC7002_A0326 was incubated with L-ornithine and 2-oxoglutarate, glutamate was produced and 2-oxoglutarate was consumed (Figure 3-7), which validated the enzyme activity of N-acetylmornithine aminotransferase. Because it was suggested that N-
acetylornithine aminotransferase could also function as GABA aminotransferase, the potential activity for GABA aminotransferase was also characterized. When the protein product from ORF slr1022 or ORF SYNPOCC7002_A0326 was incubated with GABA and 2-oxoglutarate, glutamate was produced and 2-oxoglutarate was consumed (Figure 3-8). These biochemical results established that N-acetylornithine aminotransferase is a bi-functional enzyme that has both N-acetylornithine aminotransferase and GABA aminotransferase activities. Thus, together with the glutamate decarboxylase, the complete GABA shunt exists in some cyanobacteria (e.g., *Synechocystis* sp. PCC 6803).

### 3.4.3 Metabolic study of glutamate decarboxylase expression strain

Our biochemical results validated that the GABA shunt enzyme activities exist in some cyanobacteria such as *Synechocystis* sp. PCC 6803. However, the apparent absence of glutamate decarboxylase in most cyanobacteria (e.g., *Synechococcus* sp. PCC 7002) suggested that the GABA shunt is not universally present in cyanobacteria. To investigate the possible role of the GABA shunt in the metabolic contents of cyanobacteria further, a glutamate decarboxylase expression strain of *Synechococcus* sp. PCC 7002 was constructed as described in the Materials and Methods (section 3.3.4), in which the glutamate decarboxylase (*sll1641*) from *Synechocystis* sp. PCC 6803 was expressed under the strong *cpcBA* promoter. The presence of the plasmid and the incorporation of the *sll1641* gene into *Synechococcus* sp. PCC 7002 was verified by PCR amplification of the *sll1641* operon and was further confirmed by sequencing the amplicon (Figure 3-10). Our preliminary results showed that 2-oxoglutarate level in the 7002-GAD strain reduced to about 30% of wild-type level, validated that the glutamate decarboxylase was functional in the 7002-GAD strain and that the GABA shunt might also contribute the carbon flux.
from 2-oxoglutarate to succinate. Interestingly, glutamine was accumulated to a higher level in the 7002-GAD strain compared to wild-type strain (~8-fold higher). However, the underlying mechanism is still not clear, which might involve regulation of the intracellular concentrations of both 2-oxoglutarate and glutamate, considering that they are two very important metabolite intermediates in cyanobacteria.

3.4.4 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 in the Materials and Methods, under standard growth conditions *C. fritschii* PCC 9212 grew faster when the medium was supplemented with 10 mM acetate (Figure 3-9). 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 (Figure 3-9).

As expected, *C. fritschii* PCC 9212 grew more slowly when cultures were sparged with air (Figure 3-9). 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) CO₂. This result shows that acetate can stimulate growth but certainly is not able to supplant CO₂ 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 (Figure 3-9).
3.4.5 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* strain PCC 9212 was introduced into the pAQ1Ex expression plasmid system (Xu et al., 2011), which was subsequently transformed into *Synechococcus* sp. PCC 7002. The presence of the plasmid and the incorporation of the *aceAB* genes into *Synechococcus* sp. PCC 7002 was verified by PCR amplification of the *aceAB* operon and was further confirmed by sequencing the amplicon (Figure 3-10). When the wild type and the strain carrying the *aceAB* 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 to WT (Figure 3-11). 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 (Figure 3-11). 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-CO2 conditions for WT cells (Figure 3-11). Under these conditions, cells exhibited net acetate excretion rather than excretion was observed under dark or low-CO2 conditions for WT cells (Figure 3-11), and the same was observed for *Synechococcus* 7002-glyox under dark conditions. Acetate assimilation was still observed under low CO2 conditions in strain *Synechococcus* 7002-glyox (Figure 3-11), although the assimilation rate was much slower.
compared to 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\textsubscript{2} concentration was crucial for acetate uptake and acetate uptake rate was reduced by almost 50% in the absence of CO\textsubscript{2} (Ihlenfeldt and Gibson, 1977). These observations confirm that acetate assimilation, the glyoxylate cycle and CO\textsubscript{2} fixation are closely related metabolic processes that may possibly be coordinately regulated under different growth conditions.

### 3.4.6 Gene neighborhood analysis of the glyoxylate cycle genes

In order to study the possible relationships between the glyoxylate cycle and other metabolic pathways, BLASTP analysis and gene neighborhoods surrounding the *aceAB* operon were also investigated. As mentioned before, the *aceAB* genes, encoding isocitrate lyase and malate synthase, respectively, are located in an apparent operon in *C. fritschii* PCC 9212 (Figure 3-12). BLASTP analysis showed that these two genes also occur in *C. fritschii* PCC 6912, *Cyanobacterium* PCC 7702, *Mastigocoleus testarum*, and *Tolypothrix bouteillei*. Interestingly, many of these cyanobacteria (*Chlorogloeopsis fritschii* PCC 9212, *Chlorogloeopsis fritschii* PCC 6912, *Pleurocapsa minor* PCC 7327, *Fischereilla* sp. PCC 9605, *Cyanobacterium* PCC 7702, *Mastigocoleus testarum*, and *Tolypothrix bouteillei*). Furthermore, all of these strains are able to fix nitrogen, suggesting 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 *aceAB* operon in *C. fritschii* PCC 9212 indicates that there is also an apparent operon of PHB-related genes (*phaABEC*) located
downstream (Figure 3-12). 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.

3.4.7 **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 the 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 towards glycolysis in the presence of acetate (Figure 3-12). A similar response was reported in *E. coli* cells grown in the presence of acetate (Oh et al., 2002).

### 3.4.8 Acetate increases the production of PHB

Because of the co-localization of the glyoxylate cycle genes and the genes for PHB metabolism, we determined whether the PHB content of *C. fritschii* PCC 9212 cells would be affected by the addition of acetate. PHB accumulation was firstly 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 (Figure 3-13). When acetate was supplied to the medium under the same conditions, the PHB content increased to ~15% of total cell dry weight (Figure 3-13, inset). These results indicated that the assimilated acetate that was metabolized by 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. *Pleurocapsa minor* PCC 7327, *Fischerella* sp. PCC 9605) (Gan et al., 2014a), 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 (Gan et al., 2014b). Along with this change, transcript abundances for numerous carbon transporter genes also increased. This indicated that the cells were attempting to use 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
(Gan et al., 2014b). 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 the massive metabolic and physiological changes during
the shift of growth condition to far-red light in FaRLiP strains.

3.5 Discussion

The glyoxylate cycle and the TCA cycle can both be used to metabolize acetate (i.e.,
acetyl-CoA), and these metabolic processes provide essential carbon skeletons (e.g. 2-
oxoglutarate, oxaloacetate and sometimes succinate) and reducing power (e.g., NADH) for cells.
These two cycles share many enzymes and intermediates (Figure 3-1), 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 (Zhang and Bryant, 2011)). As a result, the glyoxylate cycle can more efficiently
assimilate carbon from acetyl-CoA, 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 necessary
metabolites for gluconeogenesis and other biosynthetic processes. Thus, the glyoxylate cycle
provides an effective route for growth on fatty acids and C₂-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 (Dunn et al., 2009; Eastmond and Graham, 2001; Kunze et al., 2006). Although isocitrate lyase and malate synthase activities were reportedly detectable in birds and amphibians (Davis et al., 1990), 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 bi-functional enzyme (Kondrashov et al., 2006; Nakazawa et al., 2011). 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 (Plancke et al., 2014). However, it should be noted that acetate is ineffective as a growth substrate and is even toxic for some marine algae (Wood et al., 1999). In addition to allowing the growth of bacteria on C₂ 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 (Eastmond and Graham, 2001; Graham, 2008). 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 (Chen et al., 2000).

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 (Pickett et al., 1994). This enzyme requires ferredoxin to supply the necessary reducing power and thus primarily occurs in anaerobic bacteria. CO₂ is also required for the
growth of heliobacteria when acetate is supplied as the only organic carbon source (Tang et al., 2010). 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 (Korotkova et al., 2002). 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 (Erb et al., 2007). A third acetate-assimilation pathway, the methylaspartate cycle, was recently described in *Haloarcula marismortui* (Khomyakova et al., 2011). 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 (Khomyakova et al., 2011). 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 (Ensign, 2011). 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. Previous studies had suggested that some cyanobacteria could use acetate as the carbon and energy source (Pearce and Carr, 1967). 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 during fermentation in the dark (McNeely et al., 2011)). 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 (Eley, 1988), 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 (Knoop et al., 2013; Shastri and Morgan, 2005). However, the genes for isocitrate lyase and malate synthase are not present in *Synechocystis* sp. PCC 6803 nor the majority of other cyanobacteria. Furthermore, these enzymatic activities were not identified when more refined and sensitive methods were employed with *Synechocystis* sp. PCC 6803 (Knoop et al., 2013). 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 (You et al., 2014).

By identifying and characterizing the isocitrate lyase and malate synthase from *C. fritschii* PCC 9212, our results clearly demonstrate that the glyoxylate cycle probably does exist in a few cyanobacterial strains and that it plays an important role in acetate assimilation in those organisms. We also demonstrated that the ability to assimilate acetate could be significantly improved by introducing the *aceAB* 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 (Gimenez et al., 2003). 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 (Becker et al., 2005; Perez-Garcia et al., 2011). Our transcription profiling results showed that several putative transporter genes were up regulated...
when acetate was supplied to the medium, 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 (Boyle and Morgan, 2009; Combres et al., 1994; Perez-Garcia et al., 2011). 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 were not up regulated when acetate was supplied to the medium, indicating that there might be regulation at other levels (Miller and Allen, 1972). 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 (Bedhomme et al., 2009). 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.

Some recent FBA models for *Synechocystis* sp. PCC 6803 also included the glyoxylate shunt to investigate the possible roles of this cycle in cyanobacteria (Fu, 2009; Knoop et al., 2013; Montagud et al., 2010; Montagud et al., 2011; Shastri and Morgan, 2005). In the glyoxylate shunt, isocitrate lyase cleaves isocitrate to produce succinate and glyoxylate, and malate synthase then converts the glyoxylate into malate. FBA models suggest that the glyoxylate shunt enzymes
would be functional under some growth conditions. The analyses showed that the glyoxylate shunt is not active under photoautotrophic conditions but is predicted to be active under optimal heterotrophic conditions (Shastri and Morgan, 2005), which is consistent with $^{13}$C-MFA studies under mixo- and heterotrophic conditions (Yang et al., 2002). However, the genes for isocitrate lyase and malate synthase are not present in *Synechocystis* sp. PCC 6803 and the majority of other cyanobacteria. Although enzyme activities have reportedly been detected in several cyanobacteria, these activities were not identified when more refined methods were employed with *Synechocystis* sp. PCC 6803 (Knoop et al., 2013). These results suggest two possibilities: (1) alternative enzymes that have not yet been purified and correlated to specific genes could function in acetate metabolism in some cyanobacteria; (2) the previously detected activities were due to non-specific reactions in the biochemical assays employed. Consistent with the latter interpretation, recent isotopic tracing studies indicate that the glyoxylate shunt is not functional and that glyoxylate is mainly used for glycine synthesis (You et al., 2014).

The possible roles of the gamma-aminobutyric acid (GABA) shunt in the heterotrophic metabolism of cyanobacteria have also been investigated. From the point of view of FBA, the GABA shunt is stoichiometrically identical to the recently discovered TCA cycle variant using 2-OGDC and SSADH, and thus both variants should result in identical biomass yields. Interestingly, however, the biomass yield is lower than for the conventional cycle using 2-OGDH under respiratory metabolism conditions (Knoop et al., 2013; Shastri and Morgan, 2005). Based on the finding that autotrophic growth was similarly reduced when metabolites were forced through the 2-OGDH complex or the 2-OGDC/SSADH bypass, but not by forced flux through the GABA shunt, it has been suggested that the GABA shunt may be an evolutionary favorable solution to completing the TCA cycle (Nogales et al., 2012). This may also be the reason for the existence of the GABA shunt but not the 2-OGDC/SSADH bypass in some *Prochlorococcus* and marine *Synechococcus* species. Another possible advantage of having the TCA bypass rather than
the 2-OGDH complexes may be related to protein synthesis and assembly. 2-OGDH is a highly complex, mult-subunit enzyme compared to 2-OGDC and SSADH, as well as the enzymes that catalyze the GABA shunt. Considering the important roles of 2-oxoglutarate in the metabolism of cyanobacteria and the relative insignificance of cyclic flux through the TCA cycle during phototrophic growth because of the products (i.e., NADPH and ATP) of the light reactions, such a difference in enzyme investment may result in a trade-off between enzymatic efficiency and enzyme synthesis costs (Knoop et al., 2013).

In close accordance with the well-established role of 2-oxoglutarate in many important cellular processes (e.g. ammonia assimilation, regulation of nitrogen and carbon metabolism, precursor metabolite for heme, bilins, and chlorophylls, etc.), several observations show that 2-oxoglutarate levels in plant cells can reflect the C/N status (Foyer et al., 2011; Lancien et al., 2000; Nunes-Nesi et al., 2010), which implies that 2-oxoglutarate also plays a signaling role in land plants. Previous results showed that chemical inhibition of the 2-OGDH complex in potato tubers had a clear impact on respiration (Araújo et al., 2008). More pronounced effects were also observed following the inhibition of this enzyme in tomato roots (van der Merwe et al., 2010), which indicates that 2-oxoglutarate levels are closely correlated with respiratory activities. Furthermore, it has been suggested that the rates of respiration in the light are lower than in the dark in plants. However, indicating that nitrogen metabolism interacts closely with respiration, daytime respiration plays an important role in carbon and nitrogen metabolism and provides at least some of the 2-oxoglutarate and NADH that are required for nitrogen metabolism (Nunes-Nesi et al., 2010; Stitt et al., 2002). Interestingly, some studies indicate that the TCA cycle is almost completely inhibited in illuminated leaves (Tcherkez et al., 2005), while others have found that there is still significant TCA cycle activity in the light (Nunes-Nesi et al., 2007). Moreover, genetic manipulations of the TCA cycle and mitochondrial electron transport components have produced effects on N metabolism and related processes (Foyer et al., 2011). When illuminated
leaves of Brassica napus were incubated with $^{13}$CO$_2$ and $^{15}$N-ammonium nitrate, it was found that, while there is considerable incorporation of nitrogen into the newly synthesized glutamate and glutamine, the majority of the carbon in these metabolites is not derived from concurrent CO$_2$ assimilation (Gauthier et al., 2010). This study showed that remobilization of 2-oxoglutarate that accumulated during the previous light and dark cycle is probably sufficient to support subsequent glutamate synthesis in the light. Taken together, the results demonstrate that 2-oxoglutarate serves as a key metabolite for regulating metabolic changes and rechanneling of intermediates between the TCA cycle and nitrogen metabolism. Finally, in some cyanobacteria the newly discovered TCA cycle bypass provides an additional connection between carbon and nitrogen metabolism through the GABA shunt and succinic semialdehyde, which might serve as a key point for regulation. Although the results of such metabolic reprogramming have not yet been determined in cyanobacteria, it might be worthwhile to manipulate gene expression levels of the TCA cycle enzymes, the glyoxylate cycle and the GABA shunt, and investigate the physiological and metabolic changes that occur in cyanobacteria under different stress conditions (e.g. O$_2$ limitation, carbon limitation, and nitrogen limitation).

3.6 References


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Xiong, W., Brune, D., and Vermaas, W.F.J. (2014). The $\gamma$-aminobutyric acid shunt contributes to closing the tricarboxylic acid cycle in *Synechocystis* sp. PCC 6803. Molecular Microbiology 93, 786-796.


Figure 3-1 The TCA cycle, the glyoxylate cycle and the GABA shunt

Scheme showing the glyoxylate cycle, GABA shunt and the TCA cycle in cyanobacteria. The glyoxylate cycle and GABA shunt are drawn in black arrows to show their differences with the TCA cycle (in grey arrows). Abbreviations: 2-OG, 2-oxoglutarate; 2-OGDC, 2-oxoglutarate decarboxylase; ACO, Aconitase; CS: Citrate synthase; FUM, Fumarase; GABA, gamma-Aminobutyric acid; GABA-AT, GABA aminotransferase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; 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.
Figure 3-2 SDS-PAGE and immunoblotting analysis

Purified proteins of isocitrate lyase (ICL) and malate synthase (MS) were analyzed. Left lane for each enzyme was stained with Coomassie blue and right lane was detected by immunoblotting with antibodies to the poly-[His]₆ tag.
Figure 3-3 HPLC analysis of isocitrate lyase reaction components
A. HPLC analysis showing that isocitrate (peak 1) was converted to glyoxylate (peak 2) and succinate (peak 3), catalyzed by the purified isocitrate lyase; B. HPLC analysis showing the production of isocitrate (peak 1) from glyoxylate (peak 2) and succinate (peak 3), catalyzed by the purified isocitrate lyase. Detailed assay conditions were described in the Materials and Methods (section 3.3.5.1).
Figure 3-4 HPLC analysis of malate synthase reaction components

HPLC analysis showing the formation of malate (peak 5) from glyoxylate (peak 2) and acetyl-CoA (peak 4) catalyzed by the purified malate synthase. Detailed assay conditions were described in the Materials and Methods (section 3.3.5.2).
GAD 6803 ArgD 7002 ArgD

Figure 3-5 SDS-PAGE and immunoblotting analysis of GABA shunt proteins

Purified recombinant proteins of the glutamate decarboxylase (GAD, *sll1641*), and the acetylornithine aminotransferase from both *Synechocystis* sp. PCC 6803 (6803ArgD, *slr1022*) and *Synechococcus* sp. PCC 7002 (7002ArgD, *SYNPCC7002_A0326*) were analyzed. Left lanes for each enzyme were stained with Coomassie blue and right lanes were detected by immunoblotting with antibodies to the poly-[His]₆ tag.
HPLC analysis showing the formation of GABA from glutamate catalyzed by the purified malate synthase. Detailed assay conditions were described in the Materials and Methods (section 3.3.5.3).
HPLC analysis showing the formation of glutamate from L-ornithine catalyzed by the purified acetylornithine aminotransferase from both *Synechocystis* sp. PCC 6803 (ArgD_6803) and *Synechococcus* sp. PCC 7002 (ArgD_7002). Detailed assay conditions were described in the Materials and Methods (section 3.3.5.4).
Figure 3-8 HPLC analysis of GABA aminotransferase reaction components

HPLC analysis showing the formation of glutamate from GABA catalyzed by the purified acetylornithine aminotransferase from both *Synechocystis* sp. PCC 6803 (ArgD_6803) and *Synechococcus* sp. PCC 7002 (ArgD_7002), demonstrating that acetylornithine aminotransferase can also function as GABA aminotransferase. Detailed assay conditions were described in the Materials and Methods (section 3.3.5.5).
Figure 3-9 Acetate assimilations and growth curves of *C. fritschii* PCC 9212

Blue curves indicate the cell density and green lines indicate the acetate concentrations in the medium at different growth stages. **A:** *Chlorogloeopsis fritschii* PCC 9212 growing under normal conditions; **B:** *Chlorogloeopsis fritschii* PCC 9212 growing under low CO$_2$ conditions; **C:** *Chlorogloeopsis fritschii* PCC 9212 growing under dark conditions. The data shown are averages of three biological replicates, and the error bars show the standard deviation.
A: Verification of the presence of glyoxylate cycle genes. 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 *aceAB* genes from *C. fritschii* PCC 9212 inserted in plasmid pAQ1-Ex as described in the Materials and Methods (section 3.3.4).

B: Verification of the presence of glutamate decarboxylase gene. The template DNA was derived from wild-type *Synechococcus* sp. PCC 7002 (lane 7002), from wild-type *Synechocystis* sp. PCC 6803 (lane 6803), and from the recombinant strain 7002-GAD (lane 7002-GAD), which has the *sll1641* gene from *Synechocystis* sp. PCC 6803 inserted in plasmid pAQ1-Ex as described in the Materials and Methods (section 3.3.4).

Figure 3-10 Verification of the presence of incorporated genes
Figure 3-11 Acetate assimilation and growth of *Synechococcus* sp. PCC 7002
Blue curves indicate the cell density and green lines indicate the acetate concentrations in the medium at different times. A: *Synechococcus* sp. PCC 7002 growing 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\textsubscript{2} conditions. WT, wild type *Synechococcus* sp. PCC 7002; 7002-glyox, *Synechococcus* sp. PCC 7002 strain with glyoxylate cycle genes overexpressed from plasmid pAQ1. The data shown are averages of three biological replicates, and the error bars show the standard deviation.
Figure 3-12 Relative transcripts abundance for mRNAs in *C. fritschii* PCC 9212

A. Gene neighborhood around the genes encoding enzymes of the glyoxylate cycle. Numbers above each gene indicate the fold-difference of mRNA abundance in cells grown with acetate compared to cells grown without acetate. B. Scatter plot showing the relative abundance of all the mRNAs under growth conditions with acetate (9212A) or without acetate (9212), details of the analysis were described in the Materials and Methods part (section 3.3.7). Grey lines indicate a two-fold increase or 50% decrease in mRNA level. *aceA*, isocitrate lyase; *aceB*, malate synthase; *phaA*, acetyl-CoA acetyltransferase; *phaB*, acetoacetyl-CoA reducatase; *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.
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 standard deviation.

**Figure 3-13 Accumulation of PHB in *C. fritschii* PCC 9212**
Table 3-1 Primers used in this chapter

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<tr>
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Chapter 4  Metabolic engineering of *Synechococcus* sp. PCC 7002 to the production of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate

Publication:

**Shuyi Zhang**, Yang Liu and Donald Bryant (2015) Metabolic engineering of *Synechococcus* sp. PCC 7002 to the production of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate. Submitted for publication.
4.1 Abstract

Cyanobacteria are an important group of photoautotrophic bacteria that have been engineered and used to produce a wide range of biomaterials and biofuels, which are usually derived from important intermediates of the central metabolic pathways. In this study, the production of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate in cyanobacteria was studied, and metabolic engineering strategies to improve the yields were also investigated. The genes involved in the biosynthetic pathway for poly-3-hydroxybutyrate from Chlorogloeopsis fritschii PCC 9212 were introduced into Synechococcus sp. PCC 7002, and the resulting strain was able to accumulate ~2.5% of total cell dry weight as poly-3-hydroxybutyrate. When the ccmR gene was deleted in this strain, the yield of poly-3-hydroxybutyrate increased to ~4% of total cell dry weight. A biosynthetic pathway for the production of 4-hydroxybutyryl-CoA was also constructed and introduced into the poly-3-hydroxybutyrate-producing strain. The resulting strain was able to produce ~4.5% of total cell dry weight as poly-3-hydroxybutyrate-co-4-hydroxybutyrate, in which 4-hydroxybutyrate accounted for ~12% of the co-polymer. This is the first demonstration that poly-3-hydroxybutyrate-co-4-hydroxybutyrate can be produced in cyanobacteria and confirms that succinic semialdehyde is an important TCA cycle metabolite in cyanobacteria. This study demonstrates the potential for future metabolic engineering in cyanobacteria that is based on newly discovered metabolites.
4.2 Introduction

Due to the ever-increasing demand for sustainable energy and materials, cyanobacteria, one group of photoautotrophic bacteria that are able to assimilate CO$_2$, are projected to play important roles as resources for the production of biofuels and biomaterials. Cyanobacteria have been engineered and used to produce a wide range of biomaterials and biofuels, which are usually derived from major metabolites of the central metabolic pathways inside cells (Atsumi et al., 2008; Kumaraswamy et al., 2013; Lan and Liao, 2011; Machado and Atsumi, 2012; Oliver et al., 2013). Thus, the discovery of novel metabolic pathways should not only result in a better understanding of metabolic diversity in cyanobacteria, but could also provide new metabolic intermediates that can potentially serve as targets for future metabolic engineering purposes. We recently identified a TCA cycle variant in cyanobacteria, and this not only clarified a very long-term misunderstanding about the nature of the TCA cycle, but it also identified succinic semialdehyde, which might serve as a potential metabolic engineering target, as an important new metabolite in the TCA cycle variant (Zhang and Bryant, 2011). Indeed, many approaches have been explored to use succinic semialdehyde as the starting metabolite to produce important biomaterials, including 1,4-butanediol and polyhydroxyalkanoates (PHA) (Lee et al., 2012; Li et al., 2010; Yim et al., 2011).

A variety of bacteria have been reported to synthesize PHAs as intracellular carbon and energy storage compounds, especially when those organisms are grown under stress conditions produced by limitation for essential nutrients (e.g., nitrogen, phosphate) (Lee, 1996; Saito et al., 1996; Steinbüchel and Valentin, 1995; Zinn et al., 2001). The synthesized polyesters can be degraded by PHA depolymerases, and the decomposition products are used as nutrients for microorganisms in environments as diverse as soils and oceans (Saito et al., 1996). Thus, due to their biodegradable and biocompatible properties, potential industrial applications for PHAs have
been investigated over a long time (Chen, 2009; Martin and Williams, 2003; Park et al., 2005). Depending on the bacterial strain, specific PHA synthases, and the growth substrates, PHAs can be synthesized as homopolymers or copolymers (Gao et al., 2011; Rehm and Steinbüchel, 2005). Among the different types of PHAs synthesized in bacteria, the homopolymer of poly-3-hydroxybutyrate (hereafter P3HB) is the most common and most studied polymer (Gao et al., 2011). However, because P3HB is relatively amorphous, elastic and viscous, potential applications for P3HB are limited. On the other hand, the incorporation of 4-hydroxybutyrate (4HB) into P3HB generates a copolymer, poly-3-hydroxybutyrate-co-4-hydroxybutyrate (hereafter P3HB-co-4HB), which has significantly improved thermal and mechanical properties (Li et al., 2010). Depending on the exact co-polymer composition, the resulting biomaterials exhibit a wide range of physical properties (e.g., crystalline, plastic, elastic rubber) and have many potential industrial applications (Ishida et al., 2001; Saito et al., 1996).

P3HB-co-4HB was first found to be produced in Ralstonia eutropha when this bacterium was cultured with 4HB or 4-chlorobutyric acid as carbon sources (Doi et al., 1988), and later on its production was also found in many other bacteria, such as Alcaligenes latus, Comamonas testosteronii and Hydrogenophaga pseudoflava (Choi et al., 1999; Ishida and Inoue, 2004; Saito et al., 1996). However, carbon sources structurally related to 4HB were required in the medium to produce P3HB-co-4HB, and the high cost of using these precursors adds additional costs to the production of P3HB-co-4HB, which constrains further industrial applications of P3HB-co-4HB (Song et al., 1999; Valentin et al., 2000). Some previous studies have been performed in E. coli to produce P3HB-co-4HB from carbon sources not directly linked to 4HB (Li et al., 2010; Valentin and Dennis, 1997). However, additional organic carbon sources (e.g., glucose) are still needed in the medium or the 4HB content in the synthesized co-polymer was relatively low (Li et al., 2010; Valentin and Dennis, 1997; Valentin et al., 2000). All of these disadvantages limit the further production and utilization of P3HB-co-4HB.
Cyanobacteria are photoautotrophic bacteria that utilize sunlight as their energy source and CO\textsubscript{2} as their carbon source. Because they have adapted or can acclimate to diverse growth environments (Gan et al., 2014a; Gan et al., 2014b), they can serve as “photo-bioreactors” for the production of renewable biofuels and green chemicals (Ducat et al., 2011; Parmar et al., 2011). Some cyanobacteria have been reported to produce P3HB, and different metabolic strategies to maximize the accumulation of P3HB under various growth conditions have been explored (Panda and Mallick, 2007; Tsang et al., 2013; Wu et al., 2002). However, P3HB-co-4HB has not yet been produced in cyanobacteria, probably due to the absence of an appropriate precursor for the biosynthesis of 4HB. Succinic semialdehyde was recently discovered to be an important, new intermediate in the TCA cycle variant that occurs in cyanobacteria (Zhang and Bryant, 2011). Succinic semialdehyde can be converted to 4-hydroxybutyryl-CoA (4HB-CoA) by 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA transferase from Porphyromonas gingivalis (Yim et al., 2011). Thus, by introducing the 4-hydroxybutyrate dehydrogenase and the 4-hydroxybutyrate-CoA transferase into a cyanobacterium that produces P3HB, the biosynthesis of P3HB-co-4HB should become possible.

The production of P3HB and P3HB-co-4HB using light and CO\textsubscript{2} in cyanobacteria was explored in this study, and some metabolic engineering strategies to improve the product yield were also investigated. Chlorogloeopsis fritschii PCC 9212 has a gene cluster that encodes the biosynthetic pathway for P3HB, and P3HB production in this strain was demonstrated in our recent study (Zhang and Bryant, 2015). However, although gene knockouts have been reported (Stucken et al., 2012) in this strain, the absence of a simple and reliable system for genetic manipulation limits further metabolic engineering in this organism. By using the pAQ1Ex expression system (Xu et al., 2011), this entire gene cluster was instead transferred to Synechococcus sp. PCC 7002, which has a faster growth rate and well-established genetic modification tools. The resulting strain could accumulate ~2.5% percent of total cell dry weight.
as P3HB, and the yield was improved to ~4% when the *ccmR* gene was deleted in this strain. Additionally, the complete biosynthetic pathway to synthesize 4HB-CoA from succinic semialdehyde was constructed and introduced into the P3HB production strain of *Synechococcus* sp. PCC 7002. The resulting strain produced ~4.5% total cell dry weight as P3HB-co-4HB, in which the 4HB monomer accounted for ~12% of the co-polymer. Taken together, this work shows for the first time that P3HB-co-4HB can be produced in cyanobacteria and identifies a genetic modification that improved the yield of P3HB. Furthermore, this work nicely illustrates the possibility for future metabolic engineering of cyanobacteria based on the discovery of novel metabolites.

4.3 Materials and Methods

4.3.1 Growth conditions

Wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were grown in liquid A+ medium under standard conditions as previously described (Ludwig and Bryant, 2011). Standard conditions are defined as 38°C, an irradiance of 250 μmol photons m⁻² s⁻¹ provided by cool white fluorescent lights, and gentle sparging with 1% (vol/vol) CO₂ in air. Antibiotics were used in the following concentrations when appropriate: spectinomycin (50 μg ml⁻¹), kanamycin (100 μg ml⁻¹), gentamycin (20 μg ml⁻¹) and erythromycin (20 μg ml⁻¹).
4.3.2 Over-expression of the PHB operon in *Synechococcus* sp. PCC 7002

The *phaABEC* operon from *C. fritschii* PCC 9212 was amplified by polymerase chain reaction (PCR) and inserted onto the pAQ1-based expression system (Xu et al., 2011) by using primer set 3HBExF and 3HBExR (Table 1). The resulting plasmid was verified by DNA sequencing and was transformed into wild-type *Synechococcus* sp. PCC 7002 as previously described (Frigaard et al., 2004). Successful incorporation of the operon was confirmed by PCR using primer set 3HBExF and 3HBExR (Table 1), and the resulting PCR product was also verified by DNA sequencing. In addition, a His<sub>10</sub>-tag was added to N-terminus of the acetyl-CoA acetyltransferase (*phaA*), and immunoblotting with commercial antibodies (Rockland) to the His<sub>10</sub>-tag was also performed as previously described (Shen et al., 2002) to detect the recombinant acetyl-CoA acetyltransferase and to confirm that expression from the *phaABEC* operon was occurring. The resulting strain was denoted as WTPHB.

4.3.3 Generation of deletion mutants

The operon encoding 2-oxoglutarate decarboxylase (SynPCC7002_A2770) and succinic semialdehyde dehydrogenase (SynPCC7002_A2771) was deleted in the WTPHB strain by transformation and homologous recombination and replaced by a DNA fragment encoding an antibiotic resistance gene (*erm*, erythromycin resistance) as previously described (Frigaard et al., 2004). Primer sets A2771D1-A2771D2 and A2770D3-A2770D4 (Table 1) were used to amplify the upstream and downstream regions of the operon encoding SynPCC7002_A2771 and SynPCC7002_A2770. Full segregation of the deletion mutant was verified by PCR by comparing...
the products of template DNAs from both the wild type and the mutant strain using primer set A2771D1-A2770D4. The generated strain was denoted as Δ7071-PHB.

The coding sequence of SynPCC7002_A0171 (ecmR) was also deleted in the wild-type Synechococcus sp. PCC 7002 and the WTPHB strain, and replaced by a DNA fragment encoding an antibiotic resistance gene (aphAII, kanamycin resistance) to produce SynPCC7002_A0171 mutant strains. Transformation and selection were performed as previously described (Frigaard et al., 2004). Primer sets A0171D1-A0171D2 and A0171D3-A0171D4 (Table 1) were used to amplify the upstream and downstream regions of SynPCC7002_A0171. The complete segregation of alleles was verified by PCR by comparing the products obtained with template DNAs derived from the wild type and mutant strains using primer set A0171D1-A0171D4. The resulting strain with deletion of SynPCC7002_A0171 in the wild type Synechococcus sp. PCC 7002 was denoted as ΔA0171, and the generated strain with SynPCC7002_A0171 deleted in the WTPHB strain was denoted as ΔA0171-PHB.

4.3.4 Construction of the 4-hydroxybutyrate CoA biosynthesis pathways

In order to construct the biosynthetic pathway from succinic semialdehyde to 4HB-CoA (Figure 1), the genes encoding 2-oxoglutarate decarboxylase (SynPCC7002_A2770), 4-hydroxybutyrate dehydrogenase (gbdI) and 4-hydroxybutyryl-CoA transferase (cat2) were first assembled together on plasmid pAQ1Ex under control of the psaAB promoter (from Synechocystis sp. PCC 6803) (Xu et al., 2011). Primer set 4HBExF-4HBExR (Table 1) was used to amplify the operon encoding 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA transferase from Porphyromonas gingivalis W83, and primer set A2770ExF-A2770ExR (Table 1) was used to amplify the gene encoding the 2-oxoglutarate decarboxylase from Synechococcus sp.
PCC 7002. To avoid homologous recombination with the chromosomal copy of SynPCC7002_A2770, the operon encoding SynPCC7002_A2771 and SynPCC7002_A2770 was first deleted in the strain ΔA0171-PHB. Then, the malate dehydrogenase gene (SYNPCC7002_A2093) in the resulting strain was replaced with the fused construct by homologous recombination as described (Frigaard et al., 2004). Primer sets A2093D1-A2093D2 and A2093D3-A2093D4 (Table 1) were used to amplify the upstream and downstream flanking regions of malate dehydrogenase. Full segregation of the replacement of the malate dehydrogenase by the fused construct containing the psaAB promoter, all three genes, and the spectinomycin gene, was verified using primer set A2093D1-A2093D4. The products were sequenced to verify that no inadvertent changes had occurred during strain construction. The resulting strain was denoted as P3HB4HB.

4.3.5 Total mRNA profiling

Transcriptome profiling was performed as described (Ludwig and Bryant, 2011). The SynPCC7002_A0171 mutant and wild-type strains were grown in medium A+ under standard conditions and harvested at OD$_{730}$ = 0.7. Total RNA was extracted as described (Ludwig and Bryant, 2011). The construction of cDNA libraries and sequencing (Illumina) were performed in the Genomic Core Facility at The Pennsylvania State University. Mapping against the Synechococcus sp. PCC 7002 genome was performed using the BWA software package (Li and Durbin, 2009). The resulting alignment files were further analyzed with self-developed scripts to extract expression levels for each gene as described previously (Zhang and Bryant, 2015). In order to compare the relative expression levels of the same gene from different samples that were grown under different growth conditions, the number of aligned sequences was normalized.
relative to the total number of mRNA counts in each sample. The RNA sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP058241.

4.3.6 PHB detection

PHB quantifications were performed as previously described (Braunegg et al., 1978; Tsang et al., 2013). Aliquots (20 ml) of liquid cultures of *Synechococcus* sp. PCC 7002 were centrifuged for 10 min at 10,000 × g. The pellets were washed once with double-deionized water (20 ml). The resulting cell pellets were then lyophilized to obtain dried cells. The dried cells were put into glass tubes with sealed rubber caps. Sodium 3-hydroxybutyrate and gamma-butyrolactone (Sigma-Aldrich) were used as standards for 3HB and 4HB, respectively, and were analyzed separately following same procedures. Chloroform (1 ml) and acidified methanol (1 ml, 15% vol/vol H₂SO₄) were added to each sample as well as the standards. The tubes were heated in a 97°C water bath for 3 h to methanolyze sodium 3-hydroxybutyrate or gamma-butyrolactone into methyl 3-hydroxybutyrate or methyl 4-methoxybutyrate, respectively. After methanolysis, double-deionized water (1 mL) was added to each sample. Following phase separation, an aliquot (2 µl) of the bottom chloroform phase was collected and loaded directly onto a Shimadzu GC-17A gas chromatograph connected to a Shimadzu GCMS-QP500 mass spectrometer using a HP-5-MS 30 m column (ID: 0.25 narrow bore; film: 0.25 µm) (Agilent Technologies; Foster City, CA) for analysis as previously described (Tsang et al., 2013). The concentrations and contents of 3HB and 4HB were calculated on the basis of a standard curve generated with known concentrations of sodium 3-hydroxybutyrate and gamma-butyrolactone (Sigma-Aldrich).
4.4 Results

4.4.1 Generation and verification of overexpression strain

The *phaABEC* genes form an apparent operon in *C. fritschii* PCC 9212, and are also co-located with the *aceBA* genes for the enzymes of the glyoxylate cycle (Figure 2). The *phaABEC* operon was cloned onto the pAQ1Ex system and expressed in *Synechococcus* sp. PCC 7002 as described in the Materials and Methods. The incorporation of this operon into *Synechococcus* sp. PCC 7002 was verified by PCR as shown in Figure 2. Immunoblotting was also performed using antibodies against His$_{10}$-tag, which demonstrated the production and accumulation of the acetyl-CoA acetyltransferase (Figure 2). These results clearly demonstrate that the *phaABEC* operon was successfully incorporated into *Synechococcus* sp. PCC 7002, and verified that at least the first gene of the operon was expressed successfully. The over-expression strain had a somewhat yellowish color compared to wild-type strain. This was probably due to protein over-expression as well as the biosynthesis and accumulation of P3HB, which might shift the overall carbon and nitrogen balance of the cells.

4.4.2 P3HB production in the overexpression strain

With the successful incorporation of the *phaABEC* operon and demonstration of expression of acetyl-CoA acetyltransferase, the production of P3HB in the WTPHB strain was further investigated by GC-MS as described in the Materials and Methods. The GC-MS results showed that P3HB production occurred in the WTPHB strain but not in wild-type *Synechococcus* sp. PCC 7002, and the mass fragmentation pattern confirmed that 3HB was the only monomer in
the polymer (Figure 3). Our previous results showed that *C. fritschii* PCC 9212 could accumulate P3HB to about 5% (w/w) of cell dry weight under standard growth conditions for this cyanobacterium (Zhang and Bryant, 2015). Cells of the WTPHB strain accumulated a lower content (~2.5% of cell dry weight) of P3HB under standard conditions (Figure 4) for *Synechococcus* sp. PCC 7002. The difference presumably is due to metabolic differences in these two organisms (e.g., the glyoxylate cycle is present in *C. fritschii* PCC 9212 but not *Synechococcus* sp. PCC 7002). The glyoxylate cycle was demonstrated to play an important role in the metabolism of acetyl-CoA, which is the building block for P3HB (De Philippis et al., 1992; Ensign, 2006). However, the growth rate of wild type *C. fritschii* PCC 9212 is much slower compared to *Synechococcus* sp. PCC 7002 and *C. fritschii* PCC 9212 is currently not easily amenable to genetic analysis (Stucken et al., 2012). Thus, *Synechococcus* sp. PCC 7002 still has advantages in rapidly accumulating P3HB as well as in allowing metabolic engineering to improve the production of P3HB (see below).

### 4.4.3 A carbon concentration mechanism regulator

The biosynthesis of P3HB utilizes acetyl-CoA as the building block, and thus metabolic engineering to increase the intracellular acetyl-CoA pool should potentially improve the production of P3HB. Acetyl-CoA is an important carbon metabolite in both central carbohydrate metabolic and fatty acid biosynthesis and degradation pathways. Thus, it is likely that improved carbon fixation could potentially provide more carbon resources to the central metabolic pathways and thus increase the cellular level of acetyl-CoA. It has been reported that the LysR-type transcription factor, CcmR, controls the expression of genes encoding the carbon concentration mechanism of cyanobacteria, and it represses its own transcription along with
structural genes encoding high affinity C\textsubscript{i} transporters in \textit{Synechocystis} sp. PCC 6803 (Daley et al., 2012). This indicates that CcmR is also likely to play an extremely important role in connecting central metabolism to inorganic carbon acquisition for photosynthesis. Thus, deletion of the \textit{ccmR} gene (SYNPCC7002_A0171) in \textit{Synechococcus} sp. PCC 7002 might potentially improve carbon fixation and thus increase the acetyl-CoA pool inside cells. In order to test this idea, a SYNPCC7002_A0171 deletion mutant of \textit{Synechococcus} sp. PCC 7002 was constructed and confirmed by PCR, as shown in Figure 5.

4.4.4 RNA profiling of SYNPCC7002_A0171 mutant

Transcription profiling of the SYNPCC7002_A0171 mutant was performed in comparison with the wild-type strain. The profiling results showed that transcripts for genes encoding bicarbonate transport systems as well as the Na\textsuperscript{+}/H\textsuperscript{+} antiporter increased quite substantially, which is consistent with previously reported results (Figure 6) (Woodger et al., 2007). Along with these changes, transcript abundances for the two genes encoding glyceraldehyde-3-phosphate dehydrogenase (\textit{gap}) also changed differently. Transcripts for the \textit{gap1} gene, whose product, glyceraldehyde-3-phosphate dehydrogenase-1, is involved in glycolysis, increased about 5-fold, while transcripts for the \textit{gap2} gene, whose product is thought to be primarily used in the Calvin-Benson-Bassham cycle (Koksharova et al., 1998), decreased to about 20% of the wild-type level. These results suggested that carbon flux might be shifted toward the glycolytic pathway, which might lead to the synthesis of more acetyl-CoA. Additionally, transcripts for the \textit{nifJ} gene, which encodes pyruvate:ferredoxin (flavodoxin) oxidoreductase that catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, increased dramatically (~15-fold), which further suggested that acetyl-CoA might accumulate in this mutant.
strain. In addition to these observations, mRNA levels for the hydrogenase genes (e.g., \textit{hoxF}, \textit{hoxU}) as well as the transhydrogenase genes (\textit{pntABC}) also increased in the mutant. Collectively, these changes implied that the redox cofactors or redox potential in the mutant might change to balance changes in carbon metabolism.

4.4.5 PHB production in mutant strains

Based on the transcription profiling results, it seemed likely that the SYNPCC7002\_A0171 deletion mutant could facilitate carbon assimilation by redirecting more carbon resources to the biosynthesis of acetyl-CoA. Thus, the production of P3HB was investigated in the \(\Delta\text{A0171-PHB}\) strain. Complete deletion of SYNPCC7002\_A0171 in the strain \(\Delta\text{A0171-PHB}\) was verified by PCR as shown in Figure 7. P3HB accumulated to \(\sim4\%\) of total cell dry weight in this strain, which represented a \(\sim60\%\) increase compared to that of WTPHB strain (Figure 7). To explore further metabolic engineering strategies to improve the production of P3HB in cyanobacteria, P3HB production was also investigated in a mutant strain in which the operon encoding 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase had been deleted (Figure 7). Depletion of 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase results in accumulation of 2-oxoglutarate, and we hypothesized that the acetyl-CoA concentration might also increase because of limited metabolic flux into the TCA cycle due to the accumulation of higher levels of 2-oxoglutarate. However, no significant increase in the accumulation of P3HB was observed in \(\Delta7071\)-PHB strain compared to WTPHB strain (Figure 7). These results suggested that the TCA cycle and thus the concentration of acetyl-CoA and 2-oxoglutarate concentrations are regulated at levels that are currently still unknown in
cyanobacteria, despite the fact that acetyl-CoA and 2-oxoglutarate are important cellular metabolites.

### 4.4.6 Production of P3HB-co-4HB in cyanobacteria

As mentioned in the introduction, P3HB is relatively amorphous, elastic, and viscous, and the incorporation of some 4HB into this polymer greatly improves its physical properties. The co-polymerization of 3-HB-CoA and 4HB-CoA monomer subunits results in P3HB-co-4HB, the synthesis of which is catalyzed by the poly-hydroxybutyrate synthase (*phaEC*). The newly discovered TCA cycle variant of cyanobacteria showed that succinic semialdehyde is a potentially important metabolic intermediate (Zhang and Bryant, 2011), and thus we evaluated the ability of cyanobacteria to produce 4HB-CoA from succinic semialdehyde. To shift the metabolic flux further to the oxidative branch of the TCA cycle and increase the accumulation of 2-oxoglutarate (the precursor for succinic semialdehyde), the fused construct encoding the biosynthetic pathway of 4HB-CoA was used to replace the malate dehydrogenase gene on the chromosome of the ΔA0171-PHB strain, in which the operon encoding 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase was also deleted to avoid possible problems arising from homologous recombination. Complete segregation of the construct for the replacement of malate dehydrogenase gene by the fused construct was confirmed by PCR as shown in Figure 8. The production of P3HB-co-4HB in the resulting strain P3HB4HB was detected by GC-MS as described in the Materials and Methods. The ration of 3HB and 4HB was determined by GC analysis, and the identity of the two monomers was verified by mass fragmentation (Figure 9). In total, P3HB-co-4HB accumulated to ~4.5% of total cell dry weight, and 4HB accounted for about 12% of the monomers in the P3HB-co-4HB copolymer produced in
this strain. These results clearly demonstrate that succinic semialdehyde can be converted to 4HB-CoA by the constructed 4HB-CoA biosynthetic pathway. Furthermore, these results establish that succinic semialdehyde is an important metabolic intermediate in the TCA cycle variant that occurs in cyanobacteria.

4.5 Discussion

Cyanobacteria have been used to produce many useful biomaterials as well as biofuels, which are usually derived from important metabolites inside cells (Ducat et al., 2011; Machado and Atsumi, 2012; Zhao et al., 2015). Thus, the discovery of new metabolic pathways as well as new metabolic intermediates in cyanobacteria should not only enrich our knowledge about cyanobacterial metabolism but can also provide novel targets for potential metabolic engineering. Although the genes involved in the biosynthesis of P3HB have been described in cyanobacteria (Tsang et al., 2013) and the production of P3HB was also reported in cyanobacteria (Miyake et al., 2000; Wu et al., 2002), the production of P3HB-co-4HB had not yet been demonstrated in cyanobacteria. This study explored the production of P3HB-co-4HB, using 4HB-CoA that was derived from succinic semialdehyde, a metabolic intermediate of the recently discovered TCA cycle variant in cyanobacteria (Zhang and Bryant, 2011; Zhang and Bryant, 2014). Overall, our results demonstrated the production of both P3HB as well as P3HB-co-4HB in the cyanobacterium *Synechococcus* sp. PCC 7002, and some metabolic engineering strategies to improve the yield were also investigated.

Although previous flux balance analyses showed that, under photoautotrophic conditions in cyanobacteria, the main carbon flux occurred within the Calvin-Benson-Bassham cycle and that the TCA cycle mostly functioned as a branched pathway, it was also suggested that dark
consumption of glycogen in cyanobacteria should include some cyclic flux through the TCA cycle, possibly through 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase (Knoop et al., 2013; Steuer et al., 2012). Furthermore, recent $^{13}$C-MFA studies reported that after unlabeled glutamate was added to $^{13}$C-labeled cultures of *Synechocystis* sp. PCC 6803, substantial fractions of unlabeled 2-OG, succinate, and malate were observed, which may also be produced by the newly identified TCA bypass through succinic semialdehyde (You et al., 2014). Meanwhile, the detection of 4HB monomer in the PHA produced by the P3HB4HB strain under photoautotrophic conditions also establishes that succinic semialdehyde is not only present in *Synechococcus* sp. PCC 7002 but that it can be converted to useful biomaterials (e.g., 4HB-CoA) if appropriate enzymes are introduced into cells. Taken together, these results imply that a complete and functional TCA cycle exists in *Synechococcus* sp. PCC 7002 and probably other cyanobacteria as well, and they indicate that some flux through the TCA cycle occurs even under photoautotrophic growth conditions, which may be enhanced when succinic semialdehyde is consumed by introduced enzymes. It should also be noted that the genes responsible for the newly discovered TCA cycle bypass could readily be transferred into other bacteria (e.g., *E. coli*), when succinic semialdehyde is desired as a metabolic intermediate for the production of important biomaterials.

Although the amounts of P3HB produced by cyanobacteria are currently lower than those from *E. coli* (Li et al., 2010), the requirements of substrate glucose or other organic carbon sources in the growth medium certainly increases the costs of producing P3HB or P3HB-co-4HB in *E. coli*. Besides, the results from this study only represent initial attempts, and future metabolic engineering strategies in cyanobacteria may improve P3HB or P3HB-co-4HB yields to levels comparable to those for *E. coli*. The main building block for the production of P3HB is acetyl-CoA, and thus metabolic engineering strategies to increase the cellular acetyl-CoA pool would potentially increase the yield of P3HB. One direct method might be adding acetate to the medium,
which could be converted to acetyl-CoA directly once taken up by cyanobacteria. Our previous results have shown that adding acetate to the medium increased the P3HB content from ~5% to ~15% of total cell dry weight in *Chlorogloeopsis fritschii* PCC 9212 (Zhang and Bryant, 2015). However, addition of acetate to the growth medium of the WTPHB strain did not lead to obvious changes in the P3HB content of cells (data not shown). The reason(s) for this are unclear, but could include the absence of an efficient acetate uptake (e.g., acetate transporter) or assimilation (i.e., the glyoxylate cycle) pathway in *Synechococcus* sp. PCC 7002. Additionally, it was demonstrated that adding 2-oxoglutarate to the growth medium could increase the 4HB ratio in P3HB-co-4HB in *E. coli* (Li et al., 2010), and thus adding structurally related carbon sources to the growth medium might further improve the yields of P3HB or P3HB-co-4HB. However, it should be noted that the added carbon source(s) will certainly increase the production cost and should be balanced with the production yield in large-scale production.

Other than direct addition of acetate or 2-oxoglutarate to the medium, metabolic engineering strategies could also be used to increase the cellular concentration of acetyl-CoA. Pyruvate is oxidatively cleaved to form acetyl-CoA and CO\(_2\) with the involvement of coenzyme A to accept the acetyl group (Wolfe and O’Kane, 1953). The conversion of pyruvate to acetyl-CoA can be catalyzed by two distinct enzymes, pyruvate dehydrogenase (PDH) and pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFOR) (Arjunan et al., 2002; Schmitz et al., 1993). Many cyanobacteria possess both PDH and PFOR to accommodate changing O\(_2\) levels, especially in dark environments where O\(_2\) is rapidly consumed due to the high intrinsic respiratory activities of cyanobacteria as well as those of surrounding organisms (Schmitz et al., 2001). Thus, overexpression of one or both of these two enzymes might improve the conversion of pyruvate to acetyl-CoA and thus increase the pool of acetyl-CoA for P3HB biosynthesis.

Other approaches to enhance the beta-oxidation pathway, and thus the degradation of fatty acids, should also increase the acetyl-CoA pool. Overexpression of the long-chain-fatty-acid
CoA ligase (fadD) may result in a higher concentration of acyl-CoA and thus increase the acetyl-CoA pool (Choi and Lee, 2013). Furthermore, it was also reported in *E. coli* that the pool of acetyl-coA could be increased by overexpression of pantothenate kinase (Lin et al., 2004). The first enzyme in the biosynthesis of P3HB is acetyl-CoA acetyltransferase, which catalyzes a reversible reaction that might limit the carbon flux toward P3HB biosynthesis. Thus, other than improving the intracellular concentration of acetyl-CoA, making the first step in the conversion of acetyl-CoA to acetoacetyl-CoA irreversible could also increase the carbon flux toward P3HB biosynthesis. Recently, an acetoacetyl-CoA synthase of the thiolase superfamily (*nphT7*, from *Streptomyces* sp. strain CL190) was identified, which catalyzes the reaction that condenses acetyl-CoA and malonyl-CoA to form acetoacetyl-CoA with the release of CO₂, which makes this an essentially irreversible reaction (Okamura et al., 2010). This enzyme has been used to improve the production of 1-butanol in cyanobacteria (Lan and Liao, 2012). All of these results suggest that replacement of acetyl-CoA acetyltransferase with the newly identified acetoacetyl-CoA synthase may further improve the carbon flux toward biosynthesis of P3HB. Attempts to test this possibility are in progress.

Cyanobacteria also use glycogen as a carbon and energy source, and PHAs can serve the same two functions. Thus, metabolic engineering to reduce the metabolic flux toward biosynthesis of glycogen might potentially shift more carbon resources toward P3HB biosynthesis (Xu et al., 2013). It was reported previously that a *glgC* deletion mutant was not able to synthesize glycogen under any circumstances and had an increased content of soluble sugars (Guerra et al., 2013). Thus, deletion of the *glgC* gene in the P3HB producing strain might also improve the production of P3HB, because glycogen biosynthesis is not essential during photoautotrophic growth (Xu et al., 2013). Moreover, growing cyanobacteria under nitrogen limitation conditions could rebalance the metabolic flux between nitrogen and carbon and may favor the biosynthesis of PHB. Indeed, it was demonstrated that PHB accumulation increased
substantially under nitrogen limitation conditions in *Synechococcus* sp. MA19 (Miyake et al., 2000). Surprisingly, however, no significant changes in the accumulation of PHB contents were found when the WTPHB strain was grown under nitrogen limitation condition (data not shown). Considering that the PHB biosynthetic pathway is not present in wild-type *Synechococcus* sp. PCC 7002 but occurs in *Synechococcus* sp. MA19, this could be due to differences in the regulatory mechanisms in these two cyanobacteria. However, one should keep in mind that the growth rate would be affected under such limitation conditions and this could significantly alter the total yields of PHB.

Overall, it should be noted that the combination of all the mentioned methods might eventually improve the PHA production in cyanobacteria to the level that is comparable with *E. coli*. During the past several decades, metabolic engineering strategies in *E. coli* have become well established and studied, and *E. coli* has been used to produce many different biomaterials, ranging from small molecules to complicated natural products and related pharmaceutical analogs (Keasling, 2010; Peralta-Yahya et al., 2012; Stephanopoulos, 2007). However, because of the advantage of the low cost to grow cyanobacteria, and recently well-established genetic tools for mutagenesis and protein overexpression, one can envisage a new era in metabolic engineering of cyanobacteria to produce a wide range of biomaterials. The preliminary work toward this goal in this study provides a good example of how newly discovered metabolic pathways in cyanobacteria could lead to new applications of these bacteria. Future investigations of the metabolic diversity in cyanobacteria certainly would not only enrich our knowledge of cyanobacterial metabolism but might also make cyanobacteria a more powerful tool for providing sustainable green energy by providing new feedstocks for the production of biofuels and biomaterials in these organisms.
4.6 References


Figure 4-1 Scheme showing the biosynthesis pathways of P3HB and P3HB-co-4HB

Abbreviations: cat2, 4-hydroxybutyryl-CoA transferase; gbd1, 4-hydroxybutyrate dehydrogenase; ogdA, 2-oxoglutarate decarboxylase; phaA, acetyl-CoA acetyltransferase; phaB, acetoacetyl-CoA reductase; phaEC, poly(R)-hydroxyalkanoic acid synthase, class III, PhaE and PhaC subunits; ssaD, succinic semialdehyde dehydrogenase.
Figure 4-2 Verification of the presence of PHB biosynthesis pathway in WTPHB

A. Scheme showing that *phaABEC* form an apparent operon and co-localized with the glyoxylate cycle genes in *C. fritschii* PCC 9212. *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. B. PCR verification of the presence of *phaABEC* in the WTPHB strain. The template DNA was derived from wild-type *C. fritschii* PCC 9212 (lane 9212), and from the recombinant strain WTPHB (lane WTPHB), which has the *phaABEC* genes from *C. fritschii* PCC 9212 inserted in plasmid pAQ1-Ex as described in the Materials and Methods (section 4.3.2). C. Immunoblotting of whole cell extracts of *Synechococcus* sp. PCC 7002 wild type (WT7002) as well as WTPHB strain with antibodies to [His]-tag.
Figure 4-3 Detection and verification of the production of P3HB

A. GC-MS elution profiles showing the detection of methyl-3-hydroxybutyrate from the methanolized 3-hydroxybutyrate standard (3HB standard) as well as the methanolized dry cell pellets of WTPHB strain, but not the dry cell pellets of wild type strain *Synechococcus* sp. PCC 7002 (WT7002). B. Mass fragmentation patterns for the peaks detected in the GC-MS elution profiles, confirming that the peak detected in the WTPHB strain (panel 2) has nearly identical pattern as that from the 3-hydroxybutyrate standard (panel 1).
Figure 4-4 Accumulation of P3HB in WTPHB strain

P3HB contents of WTPHB strain were monitored as a function of batch growth under standard conditions. The bars indicate the total cell dry weight (CDW), and the black portions of the bars show the P3HB content at different growth stages. This information is also plotted to emphasize the kinetics and amount of P3HB production. The data shown are averages of three biological replicates, and the error bars show the standard deviation.
Figure 4-5 Scheme for mutant construction and verification of mutant ΔA0171

A. General scheme showing replacement of the target gene with antibiotic resistance cassettes. PCR was used to amplify the upstream and downstream flanking regions for a gene of interest using primer pairs D1-D2 and D3-D4, and these fragments were ligated to the antibiotic cassette. The resulting construction was transformed into *Synechococcus* sp. PCC 7002 to generate fully segregated mutants as described in the Materials and Methods (section 4.3.3). B. Agarose gel electrophoretic analysis of amplicons from PCR reactions using primer set A0171D1-A0171D4 and template DNA derived from wild type (WT) or transformed cells (ΔA0171), verifying the complete segregation of alleles of the gene SynPCC7002_A0171 by homologous recombination method as illustrated in panel A.
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**Figure 4-6 Relative transcripts abundance for mRNAs in *Synechococcus* sp. PCC 7002**

Grey lines indicate a two-fold increase or 50% decrease in mRNA level, details of the analysis were described in the Materials and Methods part (section 4.3.5). *bicA*, bicarbonate transporter, *BicA*; *cupA*, CO2 hydration protein; *gap1*, glyceraldehyde-3-phosphate dehydrogenase, type I; *gap2*, glyceraldehyde-3-phosphate dehydrogenase, type II; *hoxF*, hydrogenase large diaphorase subunit F; *hoxU*, hydrogenase small diaphorase subunit U; *hoxY*, hydrogenase small subunit Y; *hyp3*, CBS domain-containing protein; possibly associated with hydrogenase assembly; *mnhB*, multisubunit Na⁺/H⁺ antiporter, B subunit; *mnhC*, multisubunit Na⁺/H⁺ antiporter, subunit C; *mnhD1*, multisubunit Na⁺/H⁺ antiporter, subunit D1; *mnhD2*, multisubunit Na⁺/H⁺ antiporter, subunit D2; *napA*, Sodium/hydrogen exchanger family protein (Na⁺/H⁺ antiporter); *ndhD-III*, proton-translocating NADH-quinone oxidoreductase (NADH dehydrogenase subunit 4); *ndhF-III*,...
NADH2 dehydrogenase (plastoquinone) chain 5; nifJ, pyruvate:ferredoxin (flavodoxin) oxidoreductase; pntA, nicotinamide nucleotide transhydrogenase, chain alpha, part 1; pntB, nicotinamide nucleotide transhydrogenase, subunit beta; pntC, nicotinamide nucleotide transhydrogenase, chain alpha, part 2; ppsA, phosphoenolpyruvate synthase; sbtA, sodium-dependent bicarbonate transporter.
Figure 4-7 P3HB production in mutant strains of WTPHB

A. Agarose gel electrophoretic analysis of amplicons from PCR reactions using primer set A2771D1-A2770D4 and template DNA derived from WTPHB strain or transformed cells (Δ7071-PHB), which verify the complete segregation of the alleles of gene SynPCC7002_A2771 and SynPCC7002_A2770 after transformation and homologous recombination method as described in the Materials and Methods. B. Agarose gel electrophoretic analysis of amplicons from PCR reactions using primer set A0171D1-A0171D4 and template DNA derived from WTPHB strain or transformed cells (ΔA0171-PHB), verifying the complete segregation of alleles the gene SynPCC7002_A0171 after transformation and homologous recombination as described in the Materials and Methods Part. C. P3HB contents in mutant strains of WTPHB, lacking the SynPCC7002_A2771 and SynPCC7002_A2770 operon (Δ7071-PHB), or lacking SynPCC7002_A0171 (ΔA0171-PHB). The bars indicate the total cell dry weight (CDW), and the black portions of the bars show the P3HB contents. This information is also plotted to emphasize the differences between these strains. The data shown are averages of three biological replicates, and the error bars show the standard deviation.
Figure 4-8 Construction and verification of the 4HB-CoA biosynthetic pathway

A. Scheme showing the replacement of open reading frame SynPCC7002_A2093 (encoding malate dehydrogenase) by the fused construct containing the 4-hydroxylbutyryl-CoA biosynthesis pathway genes by homologous recombination and selection with spectinomycin (aadA gene cassette confers resistance to spectinomycin), using the promoter regions ($P_{psaAB}$) for $psaAB$ from Synechocystis sp. PCC 6803. cat2, 4-hydroxybutyryl-CoA transferase; gbd1, 4-hydroxybutyrate dehydrogenase; ogdA, 2-oxoglutarate decarboxylase. B. Agarose gel electrophoresis of PCR amplicons using primer set A2093D1 and A2093D4 to verify the introduction of the fused construct into the respective strains. Template DNAs came from the ΔA0171-PHB strain and the fully segregated mutant strain (P3HB4HB) with SynPCC7002_A2093 replaced with the fused construct.
Figure 4-9 Detection and verification of the production of P3HB-co-4HB

A. GC-MS elution profiles demonstrate the detection of both methyl-3-hydroxybutyrate and methyl-4-methoxybutyrate from methanolyzed dry cell pellets of the P3HB4HB strain. B. Mass fragmentation patterns for the 4HB peak detected in the GC-MS elution profiles, confirming that the 4HB peak detected in the P3HB4HB strain (panel 2) has a nearly identical fragmentation pattern as that of the γ-butyrolactone standard (panel 1). The other peaks in the elution profiles for P3HB4HB are derived from unrelated contaminating compounds that could not be positively identified.
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Chapter 5  
VIPP1 is required for the biogenesis of Photosystem I rather than thylakoid membranes in *Synechococcus* sp. PCC 7002

Publication:

5.1 Abstract

The biogenesis of thylakoid membranes in cyanobacteria is presently not well understood, but the *vipp1* gene product has been suggested to play an important role in this process. Previous studies in *Synechocystis* sp. PCC 6803 reported that *vipp1* (sll0617) was essential. By constructing a fully segregated null mutant in *vipp1* (SynPCC7002_A0294) in *Synechococcus* sp. PCC 7002, we show that Vipp1 is not essential. Spectroscopic studies revealed that Photosystem I (PS I) was below detection limits in the *vipp1* mutant, but Photosystem II (PS II) was still assembled and was active. Thylakoid membranes were still observed in *vipp1* mutant cells and resembled those in a *psaAB* mutant that completely lacks PS I. When the *vipp1* mutation was complemented with the orthologous *vipp1* gene from *Synechocystis* sp. PCC 6803 that was expressed from the strong *PcpcBA* promoter, PS I content and activities were restored to normal levels, and cells again produced thylakoids that were indistinguishable from those of wild type. Transcription profiling showed that *psaAB* transcripts were lower in abundance in the *vipp1* mutant. However, when the *yfp* gene was expressed from the *PpsaAB* promoter in the presence and the absence of Vipp1, no difference in YFP expression was observed, which shows that Vipp1 is not a transcription factor for the *psaAB* genes. This study shows that thylakoids are still produced in the absence of Vipp1 and that normal thylakoid biogenesis in *Synechococcus* sp. PCC 7002 requires expression and biogenesis of PS I, which in turn requires Vipp1.
5.2 Introduction

Cyanobacteria are considered to be the first oxygen-evolving photolithoautotrophs on Earth. They may have evolved as early as 2.5 billion years ago and are thought to be responsible for the oxygenation of the atmosphere (Des Marais, 2000). In cyanobacteria, photosynthesis antenna pigments capture solar energy, which is subsequently transformed into chemical energy using two reaction centers, Photosystems (PS) I and II, and an electron transport chain that connects them (Jordan, 2001). Water is oxidized to O₂ during this process, and atmospheric CO₂ is reduced to cellular biomass and carbon storage compounds such as glycogen for longer-term energy conservation. In cyanobacteria and in algal and plant chloroplasts, the photosynthetic electron transport chain is localized on intracytoplasmic membranes that form the thylakoid membrane network.

Targeting of proteins into and across the thylakoid membranes has been studied and is believed to occur through several pathways, but little is known about the origin of the thylakoid membrane system or how lipids are synthesized, transported and inserted into this membrane system (Vothknecht and Westhoff, 2001). Moreover, the relationship between the cytoplasmic and thylakoid membranes in cyanobacteria is still very poorly understood. Some researchers propose these systems are interconnected, while others maintain that they are not, and there are arguments in favor of both viewpoints. There is general agreement that the biogenesis of thylakoid membranes is a complex, multidimensional process. During this process, lipids, proteins, and pigments, as well as other cofactors must be synthesized, transported, assembled and inserted into these membranes, but few mechanistic details are available.

Several genetic studies have implicated the product of the vippl gene (Vesicle-Inducing Protein in Plastids 1) as participating in the process of thylakoid biogenesis (Kroll et al., 2001; Li
et al., 1994). Vipp1 was first described as a chloroplast-localized protein in *Pisum sativum*, and further analyses showed that Vipp1 was located on both the inner envelope membrane as well as the thylakoids (Li et al., 1994). This unique localization of Vipp1 on these two membranes led to the presumption that Vipp1 might be involved in the assembly of the thylakoid membrane system (Li et al., 1994). This presumption was supported by the characterization of a mutant of *Arabidopsis thaliana*, in which the expression of the *vipp1* gene was strongly reduced (Kroll et al., 2001). However, a fully segregated null mutant for *vipp1* could not be produced, and thus the product of *vipp1* was believed to be essential for viability (Kroll et al., 2001). The mutant plants expressed only about 20% of the Vipp1 protein levels that occur in wild type under normal growth conditions, and these knock-down mutants were incapable of photoautotrophic growth on soil (Kroll et al., 2001).

Genes similar to *vipp1* are also found in most cyanobacteria (Huang et al., 2002; Srivastava et al., 2006; Westphal et al., 2001). Recently, attempts were made to construct *vipp1* mutants of *Synechocystis* sp. PCC 6803, but although the level of Vipp1 could be lowered, in none of these studies could null mutations of the *vipp1* gene be fully segregated. These *Synechocystis* sp. PCC 6803 merodiploids had a similar phenotype to the knock-down strains of *A. thaliana*, and they exhibited a comparable loss of thylakoid membrane content and structure and also had reduced photosynthetic activity (Gao and Xu, 2009; Westphal et al., 2001). Thus, it was suggested that Vipp1 is also essential in cyanobacteria, because it apparently plays an essential role in thylakoid membrane biogenesis. However, because null alleles of *vipp1* never fully segregated in these *Synechocystis* sp. PCC 6803 strains, the results obtained from the characterization of the merodiploid strains were inconclusive and must be interpreted cautiously.

By using an indirect route for the construction of a *vipp1* mutant in the cyanobacterium *Synechococcus* sp. PCC 7002, we show here that a *vipp1* null mutant can be constructed and that
the fully segregated null mutant is viable. This mutant strain could not grow photoautotrophically, but it could be grown photoheterotrophically when supplied with glycerol under very low irradiance conditions. When this vipp1 mutant was complemented with the vipp1 gene from *Synechocystis* sp. PCC 6803 expressed from the strong $P_{cpcBA}$ promoter, the resulting strain regained the ability to grow photoautotrophically and regained all other phenotypic properties of the wild type. Characterization of these strains showed that Vipp1 is required for biogenesis of PS I, and that PS I is required for the biogenesis of “normal” thylakoid membranes in *Synechococcus* sp. PCC 7002.

5.3 Materials and Methods

5.3.1 Strains, culture conditions and transformation procedure

The wild-type strain of *Synechococcus* sp. PCC 7002 and the vipp1 mutant strain complemented with the vipp1 gene from *Synechocystis* sp. PCC 6803 (see below) were grown in liquid A+ medium under standard conditions (Ludwig and Bryant, 2011): at 38°C with an irradiance of 250 μmol photons m$^{-2}$ s$^{-1}$ provided by cool white fluorescent lights and with sparging with 1% (vol/vol) CO$_2$ in air. Mutant strains were grown under low irradiance conditions (~10 μmol photons m$^{-2}$ s$^{-1}$), and the A+ medium was supplemented with 20 mM glycerol, which served as the main carbon and energy source. For mutant strains, appropriate antibiotics were added as required at the following concentrations: spectinomycin (50 μg/mL); gentamycin (20 μg/ml); kanamycin (100 μg/ml); and erythromycin (20 μg/ml). Transformation of *Synechococcus* sp. PCC 7002 was performed as previously described (Frigaard et al., 2004).
5.3.2 Generation of vipp1 deletion mutant and a trans-complemented strain

In agreement with the results of others and despite many attempts, direct deletion of vipp1 was never successful. Thus, a different strategy was employed. Firstly, a PS I-less strain of Synechococcus sp. PCC 7002 was constructed by deleting the psaAB genes (Shen and Bryant, 1995). The vipp1 gene in this strain was then inactivated by a homologous recombination strategy by deleting a part of the gene (bp 287 to 373 of the coding sequence); the deleted region was replaced with a DNA fragment encoding aacC1, which confers resistance to gentamycin (Figure 5-1). The PCR primers used to amplify the flanking sequence regions for this approach are given in Table 5-1. The ΔpsaAB::aadA mutant strain was rescued at the psaAB locus by transformation with plasmid pAQEEmr80 as described previously (Shen et al., 2002) to produce a PsaAB⁺ Vipp1⁻ (vipp1::aacC1) mutant strain. Full segregation of the wild-type and vipp1::aacC1 alleles was verified by PCR analyses and DNA sequencing of amplicons derived from the DNA of the vipp1::aacC1 null mutant strain.

For the construction of a strain in which the vipp1::aacC1 mutation was complemented in trans, the orthologous vipp1 gene (open reading frame sll0617) from Synechocystis sp. PCC 6803 was amplified by PCR using Phusion DNA polymerase (New England Biolabs) and was introduced into plasmid pAQ1Ex-PcpcBA using primer set ExR and ExF (Table 5-1) as previously described (Xu et al., 2011). The resulting plasmid, pAQ1Ex-PcpcBA::sll0617 was transformed into the vipp1::aacC1 mutant to generate a strain in which the vipp1 null mutation was heterologously complemented by expression of the sll0617 product from the strong cpcBA (phycoecyanin) promoter (PcpcBA; also derived from Synechocystis sp. PCC 6803; see (Xu et al., 2011)). The resulting complemented mutant strain (vipp1::aacC1 pAQ1Ex-PcpcBA::sll0617)
was repeatedly streaked and grown photoautotrophically in A⁺ medium under standard irradiance conditions (i.e., 250 µmol photons m⁻² s⁻¹).

5.3.3 Pigment analysis

Chlorophyll (Chl) a, carotenoids and phycobiliproteins (PBP) concentrations were measured as described (Sakamoto and Bryant, 1997). Pigment concentrations were compared on the basis of equal cell numbers, which were determined from the optical density at 730 nm (OD₇₃₀ nm; 1.0 OD₇₃₀ nm = 1.0 ± 0.2 × 10⁸ cells ml⁻¹; see (Sakamoto and Bryant, 1997)). These measurements were made with cells that had been harvested by centrifugation from cultures grown to late exponential growth phase (OD₇₃₀ nm = ~0.6 to 0.7 ml⁻¹) and resuspended in 50 mM Tris–HCl, pH 7.0 buffer. Chl a and carotenoids were extracted from cells with 100% methanol, and their concentrations were determined as described (Sakamoto and Bryant, 1997). To determine relative PBP levels, cells were incubated at 65°C for 8 min and a difference spectrum with untreated control cells was recorded as described previously (Sakamoto and Bryant, 1997).

5.3.4 Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed as described (Shen and Bryant, 1995) on 15% (w/v) polyacrylamide slab gels (30.0:0.8 acrylamide/bisacrylamide). Equal amounts of cells (100 µl of OD₇₃₀ nm = 4) were centrifuged, and the cell pellets were collected and resuspended in 40 µl BugBuster™ protein extraction reagent (Novagen, Madison, WI). Cells were disrupted after 20 min of incubation at
room temperature. Aliquots (20 µl) of the whole cell extract were loaded to each lane. Rabbit antibodies against PsaA, PsaB, and Vipp1 were purchased from Agrisera (Vännäs, Sweden). Immunoblotting was performed as previously described (Shen et al., 2002).

5.3.5 Oxygen evolution assay

Whole-chain oxygen evolution as well as respiratory oxygen uptake in wild-type and mutant cells were measured using a Clark-type electrode as described (Nomura et al., 2006). The temperature of the measuring chamber was maintained at 38°C by a circulating water bath. Cells were washed once with fresh A+ media and adjusted to equal final cell concentration on the basis of OD$_{730}$ nm. For oxygen evolution measurements, 10 mM NaHCO$_3$ was added to the cell suspension. Respiration rates were obtained under the same conditions but without illuminating the cells.

5.3.6 Fluorescence emission spectra at 77 K

Fluorescence emission spectra at 77 K were measured with an SLM 8000C spectrofluorometer, modified by OLIS, Inc., as described (Shen and Bryant, 1995). Cells in exponential growth phase (OD$_{730}$ nm = ~0.6 to 0.7 ml$^{-1}$) were collected and resuspended in 50 mM Tris–HCl, pH 7.0 buffer. Glycerol was added to a final concentration of 60% (v/v). Cells were adjusted to a concentration of ~0.5 OD$_{730}$ nm ml$^{-1}$ and quickly frozen in liquid nitrogen. The excitation wavelength for Chl excitation was 440 nm. A long pass filter (transmitting at 600 nm)
was used at the inlet of the emission monochromator to minimize contributions from scattered light.

5.3.7 Whole-cell P700 activity measurements

Cells (final OD$_{730}$ nm = ~0.5) were collected and resuspended in 50 mM Tris–HCl, pH 8.3 buffer. The absorbance change at 700 nm was monitored by a Model JTS-10 LED pump-probe spectrometer (Bio-Logic, France). A high-power red LED (680 ± 50 nm) provided the actinic illumination. A high-power white LED, filtered through a 700 nm interference filter (Edmund Optics, Inc.), provided the measuring pulses.

5.3.8 Transmission electron microscopy

Thylakoid membranes from wild-type and mutant cells of Synechococcus sp. PCC 7002 were visualized by transmission electron microscopy of thin sections as described (Spence et al., 2004). The ultrathin sections were viewed with a JEM-1200 transmission electron microscope (JEOL Ltd, Japan). Images were captured using TIETZ Digital Image Capture software.

5.3.9 Lipid body detection

Nile-red staining was performed to detect lipid bodies using a reported method with minor modifications (Greenspan et al., 1985). Cells were harvested, washed and resuspended in 50 mM Tris–HCl, pH 8.0 buffer. Nile-red stock solution (1 µl of a 1 mg ml$^{-1}$ stock solution in
dimethyl sulfoxide) was added to an aliquot of washed cells (100 µl). After staining for 10 min, lipid bodies inside cells were visualized by fluorescence using a FluoView FV1000 confocal microscope (Olympus, Center Valley, PA) in scanning mode. The excitation wavelength was 488 nm, and an emission wavelength of 500 to 600 nm was selected for all experiments.

5.3.10 Total mRNA profiling

Transcriptome profiling was performed as described (Ludwig and Bryant, 2011). The *vipp1* mutant strain and complemented strain were first adapted to low irradiance (~10 µmol photons m⁻² s⁻¹) on medium A⁺ supplemented with glycerol (20 mM). Cells were re-inoculated and harvested at OD₇₃₀ nm = 0.7. Total RNA was then extracted as described (18). The construction of cDNA libraries and sequencing (SOLiD™) were performed in the Genomic Core Facility at The Pennsylvania State University. Mapping against the *Synechococcus* sp. PCC 7002 genome was performed using the BWA software package (Li and Durbin, 2009). The resulting alignment files were further analyzed with self-developed scripts to extract expression levels for each gene as described previously (Ludwig and Bryant, 2011). In order to compare the relative expression levels of the same gene from different samples that were grown under different growth conditions, the number of aligned sequences was normalized relative to the total number of mRNA counts in each sample. The RNA sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP035555.
5.3.11 YFP (Yellow Fluorescent Protein) signal detection

To determine whether Vipp1 plays a direct regulatory role in the transcription of the psaAB and chlLN operons, promoter regions for psaAB and chlLN were amplified and transcriptionally fused to yfp as well as aphII (conferring kanamycin resistance) separately. The primers used to amplify these promoter regions for psaAB (psaABF and psaABR) and chlLN (chlLNF, chlLNR) are listed in Table 5-1. The fused constructs were then used to replace open reading frame SYNPCC7002_A2746 (Figure 5-2) in both the vipp1 mutant and the complemented strain by homologous recombination as described (Frigaard et al., 2004). Primers used to amplify the upstream and downstream flanking regions of SYNPCC7002_A2746 are listed in Table 5-1 (A2746upF, A2746upR, A2746downF, A2746downR). Transcription profiling analyses under many different conditions showed that ORF SYNPCC7002_A2746 produces few if any transcripts under most growth conditions (Ludwig and Bryant, 2011, 2012a, b). Additionally, a deletion mutant of SYNPCC7002_A2746 has been constructed (Figure 5-2). No detectable growth phenotype was observed for this mutant strain compared to wild type strain, and thus this gene site was used as a neutral site (Figure 5-2). These strains were grown under low irradiance ($\sim$10 $\mu$mol photons m$^{-2}$ s$^{-1}$) on medium A$^+$ supplemented with glycerol (20 mM). Cells were harvested by centrifugation from exponential growth phase (OD$_{730\text{ nm}}$ = 0.6 to 0.7 ml$^{-1}$), resuspended in A$^+$ medium containing 20 Full segregation of the vipp1 mutant strain and the complemented strain containing the promoter fusions to yfp were verified using primer set A2746upF and A2746downR. The products were sequenced to verify that no inadvertent changes had occurred during strain construction. mM glycerol and adjusted to same OD$_{730\text{ nm}}$ = 0.5 ml$^{-1}$. YFP signal in these cells was detected with an SLM 8000C spectrofluorometer, modified by
OLIS, Inc. (Bogart, GA). The excitation wavelength was 488 nm and emission spectra were recorded from 500 nm to 600 nm.

5.4 Results

5.4.1 Generation of *vipp1* mutant

Genetic manipulations to generate a *vipp1* null mutant, as well as a strain in which the resulting *vipp1* mutation was complemented in *trans*, are described in the Materials and Methods (section 5.3.2). For construction of a *vipp1* null mutant, *Synechococcus* sp. PCC 7002 was first adapted to grow on glycerol as a carbon/energy source, which allows one to construct mutants lacking PS I and/or PS II (Shen and Bryant, 1995). Some observations made with *vipp1* merodiploid strains suggested that these merodiploid strains had greatly reduced levels of PS I (data not shown, but see below), and thus a strain in which the *psaAB* genes had been deleted was used for inactivation of the *vipp1* gene. After several attempts, a fully segregated *vipp1::aacC1* strain, which was verified by PCR analysis as shown in Figure 5-1, was obtained. Although the *vipp1* gene was believed to be essential in cyanobacteria (Gao and Xu, 2009; Westphal et al., 2001), the results in Figure 5-1 show that the *vipp1* product is not essential in *Synechococcus* sp. PCC 7002 and that a null mutant is viable. Because it was much easier to obtain a fully segregated *vipp1* null mutation in a PS I-less strain, it appeared likely that Vipp1 was somehow involved in the biogenesis of PS I.
5.4.2 Characterization of the \textit{vipp1} mutant strain

The \textit{vipp1} mutant strain could not grow photoautotrophically, but this strain could still grow photoheterotrophically under low irradiance conditions (~10 µmol photons m\(^{-2}\) s\(^{-1}\)) when cells were supplied with 20 mM glycerol. Immunoblotting showed that no Vipp1 protein was detectable in the mutant cells (Figure 5-3). Previous studies had shown that a deficiency in Vipp1 led to impairment of the biosynthesis of thylakoid membranes in plants and \textit{Synechocystis} sp. PCC 6803 (Gao and Xu, 2009; Kroll et al., 2001). Transmission electron microscopy (TEM) of thin-section cells was used to examine the membrane organization in the \textit{vipp1} mutant strain. As shown in Figure 5-4, cells of the \textit{vipp1} mutant had much less thylakoid membranes than the wild type (Figure 5-4). However, some vestigial thylakoid membranes were still present, and this indicated that Vipp1 is not essential for the biogenesis of thylakoid membranes. It should be noted, however, that the thylakoid membranes appeared to have a much simpler organization in the \textit{vipp1} mutant, and that in some cells the thylakoid membranes appeared to be directly connected to the cytoplasmic membrane (Figure 5-4).

5.4.3 Complementation of the \textit{vipp1} mutation \textit{in trans}

Because of the multi-step procedure employed to construct the \textit{vipp1} mutant strain, it was important to demonstrate that the resulting \textit{vipp1} mutant could be complemented to rescue a wild-type phenotype. To avoid potential problems arising from homologous recombination of \textit{vipp1} alleles, the orthologous \textit{vipp1} gene (locus tag = sll0617) from \textit{Synechocystis} sp. PCC 6803 was used for complementation instead of \textit{vipp1} from \textit{Synechococcus} sp. PCC 7002. Vipp1 from \textit{Synechocystis} sp. PCC 6803 is 54% identical and 71% similar in sequence to Vipp1 from
Synechococcus sp. PCC 7002. A plasmid was constructed in which ORF sll0617 from Synechocystis sp. PCC 6803 was placed under the control of the strong \(P_{cpcBA}\) promoter (also from Synechocystis sp. PCC 6803; (Xu et al., 2011)), and this plasmid was transformed into the vipp1 null mutant. As shown in Figure 5-1, PCR analysis using primer set p5 and p6 (Table 5-1) showed that the vipp1 gene was still interrupted in the resulting complemented strain, which was denoted as strain “Comp.” Immunoblotting showed that the complemented mutant strain accumulated much more Vipp1 protein than the wild type (Figure 5-3). The complemented strain could grow photoautotrophically (Figure 5-1) and was no longer sensitive to high light conditions. Analysis of thin sections by TEM showed that the thylakoid membranes of the Comp strain cells were indistinguishable from those in wild-type cells (Figure 5-4). These results demonstrate that vipp1 (sll0617) from Synechocystis sp. PCC 6803 can fully complement the vipp1 mutant of Synechococcus sp. PCC 7002 and could restore a wild-type phenotype (see additional results and Discussion below).

5.4.4 Pigments and oxygen evolution rate

The vipp1 mutant was noticeably bluer in color than the wild type (Figure 5-1), so we next analyzed the pigment content and oxygen evolution behavior of the vipp1 mutant and the Comp strain (Table 5-2). The Chl \(a\) content of the vipp1 mutant was reduced to about 10%, and the total carotenoid content to about 20%, of the levels in the wild type. The PBP content of the vipp1 mutant was approximately half that of the wild type (Table 5-2). The increased PBP content relative to the Chl \(a\) explains the bluish color of the cells. These values returned to nearly wild-type levels when the vipp1 mutation was complemented in \textit{trans}. Interesting, the Chl \(a\) and
carotenoid levels in the \textit{vipp1} mutant were similar to those in a PS-I less mutant, although the 
PBP content of the PS-I less mutant was essentially the same as that of the wild type (Table 5-2).

Oxygen evolution rates and respiratory oxygen uptake rates were measured for these 
strains (Figure 5-5). The measurements were either compared on the basis of equal cell number, 
as determined by the optical density at 730 nm (Figure 5-5), or on the basis of the Chl \textit{a} content 
(Figure 5-5). As shown in Figure 5-5, the \textit{vipp1} mutant evolved oxygen but this activity rapidly 
declined after several minutes of illumination (data not shown). The initial oxygen evolution rate 
on the basis of Chl \textit{a} was roughly 2.5-fold higher than that of the wild-type strain, but was only 
about 25\% of the wild-type rate on a per cell basis. These behaviors are very similar to those of a 
strain lacking PS I (Shen and Bryant, 1995). When the \textit{vipp1} mutation was complemented in 
\textit{trans}, the oxygen evolution and respiratory uptake rates for the resulting Comp strain were very 
similar to those of the wild type. Thus, PS II complexes in the \textit{vipp1} mutant and the PS I-less 
strain were assembled and were functional. The similar pigment contents and oxygen evolution 
behaviors of these two strains further suggested that the \textit{vipp1} mutant probably had fewer PS I 
complexes per cell.

\textbf{5.4.5 Low-temperature fluorescence emission spectroscopy}

Figure 5-6 shows the low temperature (77 K) fluorescence emission spectra of various 
\textit{Synechococcus} sp. PCC 7002 strains. When the excitation wavelength was 440 nm to excite Chl 
\textit{a}, three major emission peaks were observed at 685, 695, and 715 nm for the wild type. The first 
two peaks principally arise from PS II, while the emission peak at 715 nm arises from PS I (Shen 
et al., 2002). The fluorescence emission spectrum of the \textit{vipp1} mutant shows no emission peak 
from PS I and only shows emission peaks associated with PS II. The emission spectrum for the
vipp1 mutant after trans-complementation with sll0617 from Synechocystis sp. PCC 6803 was nearly indistinguishable from that of the wild type (Figure 5-6). These data strongly implicate Vipp1 in the expression or biogenesis of PS I.

5.4.6 PS I activities in whole cells

The low temperature fluorescence emission spectrum of the vipp1 mutant showed that the PS I content of this strain was severely reduced. To verify that the PS I activity was also reduced in the vipp1 mutant, photobleaching of P700 was directly measured at 700 nm in whole-cells with a pump-probe spectrophotometer as described in the Materials and Methods (section 5.3.7). As shown in Figure 5-7, photobleaching of P700 occurred when whole cells were illuminated with actinic light, and a slight increase in photobleaching occurred over a 10-sec period of illumination. The absorption change at 700 nm was fully reversible when the actinic light was switched off. No photobleaching at 700 nm was detectable for the vipp1 mutant, but the photobleaching of P700 in the trans-complemented strain was similar in magnitude and kinetics to that of the wild type. PS I complexes were isolated from the wild type and from the trans-complemented Comp strain. Time-resolved optical spectroscopy on the ms timescale showed that the PS I complexes from these two strains had similar extents of photobleaching and lifetimes of charge separation (data not shown). These spectroscopic studies show that the vipp1 mutant does not assemble functional PS I complexes. Heterologous complementation of vipp1 with ORF sll0617 from Synechocystis sp. PCC 6803 was sufficient to restore normal PS I biogenesis and functionality to the vipp1 mutant of Synechococcus sp. PCC 7002.
5.4.7 Immunoblotting to detect PS I polypeptides

Whole-cell extracts of the wild type, the *vipp1* mutant strain, and the *trans*-complemented *vipp1* mutant strain were prepared and subjected to SDS-PAGE, and the resolved proteins were transferred to membrane filters for immunoblotting. Previous studies have shown that PsaC, PsaD and PsaE, which are water-soluble and form the stromal ridge of PS I (Jordan, 2001; Shen and Bryant, 1995), do not accumulate in PS I-less mutants (Shen and Bryant, 1995). Immunoblotting showed that PsaA, PsaB, PsaC, PsaD, and PsaE do not accumulate in the *vipp1* mutant, which is consistent with all other evidence indicating that PS I is missing in the *vipp1* mutant (Figure 5-8). However, all of these proteins were detected in the wild type and in the *trans*-complemented (Comp) strain. Interestingly, the integral membrane protein, PsaL, which is responsible for trimerization of PS I (Chitnis and Chitnis, 1993), accumulated in the absence of PsaA and PsaB in the *vipp1* mutant (Figure 5-8). This observation is consistent with observations made for *Synechocystis* sp. PCC 6803, for which it was also found that PsaL accumulated in membranes independently of the levels of PsaA and PsaB (Domonkos et al., 2004).

5.4.8 Thylakoid membranes in a *psaAB* deletion mutant

Although previous studies had suggested that Vipp1 was required for thylakoid membrane biogenesis, the results shown above suggested that Vipp1 is required for *psaAB* expression or the biogenesis of PS I. The presence of vestigial thylakoid membranes in the *vipp1* mutant indicated that normal thylakoid biogenesis might require PS I assembly rather than Vipp1. A natural hypothesis arising from these observations is that a PS I-less mutant should produce vestigial thylakoids that closely resemble those in the *vipp1* mutant. Figure 5-4 shows images of
cells of a *psaAB* deletion mutant of *Synechococcus* sp. PCC 7002 that is unable to assemble any functional PS I complexes (or subcomplexes; (Shen and Bryant, 1995)). Immunoblotting showed that the PS I-less mutant cells accumulated wild-type levels of Vipp1 (Figure 5-3). Like the *vipp1* mutant, cells of the *psaAB* deletion mutant produce vestigial thylakoids that closely resemble those of the *vipp1* mutant. Therefore, these results clearly demonstrate that the capacity to produce wild-type thylakoid membranes requires the normal biogenesis of PS I rather than Vipp1.

### 5.4.9 Detection of lipid bodies

Previous studies in plant chloroplasts and *Synechocystis* sp. PCC 6803 suggested that *vipp1* might play a role in membrane biogenesis, and might more specifically affect the insertion of membrane lipids (Vothknecht et al., 2012). However, the results presented here conclusively demonstrate that cyanobacterial Vipp1 plays a role in the expression or biogenesis of PS I. Nile red is a lipid-soluble fluorescent dye that can be used to detect lipids in microorganisms (Greenspan et al., 1985). To determine whether lipid bodies were produced in the strains constructed here, Nile-red staining was performed to detect lipid bodies. The *vipp1* mutant accumulated numerous lipid bodies in the cytoplasm (Figure 5-9) but lipid bodies were not observed in wild type (Figure 5-9). This result excludes the possibility that Vipp1 is involved in the biogenesis of lipid bodies, but it suggests that Vipp1 is directly or indirectly involved in the assembly of lipids into thylakoid membranes. The inability to produce normal amounts of PS I apparently interferes with lipid insertion into the thylakoid membranes, and the lipids apparently then accumulate as lipid bodies in the cytoplasm. Consistent with the results described above, the PS I-less strain also accumulated lipid bodies in the cytoplasm that resembled those in the *vipp1* strain (Figure 5-9). When the ability to produce PS I was restored by *trans*-complementation of
the \textit{vipp1} mutation, lipids no longer accumulated as lipid bodies in the cytoplasm of the Comp strain (Figure 5-9).

\textbf{5.4.10 Vipp1 is not required for transcription of psaAB}

In the experiments described above, we showed that PS I is not detectable in cells that lack Vipp1 and that restoration of Vipp1 by \textit{trans}-complementation reverses all known phenotypic defects associated with the absence of Vipp1. Figure 5-10 shows a scatter-plot that compares the transcript abundances for each gene in the \textit{vipp1} deletion strain in comparison to their abundances in the \textit{trans}-complemented (Comp) strain. Four genes, \textit{psaAB} and \textit{chlLN}, which occur in two dicistronic operons, showed significantly lower transcript abundances in the \textit{vipp1} mutant compared to the \textit{trans}-complemented strain. These data suggested that Vipp1 might regulate \textit{psaAB} transcript levels.

To ascertain whether Vipp1 was acting as an activator or repressor of transcription of the \textit{psaAB} operon, we introduced a promoter fusion of \textit{P\textsubscript{psaAB}} to \textit{yfp} (\textit{P\textsubscript{psaAB::yfp}}) into a neutral site (SYNPCC7002\_A2746) in the \textit{Synechococcus} sp. PCC 7002 chromosome in the \textit{vipp1} mutant as well as the \textit{trans}-complemented Comp strain (Figure 5-11). The Yfp fluorescence in these two strains was equal within error (Figure 5-11). Similar results were observed for the \textit{P\textsubscript{chlLN}} fused to \textit{P\textsubscript{chlLN::yfp}} (data not shown). These results show that Vipp1 is not directly involved in regulating the transcription of \textit{psaAB}. However, it is possible that Vipp1 plays a role in PS I biogenesis that indirectly influences the stability of the \textit{psaAB} mRNA (e.g., by influencing translation of this transcript). The presence or absence of Vipp1 in cells did not cause a change in the amount of YFP expression. Because the \textit{vipp1} mutant strain can only be grown at very low irradiance levels, it is possible that the ChlLN operon, which encodes two of the three subunits of the light-
independent protochlorophyllide reductase (Suzuki and Bauer, 1992), is specifically transcribed at higher levels in the Comp strain. This strain, which is derived from the vipp1 mutant, has a much greater demand for Chl a than the parental strain because of restoration of PS I. Low irradiance growth conditions might cause a limitation for Chl a biosynthesis at the level of protochlorophyllide a reduction, which typically is catalyzed by the light-dependent protochlorophyllide a reductase (Reinbothe et al., 1996). The low light levels might lead to derepression of the light-independent protochlorophyllide a reductase, ChlNBL, in the Comp strain because of an increased demand for Chl a to assemble PS I. This would not occur in the vipp1 mutant because it does not assemble PS I and requires only 10% of the Chl found in the wild type (Table 5-2).

5.5 Discussion

The exact role of Vipp1 has been a long-standing mystery that has been investigated in several organisms over about two decades. However, its function is still unclear. A vipp1 merodiploid knock-down strain of Synechocystis sp. PCC 6803 had reduced levels of PS I (Fuhrmann et al., 2009b), and less thylakoid membranes. However, the authors of that study concluded that the reduction in thylakoid membrane content affected the ratio of PS I and PS II. Other studies suggested that Vipp1 was essential because it played a major role in the biogenesis of thylakoid membranes (Kroll et al., 2001; Vothknecht et al., 2012). However, based on the results obtained in this study in Synechococcus sp. PCC 7002, Vipp1 is clearly required for the biogenesis of PS I, and the absence of PS I leads to a greatly decreased level of thylakoids membranes in Synechococcus sp. PCC 7002 cells.
In contrast to previous studies in *Synechocystis* sp. PCC 6803, a fully segregated null mutation was constructed in the *vipp1* gene of *Synechococcus* sp. PCC 7002 in this study, and we were able to complement this mutant heterologously in *trans* with the *Synechocystis* sp. PCC6803 *vipp1* gene to restore the wild-type phenotype. These experiments clearly demonstrate that the function of Vipp1 is highly conserved, but Vipp1 is clearly not required for viability in the cyanobacterium *Synechococcus* sp. PCC 7002. Since 1996, the genomes of many cyanobacteria have been sequenced (e.g., see (Kaneko et al., 1996; Stanley et al., 2013)). Comparative analysis of these genomic data shows that some cyanobacteria, such as certain *Prochlorococcus* species, lack the *vipp1* gene. However, these cyanobacteria clearly still assemble thylakoids and produce functional PS I complexes (Dufresne et al., 2003). Thus, Vipp1 is not required for the formation of thylakoid membranes in *Prochlorococcus* spp. nor is it essential in all cases for PS I biogenesis. These observations suggest that cyanobacteria must have redundant mechanisms to assemble PS I, probably PS II, and thylakoids. This is almost certainly one of the reasons why it has been so difficult to establish mechanistic details for the biogenesis of PS I and PS II.

It has been reported that reduced expression of Vipp1 in *Synechocystis* sp. PCC 6803 resulted in a decreased PS I content and an altered PS I:PS II ratio, reduced thylakoid content, and a reduced percentage of trimeric versus monomeric PS I complexes (Fuhrmann et al., 2009b). Mutants lacking phosphatidylglycerol (PG) synthase, encoded by the *pgsA* gene, in *Synechocystis* sp. PCC6803 are not viable unless PG is added to the growth medium (Domonkos et al., 2004). When cells of a *pgsA* mutant were deprived of PG over many days, a phenotype similar to that for Vipp1 depletion was noted with respect to PS I complexes. Depletion of PG not only led to decreased PS I activity, but it also caused a depletion of PS I trimers and an increase in PS I monomers. However, PsaL was still inserted into membranes and could reassemble trimeric complexes in the absence of protein synthesis when PG was added back to cells (Domonkos et
al., 2004). The crystal structure of trimeric PS I complexes from *Thermosynechococcus elongatus* showed that three PG molecules are tightly associated with each monomeric PS I complex and thus may play a role in PS I biogenesis (Jordan, 2001). An *A. thaliana* mutant strain unable to synthesize PG was no longer able to grow photoautotrophically and had a severe reduction in Chl and thylakoid membranes (Hagio et al., 2002), which could potentially be due at least in part to a loss of functional PS I complexes. Collectively, these results suggest that a relationship exists among Vipp1, PG biosynthesis, and biogenesis of PS I complexes, and that collectively Vipp1, PG synthesis, and PS I biogenesis strongly influence thylakoid membrane structure and biogenesis.

Analysis of the *trans*-complemented *vipp1* mutant and the *psaAB* deletion mutant strains further indicated that Vipp1 is more likely involved primarily in the biogenesis of PS I complexes rather than thylakoid membrane biogenesis, because PS I complexes and activity are restored to wild-type levels in the *trans*-complemented *vipp1* mutant strain. In contrast, in the *vipp1* null mutant, no PS I complexes accumulated, and no PS I activity was detected. Previous studies also suggested that a critical Vipp1 concentration might be required for thylakoid membrane protein complex formation (Fuhrmann et al., 2009b). Vipp1 has been reported to form rod-like structures in vivo (Fuhrmann et al., 2009a), and it is possible that these Vipp1 structures could assist in the translation, transport and/or insertion of membrane-associated subunits into thylakoids. Thus, the loss of Vipp1 might interfere with one or more of these processes and therefore interfere with the insertion of the PsaA and PsaB polypeptides into the thylakoid membranes. While it seems clear that the loss of Vipp1 interferes with one or more of these processes, it certainly is not yet clear at which level Vipp1 acts to interfere with PS I biogenesis. However, the reduced level of *psaAB* transcripts in *vipp1* mutant cells suggests that Vipp1 likely acts directly or indirectly at the level of translation (see below).
In *Synechococcus* sp. PCC 7002, a *psaAB* deletion mutant lacking PS I had normal transcript levels for *vipp1* but greatly reduced thylakoid membrane content, and similarly, the *vipp1* mutant that lacks PS I had reduced thylakoid membrane content. Restoration of PS I levels in cells caused thylakoids to return to wild-type levels and overall structure, which implies that PS I plays an important role in the formation of structurally normal and functional thylakoid membranes. These observations generally agree with observations that depletion of PS II had only minor affects on thylakoid membrane formation (Nilsson et al., 1992) and that intracytoplasmic membranes were much less abundant in a mutant depleted of both PS I and PS II (van de Meene et al., 2012). It has been suggested that PS I plays a role in the early steps to form thylakoid membranes, and that PS II is then involved in forming highly ordered tubular structures of thylakoid membranes together with PS I (van de Meene et al., 2012). A direct interaction of Vipp1 with Albino3.2 protein in *Chlamydomonas reinhardtii* also implicated Vipp1 in the integration of thylakoid membrane proteins (Gohre et al., 2006). The thylakoid-localized protein, Albino3.2, belongs to the conserved YidC/Oxa1p/Alb3 protein family, and it plays an essential role during the insertion of photosystem reaction center polypeptides (such as *PsaA*, *PsbA* (D1) and *PsbD* (D2)) into the thylakoid membranes (Gohre et al., 2006; Wang and Dalbey, 2011). Overproduction of Vipp1 occurred when Albino3.2 was depleted in *C. reinhardtii* (Gohre et al., 2006). This observation suggests that Vipp1 is involved in stabilizing the membrane structure during the Albino3.2-mediated protein insertion into thylakoid membranes, and that Vipp1 may deliver photosystem polypeptides to Albino3.2 for insertion into thylakoid membranes. If *PsaA* and *PsaB* were cotranslationally inserted into thylakoids during or after Vipp1 action, this could explain why transcripts for *psaAB* were reduced in the *vipp1* mutant. Transcript levels for *yidC* were similar in *Synechococcus* sp. PCC 7002 cells in presence or absence of Vipp1. Furthermore, we observed no changes in transcript levels for genes encoding other PS I polypeptides, PS I-
specific chaperones (e.g., \textit{rubA}, \textit{ycf3}, \textit{ycf4}), or other general chaperones or proteases. Correspondingly, the transcript levels for all of these components were similar in the wild type, the \textit{vipp1} mutant, the complemented \textit{vipp1} mutant strain, and the \textit{psaAB} deletion strain.

Abundant lipid bodies were detected in the \textit{vipp1} mutant strain, which suggests that Vipp1 is not directly involved in lipid synthesis and accumulation. However, the abnormal localization of lipids in \textit{vipp1} mutant indicates that Vipp1 directly or indirectly affects the insertion of lipids into thylakoid membranes. Previous studies suggested that Vipp1 played a stimulatory role in the cpTat transport system, potentially by enhancing protein binding interactions with lipid-rich regions of thylakoid membranes (Lo and Theg, 2012). Interestingly, it appears that lipid synthesis still occurs, but normal lipid insertion into membranes is apparently greatly reduced when PS I complexes are not inserted into the membranes in \textit{Synechococcus} sp. PCC 7002. As previously observed in other organisms, it appears that there is a relationship between membrane biogenesis, lipid insertion, and PS I complex biogenesis and membrane insertion—and that Vipp1 is necessary for all of these processes to proceed normally (Vothknecht et al., 2012).

Nordhues et al. (38) recently studied the role of Vipp1 in \textit{C. reinhardtii} by RNA interference. They found that core complexes for PS I and PS II, as well as the cytochrome \textit{b}_{6}\textit{f} complex and ATP synthase were reduced 14 to 20% in Vipp1 depleted cells, but light-harvesting complex II levels increased by 30%. These authors proposed a highly speculative hypothesis that Vipp1 provides structural lipids for the biogenesis of some protein complexes of the thylakoid membrane. However, the obvious differences in their results in \textit{C. reinhardtii} and those presented here for \textit{Synechococcus} sp. PCC 7002 strongly suggest that Vipp1 may play different roles in prokaryotes and eukaryotes. Alternatively, redundant pathways may exist in some but not all
organisms that could explain the substantial phenotypic differences observed in different organisms.

Transcription profiling suggested that Vipp1 might function as a potential transcriptional regulator of the chlLN and psaAB operons. The phage shock protein A (PspA), a key transcription regulatory protein in bacteria (Darwin, 2005), has significant sequence similarity with Vipp1 and is thus is structurally related to Vipp1 as well. In other studies, the translocation of proteins by TAT complexes in E. coli was blocked in a pspA mutant, but this phenotype could be relieved by expression of the vipp1 gene from Synechocystis sp. PCC 6803 in the mutant (DeLisa et al., 2004). These findings indicate that cyanobacterial Vipp1 can functionally replace bacterial PspA. Subsequent studies suggested that the α-helical, PspA-like domain of Vipp1 plays a crucial role in forming high-molecular-mass complexes (Aseeva et al., 2004). Phylogenetic analyses of Vipp1 and PspA clearly suggest that these proteins form separate clades (Vothknecht et al., 2012), and most cyanobacteria contain a protein from each family. A possible explanation for these results is that Vipp1 evolved from cyanobacterial PspA by gene duplication and may have gained a novel function in cyanobacteria in thylakoid biogenesis or perhaps stress responses. In Synechocystis sp. PCC 6803 vipp1 expression increased under high-salt conditions (Huang et al., 2006), and in C. reinhardtii, vipp1 expression increased under high irradiance (Im and Grossman, 2002). These findings suggest that Vipp1 may play a role in responses to these stress conditions, although its expression might also increase if PS I levels increase under these same conditions. However, our results with reporter strains clearly showed that Vipp1 is not directly involved in the transcriptional regulation of either the psaAB or chlLN genes of Synechococcus sp. PCC 7002. We propose that psaAB transcripts decreased because of an effect of Vipp1 on translation or co-translational insertion of these PS I polypeptides into the thylakoid membrane. The decreased transcript levels for chlLN are probably due to the reduced demand for Chl a in the Vipp1 mutant,
which does not accumulate PS I (Table 5-2). It is also interesting the marine symbiont strain UCYN-A, which lacks PS II and cannot fix CO$_2$ but has retained PS I complexes (Tripp et al., 2010), has also retained two copies of the vipp1 gene like *Trichodesmium erythraeum*, another nitrogen-fixing marine cyanobacterium.

In conclusion, our results show that Vipp1 is not essential for viability of the cyanobacterium *Synechococcus* sp. PCC 7002, and that it is most likely involved in the biogenesis of PS I, possibly by participating in the insertion of PS I polypeptides into thylakoid membranes. Our results further suggest that normal thylakoid membrane biogenesis is dependent upon assembly of PS I but is not directly dependent on Vipp1. Further studies to elucidate the precise role of Vipp1 in PS I and lipid insertion into membranes will enhance our knowledge of the underlying mechanisms of thylakoid membrane biogenesis and photosynthetic protein assembly.

5.6 References


Figure 5-1 Construction and verification of mutant strains

A. Scheme showing the construction of a *vipp1* mutant by homologous recombination by using primer set p1 and p2 to amplify the upstream region and primer set p3 and p4 to amplify the downstream region. An antibiotic resistance cassette was ligated into restriction sites added to the appropriate ends of the flanking sequences as described in the Materials and Methods (section 5.3.2). B. Agarose gel electrophoresis of amplicons produced using primers p5 and p6 results showing the fully segregated interruption mutant of *vipp1* using primer set p5 and p6. The template DNAs were isolated from the wild type (WT), the *trans*-complemented *vipp1* mutant (Comp), and the *vipp1* mutant strain. The results clearly showed that the wild-type *vipp1* and interrupted *vipp1::aacC1* alleles had segregated completely in the *vipp1* mutant and that the *vipp1* gene was still mutated in the Comp strain. Lane M, DNA size markers. C: Cultures of equivalent
cell density (OD$_{730\text{ nm}} = 1.0$) for wild type (WT), the trans-complemented $vipp1$ mutant strain (Comp), and the $vipp1$ mutant ($Avipp1$).
Figure 5-2 Construction and verification of a neutral site platform

A: Diagram showing the construction of a SynPCC7002_A2746 mutant and positions of oligonucleotide primers (Table 5-1). B. Agarose gel electrophoresis of amplicons produced by polymerase chain reaction demonstrating complete segregation of alleles for SynPCC7002_A2746::aphII and SynPCC7002_A2746 using primer set A2746upF and A2746downR. The template DNAs were isolated from the SynPCC7002_A2746 mutant (lane 1) and wild type (lane 2). C. Comparison of growth rates for *Synechococcus* sp. PCC 7002 wild type (WT) and a neutral-site mutant strain constructed in open reading frame SynPCC7002_A2746 (ΔA2746). The growth rates were indistinguishable within experimental error. The data are the average of three biological replicates.
Antibodies to Vipp1 were used to detect Vipp1 levels in wild type (WT), *trans*-complemented \( \Delta \text{vipp1} \) mutant strain (Comp), the \( \text{vipp1} \) mutant (\( \Delta \text{vipp1} \)), and a PS I-less mutant (\( \Delta \text{psaAB} \)). Equal quantities of cells were used to produce the extracts for this experiment, so the Vipp1 levels detected in this experiment can be compared semi-quantitatively.
Figure 5-4 Thylakoid membrane morphology

Thin sections of *Synechococcus* sp. PCC 7002 cells from various strains were examined by transmission electron microscopy. Thylakoid membranes were normally assembled in wild type (WT) (Panel A) and in the *trans*-complemented *vippl* mutant strain (Comp) (Panel C). Thylakoids were greatly reduced in number and area in the *vippl* mutant (Panel B), and these cells closely resembled cells for a *psaAB* mutant (Panel D). The arrows in the lower right portion
of Panel B show thylakoids that clear appear to be directly connected to the cytoplasmic membrane. Bars indicate 500 nm.
Figure 5-5 Oxygen evolution and respiration rates
Oxygen evolution and respiration rates for wild type (WT), the trans-complemented vipp1 mutant strain (Comp), psaAB mutant (ApsaAB) and the vipp1 mutant (Avipp1) were measured based on equal cell numbers (based on OD$_{730\text{ nm}}$) (A) or equal Chl a (B). The vipp1 mutant strain as well as the psaAB mutant strain had much higher oxygen evolution rates than the WT strain when rates were compared on the basis of Chl, but much lower oxygen evolution rates when rates were compared on the basis of equal cell numbers. Note that these values were derived from the initial rates of oxygen evolution for the vipp1 and the psaAB mutant strains, because oxygen evolution rates rapidly declined for these two strains that had no PS I activity to drive the re-oxidation of the plastoquinone pool.
Fluorescence emission spectra were measured in whole cells of wild type (WT), the trans-complemented \textit{vipp1} mutant strain (Comp), and the \textit{vipp1} mutant strain (\textit{Δvipp1}). In the \textit{Δvipp1} mutant, PS I fluorescence emission at \(\sim 715\) nm was completely absent, but PS II was still assembled and exhibited normal fluorescence emission at 685 and 695 nm. The excitation wavelength was 440 nm.

**Figure 5-6 Low temperature (77K) fluorescence emission spectra**
Figure 5-7 Photobleaching of P700

Photobleaching of P700 were measured in whole cells of wild type (WT), the trans-complemented vipp1 mutant strain (Comp), and the mutant (Δvipp1). The PS I activity in the trans-complemented vipp1 mutant strain was almost the same as the WT. No P700 photobleaching activity was detected in the vipp1 mutant strain. The actinic light was turned on at 0 s and turned off after 10 seconds, and absorption difference was measured at 700 nm.
Antibodies to PsaA, PsaB, PsaC, PsaD, PsaE and PsaL were used to detect the presence of the PS I subunits in whole-cell extracts of wild type (WT), trans-complemented *vipp1* mutant strain (Comp) and *vipp1* mutant (*Δvipp1*). PsaL was the only PS I subunit detected in the *vipp1* mutant strain, but all subunits were detected in the WT control and Comp strains. Note: these immunoblots were performed with whole-cell extracts of cells scraped from plates; thus, the results are only qualitative and should not be compared quantitatively.
Figure 5-9 Microscopy images

Confocal fluorescence microscopy images (left) and differential interference contrast (DIC) images (right) of *Synechococcus* sp. PCC 7002 cells stained with Nile red. For the fluorescence images, the excitation wavelength was 488 nm and emitted light in the wavelength range from 500 to 600 nm was detected. The size bars indicate 5 µm. **A.** Cells of wild-type *Synechococcus* sp. PCC 7002. **B.** Cells of the *vipp1* mutant strain. **C.** Trans-complemented cells of the *vipp1* mutant strain. **D.** Cells of the *psaAB* mutant strain.
Figure 5-10 Relative transcript abundances

Scatter plot comparing the relative transcript abundances for mRNAs of the *vipp1* mutant to those in the *trans*-complemented *vipp1* mutant strain, details of the analysis were described in the Materials and Methods part (section 5.3.10). Transcripts for *psaA* and *psaB* were specifically higher in the *trans*-complemented *vipp1* strain, which also had enhanced mRNA levels for the *vipp1* (sll0617) gene from *Synechocystis* sp. PCC 6803. Transcript levels for *chlL* were also more abundant in the *trans*-complemented strain, but nearly all other transcripts were unchanged. The grey lines indicate 2-fold changes in transcript levels.
Figure 5-11 YFP (Yellow Fluorescent Protein) signal detection

Construction (A) and verification (B) of insertion of $P_{psaAB}$ or $P_{chlLN}$ promoters fused to yfp into a neutral site in the chromosome of *Synechococcus* sp. PCC 7002 strains, and relative YFP fluorescence emission in the resulting strains (C). **A.** Scheme showing the replacement of open reading frame SynPCC7002_A2746 by yfp reporter gene fusions by homologous recombination and selection with kanamycin ($aphII$ gene cassette confers resistance to kanamycin). Promoter regions (Pro.) for $psaAB$ or $chlLN$ were transcriptionally fused to the yfp gene, which was placed upstream from an $aphII$ gene, which encodes aminoglycoside phosphotransferase II and confers resistance to kanamycin. **B.** Agarose gel electrophoresis of PCR amplicons using primer set A2746upF and A2746downR to verify the introduction of the promoter-yfp-aphII constructions in the respective strains. Template DNAs came from the wild-type strain (WT), the vipp1 mutant,
and the trans-complemented vipp1 mutant strain (Comp). C. Fluorescence emission spectra showing YFP emission in the constructed reporter strains. The control spectrum shows fluorescence emission from the vipp1 strain, which does not contain the yfp gene. Very similar levels of YFP fluorescence emission were detected in the vipp1 and Comp strains, which carried the yfp gene fused to the P_{psaAB} promoter. These data indicate that Vipp1 does not modify transcription from P_{psaAB} promoter.
Table 5-1 Primers used in this chapter

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Table 5-2 Pigment content of *Synechococcus* sp. PCC 7002 strains

Pigments were measured in wild type (WT), *trans*-complemented *vipp1* mutant strain (Comp), the *psaAB* mutant (*ΔpsaAB*), and the *vipp1* mutant (*Δvipp1*). The data shown are averages and standard deviations for three biological replicates.

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<td>Carotenoid</td>
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Chapter 6  Discussion

6.1 Concluding remarks

Cyanobacteria, algae and plants are oxygenic photosynthetic organisms that are projected to play a pivotal role in supplying future renewable energy needs, and thus our ability to understand and utilize these organisms efficiently is extremely important. This capability is directly dependent on fundamental studies to understand key metabolic interactions and regulatory processes in these organisms. As such, the identification of a complete, non-canonical, TCA cycle (via the 2-OGDC/SSADH bypass) in most cyanobacteria from this thesis work not only corrects a long-held misconception that these organisms have an incomplete TCA cycle due to the absence of 2-OGDH, but also provides much useful knowledge illustrating the occurrence and physiological functions of the TCA variants in these bacteria.

It has been suggested that one possible function of the TCA cycle variant is associated with responses to low oxygen conditions (Green et al., 2000; Tian et al., 2005). Accordingly, one example of the plasticity of the TCA cycle in response to a low oxygen environment was provided by the metabolic responses to waterlogging in the roots of Lotus japonicas, which led to the modular operation of a split TCA cycle (Rocha et al., 2010). Another example of the physiologically diverse uses of the TCA cycle is provided by Pseudomonas fluorescens, which is able to generate crucial metabolites such as oxalate, ATP and NADPH, and is able to diminish the synthesis of NADH and CO₂ evolution under aluminum stress (Singh et al., 2009). Under aluminum-stress conditions, expression levels of isocitrate lyase and acylating glyoxylate dehydrogenase are upregulated, leading to enhanced synthesis of oxalate and NADPH.
Furthermore, activities of succinyl-CoA synthetase and oxalate CoA-transferase also increase, which provide an effective route for ATP synthesis. These modifications of the TCA cycle appear to be of crucial significance in the adaptation to oxidative stress owing to production of increased amounts of the antioxidant NADPH and decreased amounts of NADH (Mailloux et al., 2007).

Furthermore, the biochemical and metabolic characterizations of glyoxylate cycle as well as the GABA shunt in this thesis work also clarified the mystery of the occurrence of these pathways in cyanobacteria. The glyoxylate cycle is demonstrated to play an important role in the assimilation of acetyl-CoA and is possibly involved in the balance between nitrogen and carbon metabolism. Compared to the classical TCA cycle, one notable difference is that the GABA shunt and the 2-OGDC/SSADH bypass do not produce guanosine triphosphate by substrate-level phosphorylation. Another important point is that the replacement enzymes in these variants produce NADPH rather than NADH, which implies that the bypass reactions might be regulated by sensing the NADPH:NADP⁺ ratio. Additionally, 2-oxoglutarate has also been shown to be involved in the detoxification of reactive oxygen species (Mailloux et al., 2007). These results all indicate that the newly discovered TCA cycle shunt and the GABA shunt might play important roles in responding to the changes of carbon sources and oxygen levels in the environment, possibly by controlling the concentrations of 2-oxoglutarate and NADPH inside the cell.

Besides, these discoveries should also help to understand carbon–nitrogen balance better in algae and plants. The vast amounts of rapidly accumulating genome sequence data from prokaryotic and eukaryotic sources will continue to provide opportunities to study the molecular evolution of individual enzymes as well as of complete pathways with multiple enzymatic reactions. Given the subcellular compartmentation inherent in eukaryotes, studies of the transport processes linking organellar metabolic processes to their interactions with nuclear-encoded genes are also of high interest. Additionally, it will be worthwhile to investigate the metabolic rationale for the acquisition and incorporation of new metabolic pathway genes/enzymes during evolution,
because this knowledge could help those attempting to maximize photosynthetic efficiency in engineered cyanobacteria and photosynthetic eukaryotes. Considering the mosaic distribution of TCA cycle, glyoxylate cycle as well as the GABA shunt, in cyanobacteria, algae and plants, it will also be interesting to attempt to define the evolutionary events that produced this diversity in future research.

Additionally, these discoveries not only provide interesting insights into metabolic and pathway evolution and but also provide new opportunities for biotechnological applications through metabolic engineering by utilizing the newly discovered metabolite intermediates. Indeed, cyanobacteria have been used to produce many useful biomaterials derived from important metabolites inside cells (Ducat et al., 2011; Machado and Atsumi, 2012). This thesis study also explored and demonstrated the production of P3HB-co-4HB in cyanobacteria that could be derived from succinic semialdehyde, an important new metabolic intermediate of the recently discovered TCA cycle variant in cyanobacteria (Zhang and Bryant, 2011). These initial attempts point to potential for future metabolic engineering in cyanobacteria based on newly identified metabolites. Furthermore, in order to take full advantage of cyanobacteria for the production of green biomaterials, it is worthwhile to study the conversion of light to chemical energy by the process of photosynthesis that produces the reducing power (e.g., NADPH) and chemical energy (e.g., ATP), which are required for carbon dioxide fixation and many other cellular metabolisms. However, the underlying molecular mechanisms for assembly or stability of PS I are still not clear, despite the fact that several cofactors have been found to be involved in the incorporation or biogenesis of PS I and thylakoid membrane (Bartsevich and Pakrasi, 1997; Boudreau et al., 1997; Naver et al., 2001). As such, this thesis work also functionally characterized the role of vipp1 in cyanobacteria, demonstrating that vipp1 is involved in the biogenesis of PS I but not thylakoid membrane biogenesis directly, thus clarifying another long misunderstanding. The illustration of functions of vipp1 gene in thylakoid membrane biosynthesis
and photosynthetic protein assembly may also help us to explore new approaches in metabolic engineering cyanobacterial strains for biofuel applications with high efficiency in conversion of sunlight into chemical energy through photosynthesis.

Overall, it is possible that the ultimate dream of industrialized photo-bioreactors for green energy production could be achieved in cyanobacteria in the near future, through the combination of maximizing both the metabolic engineering capacities and photosynthesis efficiencies. During the past several decades, well-established metabolic engineering strategies in *E. coli* have enabled us to produce many different biomaterials, ranging from small molecules to complicated natural products and related pharmaceutical analogs. However, considering the facts that cyanobacteria have many advantages such as their low cost for growth and well-established genetic tools for mutagenesis and protein overexpression, one should envisage a new era to produce a wide range of biomaterials from metabolically engineered cyanobacteria. My thesis work toward this goal not only demonstrates how newly discovered metabolic pathways in cyanobacteria could facilitate applications of these bacteria for the production of specific products, but also provides a good example of how to study the metabolic diversity and thus applications in other bacteria in the future. Thus, it is certainly the case that future investigations of the metabolic diversities as well as photosynthesis mechanisms in cyanobacteria will not only enrich our knowledge but also provide metabolic engineering approaches to make cyanobacteria more powerful platforms for providing sustainable green energy to meet the exponentially increasing demands of mankind.

6.2 References


Appendix A: Inactivation of nitrate reductase increases NAD(P)H availability for hydrogen production in *Synechococcus* sp. PCC 7002

Anticipated publication:

Introduction

Cyanobacteria are naturally capable of utilizing solar energy to reduce inorganic carbon (CO$_2$, HCO$_3^-$, and CO$_3^{2-}$) and produce biomass. The resulting biomass can be converted into biofuels in the form of hydrogen, biodiesel or ethanol through biological, physical or chemical processes (Dismukes et al., 2008; Parmar et al., 2011). Like many other cyanobacteria, *Synechococcus* sp. PCC 7002 harbors a pentameric Hox [NiFe] hydrogenase, which catalyzes reversible hydrogen (H$_2$) production using NAD(P)H as the electron source (Aubert-Jousset et al., 2011; Carrieri et al., 2011; Cournac et al., 2004; Ghirardi et al., 2007). All these features make *Synechococcus* sp. PCC 7002 a good cyanobacterial model for studying the parameters that affect biohydrogen production.

The cyanobacterial Hox hydrogenase is rapidly inactivated by oxygen at atmospheric concentrations (Cournac et al., 2004), but is reversibly reactivated by NADH and NADPH under anoxic conditions. When oxygen is absent, the production rate and yield of H$_2$ by the Hox hydrogenase are heavily dependent on the intracellular level of reduced NAD(P)H (Ananyev et al., 2012). Multiple genetic engineering strategies have been applied to increase the NAD(P)H availability to produce a higher H$_2$ yield in *Synechococcus* sp. PCC 7002. Overexpression and knockout strains for the glyceraldehyde 3-phosphate dehydrogenase (*gap1*) gene had 3-fold and 2.3-fold higher H$_2$ yields, respectively (Kumaraswamy et al., 2013). A strain that overexpressed *gap1* had an increased overall glycolysis rate, and therefore produced more NADH. The *gap1* knockout mutant rerouted more carbon flux into the OPP pathway, which has a higher reductant equivalent yield per glucose consumed than glycolysis. By knocking out the *ldhA* gene, which encodes D-lactate dehydrogenase, an enzyme that competes with the Hox hydrogenase for NADH, a 5-fold increased H$_2$ was observed (McNeely et al., 2010).
During autofermentation, nitrate reduction is another important pathway competing with the Hox hydrogenase for reductant in *Synechococcus* sp. PCC 7002. Once nitrate is transported into the cell by nitrate permease (NrtP), nitrate reductase (encoded by *narB*) reduces nitrate into nitrite at the cost of two electrons per nitrate. Nitrite will either be further reduced to ammonia by nitrite reductase (encoded by *nirA*) at the cost of six electrons per nitrite or excreted out of the cell (Flores et al., 2005; Sakamoto and Bryant, 1997). Therefore, elimination of nitrate reduction activity would potentially improve the dark H$_2$ yield in *Synechococcus* sp. PCC 7002 in the presence of nitrate. Previously, a *narB* knockout mutant of the cyanobacterium, *Synechocystis* sp. PCC 6803 was reported to show similar or lower H$_2$-evolving capacity compared to WT in the presence of nitrate (Baebprasert et al., 2011). In this study, we aimed to generate a knockout mutant of *narB* in *Synechococcus* sp. PCC 7002, and we studied its autofermentative metabolism in the presence or absence of nitrate, while accounting for the glycogen catabolic rate, catabolic pathways involved, H$_2$ yield, redox-poise, organic acids and CO$_2$ excretion.

**Results and discussion**

Wild-type *Synechococcus* sp. PCC 7002 was first adapted to medium A containing 5 mM urea but no nitrate (Stevens et al., 1973), and then the corresponding *narB* coding sequence (SynPCC7002_A1314) was deleted using a homologous recombination strategy (Figure A1) and replaced with a DNA fragment encoding the aminoglycoside phosphotransferase II (*aphII*), which confers kanamycin resistance. Transformation and selection were performed as previously described (Frigaard et al., 2004). Complete segregation of the *narB* and *narB::aphII* alleles was verified by PCR with template DNAs derived from the wild type and transformant strains (Figure A1). The primers used are listed in Table A1. The *ΔnarB* mutant was also phenotypically confirmed by its inability to grow photoautotrophically when nitrate was the only nitrogen source. The *ΔnarB* strain was maintained in liquid medium supplemented with 100 µg/ml kanamycin.
Intracellular metabolite extraction and hydrogen production analysis of the ΔnarB as well as the WT strain under different growth conditions were performed by our collaborators in Dr. Charles Dismukes’s group at Rutgers University.

Due to absence of nitrate reductase, the ΔnarB mutant was able to take up nitrate but unable to reduce nitrate to nitrite or further to ammonia. The ΔnarB mutant eliminated the major electron-competing enzyme in presence of nitrate, leaving more electrons available for fermentative products, such as H₂ and D-lactate. In the absence of nitrate, the ΔnarB mutant produced similar amount of H₂ as the WT (Figure A2). When the fermentation was conducted in the presence of nitrate, the ΔnarB mutant produced 14 ± 3 mol H₂/10¹⁷ cells, while WT fermented in the presence of nitrate produced only 2 ± 0.5 mol H₂/10¹⁷ cells. In the absence of nitrate, WT produced 15 ± 3 mol H₂/10¹⁷ cells, and the ΔnarB produced 12 ± 2 mol H₂/10¹⁷ cells. Comparatively, the ΔnarB mutant yielded 6-fold more H₂ than WT when fermented in the presence of nitrate.

These results clearly demonstrate that elimination of the narB-encoded nitrate reductase in Synechococcus sp. PCC 7002 resulted in elevated fermentative H₂ yields in the presence of nitrate. Nonetheless, H₂ production only accounted for a fraction of the total reductant pool in the ΔnarB strain. To reroute reducing equivalents further and effectively to the bidirectional NiFe-hydrogenase and to produce more H₂, the narB mutation could be combined with other mutations that are known to enhance H₂ yields during fermentation (e.g., gap1, ldhA).

References


Kumaraswamy, G.K., Guerra, T., Qian, X., Zhang, S., Bryant, D.A., and Dismukes, G.C. (2013). Reprogramming the glycolytic pathway for increased hydrogen production in cyanobacteria:
metabolic engineering of NAD$^+$-dependent GAPDH. Energy & Environmental Science 6, 3722-3731.


Figure A1 Generation and verification of narB mutant

A: Schematic representation of the homologous recombination methods. The upstream and downstream flanking regions for the gene of interest (narB) were amplified by PCR, using primer pairs P1-P2 and P3P; and these fragments were ligated to the antibiotic cassette (aphII). The resulting construction was transformed into Synechococcus sp. PCC 7002 to generate fully segregated deletion strain. B: The ΔnarB deletion mutant was verified by PCR amplification with primers P1 and P4 (Fig. 1), using template DNA derived from WT or transformed cells. M: DNA ladder. Lane WT, contained template DNA from the WT strain. Lane narB contained template DNA from antibiotic-resistant strain in which narB had been replaced by aphII as described above. Size difference indicates the deletion of narB.
Figure A2 H$_2$ evolution of wild type and ΔnarB strains

H$_2$ evolutions were measured in the WT and ΔnarB strains of *Synechococcus* sp. PCC 7002 after 2 days of autofermentation. “+NO3” or “-NO3” indicates presence and absence of nitrate respectively in the fermentative medium.
Table A1: Primers used in this study

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Appendix B: Construction and purification of [His]$_6$-tagged Photosystem I 
from *Synechococcus* sp. PCC 7002

**Introduction**

In order to fulfill the increasing demand for sustainable energy, the direct conversion of solar energy to a liquid or gaseous fuel has been accomplished by using metabolically engineered photoautotrophic organisms, such as cyanobacteria and algae (Angermayr et al., 2009; Atsumi et al., 2009; Berla et al., 2013; Chu and Majumdar, 2012; Dismukes et al., 2008; Fairley, 2011). However, these strategies to produce biofuels (e.g. ethanol, hydrogen) are inextricably linked to complicated biochemical pathways within the cell and the efficiency in utilizing these pathways still need more investigations (Kumaraswamy et al., 2013; Xu et al., 2013). Because of these challenges, direct usage of purified photosynthetic components (e.g. PS I) for the generation of solar fuels is considered to be a more direct and efficient route (Iwuchukwu et al., 2010).

Several approaches have been reported for direct solar H$_2$ production using PS I ((Lubner et al., 2009)). One strategy was using noble metals (Pt and Au) or Pt nanoparticles to accept electrons from PS I and synthesize H$_2$ (Grimme et al., 2008; Millsaps et al., 2001). In another system, the membrane bound [NiFe]-H$_2$ase (MBH) from *Ralstonia eutropha* was fused to the PsaE subunit of PS I to generate light-induced H$_2$ (Ihara et al., 2006). H$_2$ production rate was found to be orders of magnitude higher when the PS I-MBH fusion protein is bound to an electrode (Krassen et al., 2009). Recently, a PS I (from *Synechococcus* sp. PCC 7002)-HydA (from *Clostridium acetobutylicum*) nanoconstruct capable of light driven hydrogen evolution was also devised, in which 1,6-hexanedithiol was used as the linker between these two protein complex (Lubner et al., 2011).
All these approaches will need large amounts of purified PS I complexes. PS I complexes are usually purified by a combination of ultracentrifugation with sucrose density gradient and/or multiple ion-exchange column chromatography steps, which are very time consuming. In addition to this, purified PS I monomers are always contaminated with PS II complexes, making it even more inconvenient if only PS I monomers are needed. Furthermore, it was demonstrated that anchoring PS I monomers on the surface of an electrode could greatly improve the electron transfer efficiency between the hybrid complex (Krassen et al., 2009). Thus, it would be ideal to have the purified PS I complex ‘tagged’ for future attachment onto the electrode surface.

In this study, we aimed to construct a genetically modified *Synechococcus* sp. PCC 7002 strain, expressing the [His]_{10}-tagged PS I complex. More specifically, ten histidines will be introduced to the C-terminus of PsaJ, one of the trans-membrane subunits of PS I. By constructing this mutant strain, modified PS I complexes can be purified without contamination from PS II by the Ni^{2+} affinity column chromatography. This [His]_{10}-tagged PS I can also be readily attached to the supporting electrode surface (e.g. Ni-NTA terminated gold surface) to accept electrons more efficiently for the production of hydrogen.

**Results and discussions**

In order to generate [His]_{10}-tagged PsaJ and thus the [His]_{10}-tagged PS I in *Synechococcus* sp. PCC 7002, the *psaJ* gene (SYNPCC7002_A1009) in the wild type was first deleted and replaced by a DNA fragment encoding an antibiotic resistance gene (*erm*, erythromycin resistance) by using homologous recombination method as described (Frigaard et al., 2004). Primers used in the homologous recombination method are listed in Table B1. The generated fully segregated *psaJ* mutant was further verified by polymerase chain reaction (PCR) using primer set psaJ1 and psaJ4 (Table B1), as shown in Figure B1. The *psaJ* gene was also amplified by PCR with Phusion DNA polymerase (New England Biolabs) using primer set psaJExF and psaJExR, and cloned into
plasmid pAQ1Ex-\(P_{\text{cpcBA}}\) (Xu et al., 2011). The generated plasmid pAQ1Ex-\(P_{\text{cpcBA}::\text{psaJ}}\) was then transformed into the \(\text{psaJ}\) mutant to produce strains overproducing the \([\text{His}]_{10}\)-tagged PsaJ and successful incorporation of the modified \(\text{psaJ}\) gene into the mutant strain was further verified by PCR using primer set \(\text{psaJExF}\) and \(\text{psaJExR}\), as shown in Figure B2.

In order to purify the \([\text{His}]_{10}\)-tagged PS I complex, thylakoid membranes from the overexpression strain were first prepared as previously described (Shen et al., 2002). The thylakoid membranes were solubilized with 1.0% (w/v) \(n\)-dodecyl \(\beta\)-D-maltoside (DM) at a concentration of 1 mg/mL Chl in buffer A (50 mM HEPES-NaOH, pH 7.8, 10 mM \(\text{MgCl}_2\), 5 mM \(\text{CaCl}_2\), 25% (w/v) glycerol) at 4 °C for 40 min, with gentle stirring, after which the sample was centrifuged at 22,000 g for 10 min. The resulting supernatant was then loaded on column containing \(\text{Ni}^{2+}\)-NTA affinity resin (Goldbio), which was pre-equilibrated with buffer A containing 0.04% DM and 5 mM imidazole. The column was subsequently washed with one volume buffer A containing 0.04% DM and 5 mM imidazole, and then with buffer A containing 0.04% DM. PS I bound to the column was eluted with buffer A containing 0.04% DM and 100 mM imidazole. The obtained PS I complexes were dialyzed, concentrated and further separated into monomers and trimers by ultracentrifugation at 140,000 g for 14 h at 4°C on sucrose gradient (5% to 30%).

Separation of the purified PS I monomers and trimers on a sucrose gradient is shown in Figure B3. This result demonstrated that the purified PS I monomers had formed a well-separated band on the gradient, and no PS II complexes were associated with the PS I monomers. However, the obtained PS I monomer fraction had a much lower yield compared with the trimers, with a ratio of 1:40. PsaL was previously shown to be important for the trimerization of PS I complexes (Chitnis and Chitnis, 1993), and thus deletion of \(\text{psaL}\) should only result in PS I monomers. To increase the yield of PS I monomers further, the \(\text{psaL}\) gene (SYNPCC7002_A2620) was also deleted in the overexpression strain by using the homologous recombination methods previously
described (see above). The PS I complexes were purified and the resulting sucrose gradient is shown in Figure B3. The results clearly show that the PS I trimers were not formed and only PS I monomers were obtained, and this strain thus could be used to express large amounts of [His]_{10}-tagged PS I monomers. Overall, this PsaL-less, overexpression strain producing [His]_{10}-tagged PsaJ will facilitate the purification of PS I monomers for future applications in constructing hybrid complexes for the production of hydrogen.

References:


Figure B1 Generation and verification of \textit{psaJ} mutant

\textbf{A}: Schematic representation of the homologous recombination methods. The upstream and downstream flanking regions for the \textit{psaJ} were amplified by PCR, using primer pairs \textit{psaJ1}-\textit{psaJ2} and \textit{psaJ3}-\textit{psaJ4}; and these fragments were ligated to the antibiotic cassette (\textit{erm}). The resulting construction was transformed into \textit{Synechococcus} sp. PCC 7002 to generate fully segregated deletion strain. \textbf{B}: The \textit{psaJ} deletion mutant was verified by PCR amplification with primers \textit{psaJ1} and \textit{psaJ4}, using template DNA derived from WT or \textit{psaJ} mutant cells. M: DNA ladder. Lane WT, contained template DNA from the WT strain. Lane \textit{psaJ} contained template DNA from antibiotic-resistant strain in which \textit{psaJ} had been replaced by \textit{erm} as described above. Size difference indicates the deletion of \textit{psaJ}. 
Figure B2 Verification of the PsaJ overexpression strain and psaL mutant

A: The *psaJ* over-expression strain was verified by PCR amplification with primers psaJExF and psaJExR, using template DNA derived from WT or *psaJ* overexpression cells. M: DNA ladder; psaJ: *psaJ* mutant strain; WT: wild type *Synechococcus* sp. PCC 7002; psaJEx: PsaJ overexpression strain. 

B: The *psaL* mutant was generated using the same homologous recombination method as illustrated in Figure B1A, using primer set psaL1-psaL2 and psaL3-psaL4. The *psaL* deletion mutant was verified by PCR amplification with primers psaL1 and psaL4, using template DNA derived from WT or mutant cells. M: DNA ladder; WT: wild type *Synechococcus* sp. PCC 7002; psaL: *psaL* deletion strain. Size difference indicates the deletion of *psaL*. 
Figure B3 Sucrose gradient of PS I complex

Sucrose gradients showing the purified PS I complex from the PsaJ overexpression strain (A) and the PsaJ overexpression strain with psaL deleted (B). Two bands representing PS I monomers and trimers were observed in the PsaJ overexpression strain. No trimer fraction of PS I complexes but only a PS I monomer fraction was observed for PS I from the PsaJ overexpression strain when psaL was deleted.
### Table B1 Primers used in this study

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Appendix C: Publications
Closely related genes occur in the genomes of some methanogens and other anaerobic bacteria, discussed as a contributing factor to explain why cyanobacterial genomes except those of 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. These genes are present in all semialdehyde dehydrogenase were identified in the cyanobacterium because they lack 2-oxoglutarate dehydrogenase and thus cannot convert 2-oxoglutarate to succinyl-CoA, in various cyanobacteria (1). This observation, as well as the fact that mutants of Synechocystis sp. PCC 6803 [e.g., (8)] and SynPCC7002_A2771 were separately expressed in Escherichia coli, and the resulting soluble proteins were purified (16). When the product of SynPCC7002_A2771 was incubated with SSA, succinate was produced and nicotinamide adenine dinucleotide phosphate (NADP+) was reduced (Fig. 1A). When the products from SynPCC7002_A2770 and SynPCC7002_A2771 were incubated with 2-OG, 2-OG was quantitatively converted to succinate (Fig. 1B). These observations establish that SynPCC7002_A2770 and SynPCC7002_A2771 encode an enzyme similar to, but phylogenetically distinct from, acetolactate synthase (IVb). Because acetolactate synthase is a TPP-dependent enzyme, like 2-OGDH and 2-OGDC (14), it seemed possible that these two open reading frames might encode the enzymes replacing 2-OGDH. An examination of transcription data for these genes under many different growth conditions (15) strengthened this hypothesis, because the transcript levels of these two genes varied coordinately with those for other genes encoding enzymes of the TCA cycle.

The Tricarboxylic Acid Cycle in Cyanobacteria

Shuyi Zhang1 and Donald A. Bryant1,2,∗

It is generally accepted that cyanobacteria have an incomplete tricarboxylic acid (TCA) cycle because they lack 2-oxoglutarate dehydrogenase and thus cannot convert 2-oxoglutarate to succinyl–coenzyme A (CoA). Genes encoding a novel 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase were identified in the cyanobacterium Synechococcus sp. 7002. Together, these two enzymes convert 2-oxoglutarate to succinate and thus functionally replace 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. These genes are present in all cyanobacterial genomes except those of Prochlorococcus and marine Synechococcus species. Closely related genes occur in the genomes of some methanogens and other anaerobic bacteria, which are also thought to have incomplete TCA cycles.

The tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle, has two functions in bacteria: It oxidizes two-carbon units derived from acetyl-coenzyme A (CoA) producing carbon dioxide and the reduced form of nicotinamide adenine dinucleotide (NADH), which provides the electrons for oxidative phosphorylation, and it provides essential precursor metabolites [e.g., oxaloacetate, 2-oxoglutarate (2-OG), and in some species succinate] that are required for biosynthesis of cellular components. In 1967 two groups reported the failure to detect 2-oxoglutarate dehydrogenase (2-OGDH), which converts 2-oxoglutarate to succinyl-CoA, in various cyanobacteria (1, 2). During the ensuing 44 years, it has become common knowledge that these organisms have an incomplete TCA cycle [see, e.g., comments in (3, 4)]. The absence of 2-OGDH has also frequently been discussed as a contributing factor to explain why most cyanobacteria are obligate photolithoautotrophs (5–7). Consistent with these initial observations, no fully sequenced cyanobacterial genome encodes the genes for 2-OGDH (7). The incomplete TCA cycle has recently been incorporated into metabolic models for Synechocystis sp. PCC 6803 [e.g., (8–10)].

Alternatives to 2-oxoglutarate dehydrogenase are known to participate in the TCA cycles of a few organisms, including those of Euglena gracilis mitochondria (11) and Mycobacterium spp. (12). In the latter organism, a thiamine pyrophosphate (TPP)-dependent enzyme, 2-oxoglutarate decarboxylase (2-OGDC), is structurally related to the large subunit of 2-OGDH and produces succinic semialdehyde (SSA). SSA is subsequently oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH). Although cyanobacteria lack homologs of this type of 2-OGDC, Synechococcus sp. PCC 7002 and many other cyanobacteria encode homologs of SSADH. This observation, as well as the fact that mutants of Synechocystis sp. PCC 6803 lacking succinate dehydrogenase still synthesize succinate (13), strongly suggested that cyanobacterial genomes encode a previously unrecognized 2-OGDC. A gene neighborhood analysis revealed that the gene encoding SSADH (SynPCC7002_A2771) occurred as part of an apparent operon comprising two genes in most cyanobacteria, in which one of the genes (SynPCC7002_A2770) encodes an enzyme similar to, but phylogenetically distinct from, acetolactate synthase (IVb).

Peptides from SynPCC7002_A2770 and SynPCC7002_A2771 were detected in the soluble proteome of Synechococcus sp. PCC 7002 (17). To investigate the function of these two
enzymes in *Synechococcus* sp. PCC 7002, we constructed deletion mutants lacking either SynPCC7002_A2770 or SynPCC7002_A2771 (16). A deletion mutant for SynPCC7002_A1094, encoding the SdhB iron-sulfur subunit of succinate dehydrogenase, was also constructed. This *sdhB* mutant strain was transformed with plasmids designed to overexpress the genes for 2-OGDC or SSADH (16). Figure 2A shows the results of enzymatic assays for SSADH activity. SSA oxidation activity was present in whole-cell extracts of wild-type cells, but no activity was detected in the SynPCC7002_A2771 deletion mutant. The coupled assay for the conversion of 2-OG to succinate in the presence of NADPH was used to demonstrate the activity of 2-OGDC. Whole-cell extracts of wild-type cells exhibited this activity, but no activity was detected in extracts prepared from either of the deletion mutants (Fig. 2B). These data establish that both 2-OGDC and SSADH are present and active in wild-type *Synechococcus* sp. PCC 7002 cells grown under standard photoautotrophic conditions.

To assess the impact of the deletion mutations on growth, we grew cells under constant light or a 12-hour light:12-hour dark regime. During constant illumination, mutants lacking 2-OGDC and SSADH grew at a slower rate than the wild type (Fig. 3 and fig. S4); similar results were obtained when cells were grown on a light-dark cycle (fig. S4B). Relative to the wild type (doubling time = 4.05 ± 0.3 hours), the growth rate of the mutant lacking SSADH was about 17% slower (doubling time = 4.77 ± 0.13 hours), whereas that of the mutant lacking 2-OGDC was about 30% slower (doubling time = 5.23 ± 0.15 hours). A growth rate difference of 30% implies that, starting from equal cell numbers and allowing for 30 generations for the wild type (~120 hours), the mutant cells would represent only ~0.5% of the population. The *sdhB* mutant had a growth rate that was indistinguishable from that of the wild type (doubling time = 4.09 ± 0.25 hours). This result is similar to that obtained with a *Synechocystis* sp. PCC 6803 mutant lacking succinate dehydrogenase, which also grew photoautotrophically with a doubling time similar to that of the wild type (13).

Strains overproducing SynPCC7002_A2770 and SynPCC7002_A2771 in the *sdhB* mutant background were also studied in constant light (Fig. 3). The largest effect was observed for a mutant overproducing 2-OGDC, which grew much slower than the wild-type strain (doubling time = 9 hours). Similar results were obtained when these strains were grown on a light-dark cycle (fig. S4B). However, the overproduction strains were unstable, and evidence for more rapidly growing suppressor mutants was readily observed when the overproduction strains were streaked on plates. Because the variants grew faster, the growth rates reported here probably underestimate the effects of overexpressing these genes. 2-OG is one of the most important metabolites in cyanobacterial cells and is important for ammonia assimilation, for regulation of nitrogen and carbon metabolism, and as a precursor metabolite for heme and chlorophyll biosynthesis (18, 19). Thus, it was not surprising that
overproduction of an enzyme that can lower the intracellular levels of this critical metabolite had a severely negative impact on cell growth rate. It is also possible that SSA could accumulate and cause some toxicity effects in this mutant. Overproduction of SSADH had a noticeable but much smaller effect on cell growth (Fig. 3). In this case, SSA production presumably is still restricted by the amount of 2-OGDC in cells.

Collectively, these data demonstrate that *Synechococcus* sp. PCC 7002 has two enzymes that replace the activities of 2-OGDH and succinyl-CoA synthetase in the TCA cycle. The replacement enzymes produce NADPH (the reduced form of NADP⁺) rather than NADH and do not synthesize guanosine triphosphate by substrate-level phosphorylation (Fig. 1D). Although mutants lacking 2-OGDH and SSADH grew well under laboratory conditions as pure cultures, they would not survive for long in competition with the wild type. Nonetheless, our observations are consistent with the ability of cyanobacteria to produce energy phototrophically rather than by respiration (except during periods of darkness), and suggest that the primary function of the TCA cycle is to produce precursor metabolites for growth. Database searches showed that homologs of *Synechococcus* sp. PCC 7002 and *Synechococcus* sp. PCC 7002 occur in all cyanobacterial genomes except those of *Prochlorococcus* and marine *Synechococcus* spp., and also occur in many other bacteria, including some anaerobes (e.g., *Methanosarcina* and *Clostridium* spp.) that have been reported to have incomplete TCA cycles due to the absence of 2-OGDH.

The data presented here correct a misconception about the completeness of the cyanobacterial TCA cycle that has persisted for more than four decades. This study illustrates how the misinterpretation of negative results, whether the misannotation of a gene (20) or the failure to detect an enzyme by assay (1, 2), can have a powerful, long-lasting impact on a field. References and Notes


## Host Proteasomal Degradation Generates Amino Acids Essential for Intracellular Bacterial Growth

Christopher T. D. Price, Tasneem Al-Quadan, Marina Santic, Ilan Rosenshine, Yousef Abu Kwaik

*Legionella pneumophila* proliferates in environmental amoeba and human cells within the *Legionella*-containing vacuole (LCV). The exported AnkB F-box effector of *L. pneumophila* is anchored into the LCV membrane by host-mediated farnesylation. Here, we report that host proteasomal degradation of Lys⁴⁸-linked polyubiquitinated proteins, assembled on the LCV by AnkB, generates amino acids required for intracellular bacterial proliferation. The severe defect of the *ankB* null mutant in proliferation within amoeba and human cells is rescued by supplementation of a mixture of amino acids or cysteine, serine, pyruvate, or citrate, similar to rescue by genetic complementation. Defect of the *ankB* mutant in intrapulmonary proliferation in mice is rescued upon injection of a mixture of amino acids or cysteine. Therefore, *Legionella* promotes eukaryotic proteasomal degradation to generate amino acids needed as carbon and energy sources for bacterial proliferation within evolutionarily distant hosts.

Although bacterial acquisition of nutrients in vivo is one of the most fundamental aspects and is a prerequisite for bacterial infections (1, 2), it is not known whether intracellular pathogens possess specific virulence strategies to modify cellular processes to obtain sources of carbon, nitrogen, and energy. In the amoeba host and in human alveolar macrophages, the *Legionella pneumophila*-containing vacuole (LCV) is remodeled into a rough endoplasmic reticulum (RER)-derived vacuole that evades lysosomal fusion (3). The Dot/Icm type

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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6062/1551/DC1

Materials and Methods

Figs. S1 to S4

Table S1

References

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*Legionella pneumophila* proliferates in environmental amoeba and human cells within the *Legionella*-containing vacuole (LCV). The exported AnkB F-box effector of *L. pneumophila* is anchored into the LCV membrane by host-mediated farnesylation. Here, we report that host proteasomal degradation of Lys⁴⁸-linked polyubiquitinated proteins, assembled on the LCV by AnkB, generates amino acids required for intracellular bacterial proliferation. The severe defect of the *ankB* null mutant in proliferation within amoeba and human cells is rescued by supplementation of a mixture of amino acids or cysteine, serine, pyruvate, or citrate, similar to rescue by genetic complementation. Defect of the *ankB* mutant in intrapulmonary proliferation in mice is rescued upon injection of a mixture of amino acids or cysteine. Therefore, *Legionella* promotes eukaryotic proteasomal degradation to generate amino acids needed as carbon and energy sources for bacterial proliferation within evolutionarily distant hosts.
Extensive remodeling of a cyanobacterial photosynthetic apparatus in far-red light

Fei Gan,1 Shuyi Zhang,1 Nathan C. Rockwell,2 Shelley S. Martin,2 J. Clark Lagarias,2 Donald A. Bryant1,3*

Cyanobacteria are unique among bacteria in performing oxygenic photosynthesis, and are ecologically important primary producers (1). Marine cyanobacteria, mostly Prochlorococcus, Synechococcus, and Trichodesmium species, account for ≥25% of the net primary productivity in oceans, and terrestrial cyanobacteria also contribute substantially to global photosynthesis (2, 3). The estimated cyanobacterial biomass in terrestrial, endolithic, and freshwater lake ecosystems is ~40% of that in oceans, but this estimate would increase if polar and subarctic soils, topsoils in subhumid climates, and shallow marine and freshwater benthic environments were included (2). Because approximately equal solar irradiance reaches Earth’s surface in the wavelength ranges 600 to 700 nm and 700 to 800 nm (18.4% versus 14.9% of the total irradiance between 400 and 1100 nm), the capacity to use far-red light to perform oxygenic photosynthesis in terrestrial niches could thus have important consequences in natural and engineered systems. Cyanobacteria mainly use three large, multi-subunit complexes to harvest and convert light into stable, energy-rich compounds: photosystem (PS) I, PS II, and phycobilisomes (PBSs) (1, 4–7). Cyanobacteria have evolved many mechanisms to maximize their photosynthetic efficiency in response to the incident irradiation. Examples include adjusting the total cellular chlorophyll (Chl) content and the ratio of PS II to PS I (“intensity adaptation”) (8); state transitions that redistribute light energy transfer from PBSs to PS I and PS II (9, 10); and nonphotochemical quenching by orange carotenoid-binding protein (11). Although cyanobacteria were once thought to have simple pigmentation (Chl α, β-carotene, and phycobiliproteins), it is now recognized that some cyanobacteria synthesize Chls b, d, or f (12), many functionally distinct carotenoids (13), and spectrally diverse phycobiliproteins (7). Limitation for iron and other nutrients causes changes in light-harvesting proteins (14–16).

One of the best-characterized acclimative responses in cyanobacteria, complementary chromatic acclimation (CCA; formerly “adaptation”), was discovered more than 100 years ago. Gaidukov (17, 18) observed that Oscillatoria sancta is redish brown when grown in green light but blue-green when grown in red light, and he correctly surmised that the color differences were due to altered pigment synthesis (fig. S1). CCA results from compositional remodeling of the peripheral rods of PBSs (19, 20) and occurs through transcriptional and posttranscriptional regulation of specific phycobiliprotein genes. Genes encoding phycoerythrin and its associated bilin reductase, bilin lyses, and linker proteins are expressed in cells grown in green light, whereas those for “incubile” phycoerytin and associated proteins are expressed in cells grown in red light (19–22). Central regulatory elements controlling CCA include two response regulators (RcaF and RcaC) and a phytochrome-related sensor histidine kinase, RcaE (19, 20, 23). Because PBSs are primarily, but not exclusively, associated with energy transfer to PS II (9, 10), CCA enhances the overall rate of photosynthesis when the incident irradiation is complementary to cell coloration.

Using a systems-biology approach—including genome sequencing, comparative genomics, transcription profiling (RNA-seq), biochemical and spectroscopic analyses, and proteomics—we show that Leptolyngbya strain JSC-1 (hereafter, JSC-1) specifically remodels its photosynthetic apparatus in response to far-red illumination. These changes are probably controlled by a red/far-red-responsive phytochrome. JSC-1 specifically synthesizes both Chl d and Chl f, in addition to Chl e, when cells are grown with light wavelengths ≥700 nm. Halomicronema hongdechloris, a cyanobacterium isolated from a stromatolite from Shark Bay, Australia, also synthesizes Chl f when cells are grown in far-red light (25, 26). However, JSC-1 additionally undergoes an extensive acclimative response, in which the expression of >40% of the genome changes more than twofold after a shift from white light to far-red light. The resulting changes in gene expression lead to the replacement of most of the core proteins of PS I and PS II and to structural remodeling of PBS core substructures. We have named this global acclimative response “far-red light photosynthesis” (FarLIP), and we show here that FarLIP substantially improves photosynthetic performance in far-red light.

The JSC-1 genome has a 21-gene cluster that encodes paralogs of most of the core subunits of PS I, PS II, and PBSs (fig. S2). Similar clusters occur in 12 other cyanobacteria, including members of all five sections of the taxon Cyanobacteria (27) (fig. S3). All but one of these clusters contain six genes encoding subunits of PS I: psaA2, psaB2, psaL2, psaI2, psaF2, and psaJ2 (in the remaining strain, psaF2 and psaJ2 are adjacent and probably cotranscribed with psaB3). The psaA2 and psaB2 genes are considerably diverged from the psaA1 (77% identity, 85% similarity), psaB1 (85% identity, 92% similarity), and psaB3 (82% identity, 90% similarity) genes, respectively. The other psa genes in this cluster (psaL2, psaJ2, psaP2, and psaK2) are similarly distantly related to paralogous psa genes (psaL1, psaJ1, psaK1, and psaP1) encoded elsewhere in the genome (see table S1 for a list of genes for subunits of PS I, PS II, PBSs, and related proteins). Transcriptional analyses (tables S1 and S2) showed that the psaA2, psaB2, psaL2, psaI2, psaF2, and psaJ2 genes are not expressed in cells grown under standard growth conditions for many cyanobacteria (28). Note that this locus also includes genes for a knotless phytochrome (rfpA4), a DNA-binding response regulator (rfpB) with two CheY receiver domains and a winged-helix DNA binding domain, and a cheY-like gene (rfpC), which form an apparent operon upstream from psaA4 (fig. S2) (rfp stands for regulator of far-red photoacclimation). Phytochromes are widespread red/far-red photoreceptors (29), but orthologs of rfpA4 only occur in 12 other cyanobacteria, within photosynthesis gene clusters similar to that in JSC-1 (fig. S3). On the basis of phylogenetic analyses and conserved domain architecture, these photoreceptors form a distinctive subfamily among knotless phytochromes (Fig. 1). From these observations and biochemical studies described below, we...
hypothesize that the RfpA photoreceptor controls gene expression from this cluster. Expression of rfpA or its GAF domain in an engineered, phycocyanobilin-producing strain of *Escherichia coli* (28, 30) yielded far-red–absorbing (P<sub>fr</sub>) species under respective far-red or red illumination (fig. S6A). The *P*<sub>fr</sub> form of cyanobacterial phytochromes is initially synthesized in vivo (31, 32). RfpA is converted to the *P*<sub>r</sub> form under a broad range of light wavelengths, but only far-red light (λ ≥ 700 nm) specifically regenerates the *P*<sub>r</sub> form (fig. S6B). Because genetic tools are not available to produce an *rfpA* mutant in JSC-1, the postulated role of RfpA in sensing far-red light and controlling expression of the 21-gene photosynthesis gene cluster cannot be verified by reverse genetics. However, RfpA specifically senses far-red light, exhibits higher transcript abundance in cells grown in far-red light, and is uniquely colocalized with genes that are only expressed in far-red light (figs. S2, S3, S5, and S6 and table S1). The JSC-1 genome contains other phytochromes and related photosensors, but transcript levels only increase in far-red light for the knotted phytochrome CYJSC1_DRAFT_40400 (Fig. 1 and table S2). Calothrix sp. PCC 7507 and *Synechococcus* sp. PCC 7335 have RfpA orthologs (fig. S3) but lack Cph1 orthologs. Thus, the distribution and photochemical properties of RfpA strongly support our proposal that RfpA controls the expression of the 21-gene cluster.

To examine the photobiology more closely, JSC-1 cells were grown under six light conditions (28): (i) white fluorescent light (WL), (ii)
light, and FR. Light and FR (Fig. 2) showed that they had gained absorption at ~700 to 750 nm ($\lambda_{\text{max}} \sim 706$ nm) that was not present in cells grown in WL, GL, or 645-nm light. Low-temperature fluorescence emission spectra at 77 K (fig. S8) of cells grown under the first three listed conditions had emission maxima at 683 nm and 695 from PS II and a strong emission maximum at 725 nm from PS I. These spectra are typical of those for cells synthesizing Chl $a$ and having a relatively high PS I:PS II ratio (24). JSC-1 cells grown in 710-nm light or FR have weak fluorescence emission at 683, 695, and 717 nm and strong emission at 745 nm, in which they resemble cells synthesizing Chl $f$ (25, 26).

Pigments extracted from these cells were subjected to reversed-phase high-performance liquid chromatography. Only Chl $a$ (figs. S9 and S10, peak 3) and carotenoids were observed in pigment extracts from cells grown in WL or 645-nm light. Cells grown in FR or 710-nm light still produced Chl $a$ as the major Chl, but they also synthesized a pigment with an absorption spectrum identical to that of Chl $f$ (figs. S9 and S10, peak 2) (33, 34). This pigment was confirmed to be Chl $f$ by converting it to the corresponding pheophytin by removing the Mg$^{2+}$ ion (figs. S11 and S12) and subsequent mass spectrometry (MS) and tandem MS (MS-MS) analyses (figs. S13 and S14). Another Chl, which was slightly more hydrophilic than Chl $f$ (figs. S9 and S10, peak 1), was also detected. The absorption properties (figs. S10 and S12) of this Chl, as well as MS and MS-MS analyses (figs. S15 and S16) of the corresponding pheophytin, showed that it was Chl $d$ (33, 34). Therefore, JSC-1 synthesizes three Chls: Chl $a$, Chl $d$, and Chl $f$.

Preliminary reverse transcription polymerase chain reaction experiments confirmed that genes of the psaA operon (fig. S2) are transcribed in cells grown in FR (table S2). Cells were therefore grown in WL, transferred to FR for 24 hours, and transcription profiling (RNA-seq) was performed (28). Transcript levels for the photosynthesis-related genes in the 21-gene cluster (fig. S2) increased from 3-fold to 278-fold in cells grown in FR (Fig. 3 and table S1), and transcript levels increased at least twofold for ~900 genes (table S2). Transcript levels decreased by more than 50% for ~2000 genes in FR (table S2), including most of the paralogous genes encoding core subunits of photosynthetic complexes (Fig. 3 and table S1). The transcriptional changes during acclimation to FR are surprisingly extensive and exceed those for heterocyst (1036 genes) or hormogonia (1762 genes) differentiation in *Nostoc punctiforme* (35).

The transcription profiling data indicated that most core polypeptides of PS I, PS II, and PBSs should be replaced by products of the 21-gene cluster when cells are grown in FR. To verify that this was the case, we analyzed the proteins of this was the case, we analyzed the proteins of the paralogous genes encoding core subunits of PS I, PS II, and PBSs, respectively, that are preferentially expressed in WL. The parallel lines indicate a twofold increase or decrease by half in transcript abundance.

Fig. 3. Transcription of the 21-gene cluster in JSC-1 in far-red light is part of an extensive acclimative response. Scatter plot showing relative transcript levels for JSC-1 cells 24 hours after a shift from WL to FR. The circled stars indicate the relative transcript levels for the genes in the 21-gene cluster shown in fig. S2. The magenta, pale green, and aqua triangles indicate relative transcript levels for paralogous genes encoding subunits of PS I, PS II, and PBSs, respectively, that are preferentially expressed in WL.

Fig. 4. PS I and PS II complexes are remodeled under far-red light. (A) Sucrose density gradient centrifugation for isolation of Chl-containing complexes from thylakoid membranes of JSC-1 cells grown in WL, 645-nm light, 710-nm light, and FR after solubilization with n-dodecyl-$\beta$-d-maltoside. (B) Absorption spectra of gradient fractions containing PS I trimers (WL, solid black line; 645-nm light, solid red line) and a mixture of PS I monomers and PS II from WL (dashed black line); 645-nm light (dashed red line); FR (solid blue line); and 710-nm light (dashed blue line). (C) Low-temperature fluorescence emission spectra for the same fractions as in (B).

green-filtered fluorescent light (GL), (iii) red-filtered fluorescent light (RL), (iv) 645-nm or (v) 710-nm light provided by light-emitting diodes (LEDs), and (vi) far-red light (FR) produced from filtered tungsten light ($\lambda > 690$ nm) (see fig. S7). The absorption spectra of cells grown in 710-nm light and FR (Fig. 2) showed that they had gained absorption at ~700 to 750 nm ($\lambda_{\text{max}} \sim 706$ nm) that was not present in cells grown in WL, GL, or 645-nm light. Low-temperature fluorescence emission spectra at 77 K (fig. S8) of cells grown under the first three listed conditions had emission maxima at 683 nm and 695 from PS II and a strong emission maximum at 725 nm from PS I. These spectra are typical of those for cells synthesizing Chl $a$ and having a relatively high PS I:PS II ratio (24). JSC-1 cells grown in 710-nm light or FR have weak fluorescence emission at 683, 695, and 717 nm and strong emission at 745 nm, in which they resemble cells synthesizing Chl $f$ (25, 26).

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PS I monomers and PS II dimers, and (ii) a lower green fraction containing PS I trimers (Fig. 4A). Gradients prepared with solubilized membranes from JSC-1 cells grown in FR and 710-nm light differed dramatically. No PS I trimers were observed, and only a single fraction containing PS I monomers and PS II dimers was observed (Fig. 4A). The complexes derived from cells grown in WL or 645-nm light had absorption (Fig. 4B) and 77K fluorescence emission maxima (Fig. 4C) expected for PS I (725 nm) and PS II (685 and 695 nm) complexes containing Chl \textsubscript{a}. However, complexes isolated from cells grown in FR and 710-nm light had additional absorption features at ~720 nm (Fig. 4B) and had fluorescence emission maxima at 745 nm (Fig. 4C). These spectra demonstrate that both PS I and PS II complexes of cells grown in FR and 710-nm light contain Chl \textsubscript{f}.

Proteins associated with isolated photosynthetic complexes were analyzed by trypsin digestion and mass spectrometry of the resulting peptides.

Fig. 5. Phycobilisomes isolated from cells grown in far-red light have enhanced absorption from 700 to 750 nm. (A) Sucrose gradients showing phycobilisomes isolated from cells grown in 645-nm light and 710-nm light. (B) Absorption spectra (solid lines) and fluorescence emission spectra (dashed lines) for PBS fractions from cells grown in 645-nm light (red lines) and 710-nm light (blue lines).

Fig. 6. Diagrams summarizing the remodeling of components of PBSs, PS I, and PS II, during FaRLiP. (A) Remodeling of PBS cores in FR. Except ApcF and ApcC (see table S1), all allophycocyanin-related components (shown in aqua) composing the PBS core substructure are replaced in FR. ApcE1 has four REP (linker) domains and should assemble a pentacylindrical substructure in cells grown in WL, GL, or RL (top). ApcE2 has only two REP (linker) domains and should assemble a bicylindrical core substructure in FR (7, 38) (bottom). PBSs assembled in FR contain some phycoerythrin (table S1; shown as red disks; phycocyanin is shown in blue). This may occur because FR cannot efficiently photoconvert the CCA photo-receptor into its green-absorbing (Pg) form (23). Remodeling of PS I (B) and PS II (C) illustrated using the x-ray structures of PS I [Protein Data Bank (PDB) = 1JB0] (47) and PS II (PDB = 3BZ2) (48) from *Thermosynechococcus elongatus*. The view of PS I is from the luminal side, so PsaC, PsaD, and PsaE are not visible. The view of PS II is from the cytoplasm-facing side. Each subunit is shown in a different color (top), and subunits replaced in FR are shown in red (bottom). Subunit Ycf12 of PS II is not encoded in the draft genome of JSC-1.
irradiation between 700 and 750 nm. Additionally, JSC-1 alters relative transcript levels for ~40% of the JSC-1 genome, which leads to extensive modification of cellular metabolism (table S2). The transcription changes and global replacement of core components of PS I, PS II, and PBSs during FaRLIP are distinct from the incorporation of PBSa variants in PS II complexes of Synechocystis sp. PC6803 and other cyanobacteria in response to high light intensity or anoxic conditions (40) and from reported transcription changes for pbsD5 and pbsE2 of Anacystis sp. A153 cells grown in FR or WL at very low irradiance (41).

There is no overlap between the structural remodeling of photosynthetic complexes that occurs during CCA and FaRLIP, and it is noteworthy that JSC-1 performs both acclimation responses (see fig. S1) (24). We assume that PS I, PS II, and PBS subunits produced during FaRLIP have adapted through evolution to perform photosynthesis more efficiently when some Chl a molecules are replaced by Chl f (and Chl d) in cells growing in FR. Indeed, JSC-1 cells that have acclimated to 710-nm light have 40% greater oxygen evolution with far-red actinic light than cells acclimated to 645-nm light, although the two types of cells have identical light saturation behavior when the actinic light is WL (fig. S18).

This enhanced photosynthetic performance in FR would be ecologically important whenever cells grow in light that is strongly filtered by Chl a absorbance—for example, in mats, stromatolites, cyanobacterial blooms, or the shade of plants. FaRLIP should also benefit organisms living in sandy soils, because far-red light penetrates deeper soils, because far-red light penetrates deeper than visible wavelengths (42). The 730 nm:650 nm ratio shifts from ~0.84 at the soil surface to 2.8 at a depth of 6 mm (43), and this could be further enhanced by Chl a absorption filtering. Thus, FaRLIP could have a substantial impact on cyanobacterial photosynthesis in soil crusts (two organisms in fig. S3 are soil isolates).

Our results show that it is possible for cyanobacteria to retain paralogous copies of genes for functionally specialized photochemical reaction centers. This observation has important implications for the evolution of type 1 and type 2 reaction centers during the evolution of photosynthesis, as well as for the extension of Chl biosynthetic pathways (44). If type 1 reaction centers evolved first, a likely mechanism for the origin of type 2 reaction centers would be adaptive radiation and functional divergence (44–46). As shown in this study, functionally distinct and divergent PS I and PS II reaction centers are formed in strain JSC-1 during growth in FR. Finally, our findings could have important implications for introducing the capacity to utilize FR into plants.

REFERENCES AND NOTES

27. Materials and methods are available as supplementary materials on Science Online.

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SUPPLEMENTARY MATERIALS
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PLANETARY DYNAMICS

Chaotic dynamics of stellar spin in binaries and the production of misaligned hot Jupiters

Natalia I. Storch, Kassandra R. Anderson, Dong Lai*

Many exoplanetary systems containing hot Jupiters are observed to have highly misaligned orbital axes relative to the stellar spin axes. Kozai-Lidov oscillations of orbital eccentricity and inclination induced by a binary companion, in conjunction with tidal dissipation, constitute a major channel for the production of hot Jupiters. We demonstrate that gravitational interaction between the planet and its oblate host star can lead to chaotic evolution of the stellar spin axis during Kozai cycles. As parameters such as the planet mass and stellar rotation period are varied, periodic islands can appear in an ocean of chaos, in a manner reminiscent of other dynamical systems. In the presence of tidal dissipation, the complex spin evolution can leave an imprint on the final spin-orbit misalignment angles.

About 1% of solar-type stars host giant planets with periods of ~3 days (7). These “hot Jupiters” could not have formed in situ, given the large stellar tidal gravity and radiation fields close to their host stars. Instead, they are thought to have formed beyond a few astronomical units (AU) and migrated inward. However, the physical mechanisms of the migration remain unclear. In the past few years, high stellar obliquities have been observed in many hot Jupiter systems; that is, the spin axis of the host star and the planetary orbital angular momentum axis are misaligned (2–7). Planet migration in protoplanetary disks (8, 9) is usually expected to produce aligned orbital and spin axes (however, see (10–14)), so the observed misalignment suggests that other formation channels may be required, such as strong planet-planet scatterings (15, 16), secular interactions or chaos among multiple planets (17, 18), and the Kozai-Lidov effect induced by a distant companion (19–22). Other observations suggest that multiple formation channels of hot Jupiters may be required (23–25).

In the “Kozai + tide” scenario, a giant planet initially orbits its host star at a few AU and experiences secular gravitational perturbations from a distant companion (a star or planet). When the companion’s orbit is sufficiently inclined relative to the planetary orbit, the planet’s eccentricity undergoes excursions to large values while the orbital axis precesses with varying inclination. At periastron, tidal dissipation in the planet reduces the orbital energy, leading to inward migration and circularization of the planet’s orbit.

As the planet approaches the star in a Kozai cycle, the planet-star interaction torque due to the rotation-induced stellar quadrupole makes the stellar spin axis and the planetary orbital angular momentum axis precess around each other. Although the equations for such precession in the context of triple systems are known (21, 26), previous work on the “Kozai + tide” migration either neglected such spin-orbit coupling or included it without systematically examining the spin dynamics or exploring its consequences for various relevant parameter regimes (19–22, 27). However, the stellar spin has the potential to undergo rich evolution during the Kozai migration, which may leave its traces in the spin-orbit misalignments in hot Jupiter systems. Indeed, there are several examples of chaotic spin-orbit resonances in the solar system. For instance, Saturn’s satellite Hyperion experiences chaotic spin evolution due to resonances between spin and orbital precession periods (28). The rotation axis of Mars also undergoes chaotic variation as a result of resonances between the spin precession and a combination of orbital precession frequencies (29, 30).

We demonstrate here that gravitational interaction between the stellar spin and the planetary orbit can indeed induce a variety of dynamical behavior for the stellar spin evolution during Kozai cycles, including strongly chaotic behavior (with Lyapunov times as short as a few million years) and perfectly regular behavior in which the stellar spin stays aligned with the orbital axis at all times. We show that in the presence of tidal dissipation, the memory of chaotic spin evolution can be preserved, leaving an imprint on the final spin-orbit misalignment angles.

Kozai cycles and spin-orbit coupling

We consider a planet of mass $M_p$ initially in a nearly circular orbit around a star of mass $M_*$. For simplicity, we choose a semimajor axis $a$ with a distant binary companion of mass $M_b$, semimajor axis $a_b$, and eccentricity $e_b$, which we set to 0. In that case, the planet’s initial orbital inclination relative to the binary axis, denoted by $i_b$, falls within the range $[40^\circ, 140^\circ]$, the distant companion induces cyclic variations in planetary orbit inclination and eccentricity, with a maximum eccentricity of $e_{\text{max}} = \sqrt{1 - (5/3) \cos^2 \theta_b}$ (31, 32). These Kozai cycles occur at a characteristic rate given by

$$\Gamma_k = \frac{n}{2\pi} \frac{M_p}{M_*} \chi \left( \frac{a}{a_b} \right)^3$$

where $\chi$ is the initial eccentricity. Because of the rotation-induced stellar quadrupole, the

$$\Omega_{\text{pl}} = \frac{3}{4} \gamma k \cos \theta_b \sqrt{1 - e_0^2} \int \frac{1 - e_0^2}{1 - e^2} \sin \theta_0 \cos \theta_0$$

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(34), where $e_0$ is the initial eccentricity. Because of the rotation-induced stellar quadrupole, the
Vipp1 Is Essential for the Biogenesis of Photosystem I but Not Thylakoid Membranes in Synechococcus sp. PCC 7002*

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Background: Vipp1 was previously thought to be essential for viability and biogenesis of thylakoid membranes.

Results: A vipp1 null mutant of cyanobacterium Synechococcus sp. PCC 7002 is viable and assembles thylakoid membranes but lacks Photosystem I.

Conclusion: Vipp1 is not essential but is required for biogenesis of Photosystem I.

Significance: Normal thylakoid biogenesis and structure requires Photosystem I but not Vipp1.

The biogenesis of thylakoid membranes in cyanobacteria is presently not well understood, but the vipp1 gene product has been suggested to play an important role in this process. Previous studies in Synechocystis sp. PCC 6803 reported that vipp1 (sl0617) was essential. By constructing a fully segregated null mutant in vipp1 (SynPCC7002_A0294) in Synechococcus sp. PCC 7002, we show that Vipp1 is not essential. Spectroscopic studies revealed that Photosystem I (PS I) was below detection limits in the vipp1 mutant, but Photosystem II (PS II) was still assembled and was active. Thylakoid membranes were still observed in vipp1 mutant cells and resembled those in a psaAB mutant that completely lacks PS I. When the vipp1 mutation was complemented with the orthologous vipp1 gene from Synechocystis sp. PCC 6803 that was expressed from the strong P_{psaBA} promoter, PS I content and activities were restored to normal levels, and cells again produced thylakoids that were indistinguishable from those of wild type. Transcription profiling showed that psaAB transcripts were lower in abundance in the vipp1 mutant. However, when the yfp gene was expressed from the P_{psaBA} promoter in the presence and the absence of Vipp1, no difference in YFP expression was observed, which shows that Vipp1 is not a transcription factor for the psaAB genes. This study shows that thylakoids are still produced in the absence of Vipp1 and that normal thylakoid biogenesis in Synechococcus sp. PCC 7002 requires expression and biogenesis of PS I, which in turn requires Vipp1.

Cyanobacteria are considered to be the first oxygen-evolving photolithoautotrophs on Earth. They may have evolved as early as 3.5 billion years ago and are thought to be responsible for the oxygenization of the atmosphere, which began ~2.5 billion years ago (1). In cyanobacteria, photosynthesis antenna pigments capture solar energy, which is subsequently transformed into chemical energy using two reaction centers, Photosystem (PS)² I and PS II, and an electron transport chain that connects them (2). Water is oxidized to O₂ during this process, and atmospheric CO₂ is reduced to cellular biomass and carbon storage compounds, such as glycogen, for longer term energy conservation. In cyanobacteria and in algal and plant chloroplasts, the photosynthetic electron transport chain is localized on intracytoplasmic membranes that form the thylakoid membrane network.

Targeting of proteins into and across the thylakoid membranes has been studied and is believed to occur through several pathways, but little is known about the origin of the thylakoid membrane system or how lipids are synthesized, transported, and inserted into this membrane system (3). Moreover, the relationship between the cytoplasmic and thylakoid membranes in cyanobacteria is still very poorly understood. Some researchers propose that these systems are interconnected, whereas others maintain that they are not, and there are arguments in favor of both viewpoints. There is general agreement that the biogenesis of thylakoid membranes is a complex, multidimensional process. During this process, lipids, proteins, and pigments, as well as other cofactors, must be synthesized, transported, assembled, and inserted into these membranes, but few mechanistic details are available.

Several genetic studies have implicated the product of the vipp1 (vesicle-inducing protein in plastids 1) gene as participating in the process of thylakoid biogenesis (4, 5). Vipp1 was first described as a chloroplast-localized protein in Pisum sativum, and further analyses showed that Vipp1 was located on both the inner envelope membrane and the thylakoids (5). This unique localization of Vipp1 on these two membranes led to the presumption that Vipp1 might be involved in the assembly of the thylakoid membrane system (5). This presumption was supported by the characterization of a mutant of Arabidopsis thaliana, in which the expression of the vipp1 gene was strongly

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‡ This article contains supplemental Table S1.

§ The abbreviations used are: PS, Photosystem; Chl, chlorophyll; PBP, phyco-biliproteins; PG, phosphatidylglycerol; Comp, trans-complemented vipp1 mutant strain.
reduced (4). However, a fully segregated null mutant for vipp1 could not be produced, and thus the product of vipp1 was believed to be essential for viability (4). The mutant plants expressed only about 20% of the Vipp1 protein levels that occur in wild-type under normal growth conditions, and these knock-down mutants were incapable of photoautotrophic growth on soil (4).

Genes similar to vipp1 are also found in most cyanobacteria (6–8). Recently, attempts have been made to construct vipp1 mutants of Synechocystis sp. PCC 6803, but although the level of Vipp1 could be lowered, in none of these studies could null mutations of the vipp1 gene be fully segregated. These Synechocystis sp. PCC 6803 merodiploids had a phenotype similar to that of the knockdown strains of A. thaliana, and they exhibited a comparable loss of thylakoid membrane content and structure and also had reduced photosynthetic activity (6, 9). Thus, it was suggested that Vipp1 is also essential in cyanobacteria, because it apparently plays an essential role in thylakoid membrane biogenesis. However, because null alleles of vipp1 never fully segregated in these Synechocystis sp. PCC 6803 strains, the results obtained from the characterization of the merodiploid strains were inconclusive and must be interpreted cautiously.

By using an indirect route for the construction of a vipp1 mutant in the cyanobacterium Synechococcus sp. PCC 7002, we show here that a vipp1 null mutant can be constructed and that the fully segregated null mutant is viable. This mutant strain could not grow photoautotrophically, but it could be grown photoheterotrophically when supplied with glycerol under very low irradiance conditions. When this vipp1 mutant was complemented with the vipp1 gene from Synechocystis sp. PCC 6803 expressed from the strong P_{cpcBA} promoter, the resulting strain regained the ability to grow photoautotrophically and regained all other phenotypic properties of the wild type. Characterization of these strains showed that Vipp1 is required for biogenesis of PS I and that PS I is required for the biogenesis of "normal" thylakoid membranes in Synechococcus sp. PCC 7002.

**EXPERIMENTAL PROCEDURES**

**Strains, Culture Conditions, and Transformation Procedure—**

The wild-type strain of Synechococcus sp. PCC 7002 and the vipp1 mutant strain complemented with the vipp1 gene from Synechocystis sp. PCC 6803 (see below) were grown in liquid A medium under standard conditions (10): at 38 °C with an irradiance of 250 μmol photons m⁻² s⁻¹ provided by cool white fluorescent lights and with sparging with 1% (v/v) CO₂ in air. Mutant strains were grown under low irradiance conditions (∼10 μmol photons m⁻² s⁻¹), and the A⁻ medium was supplemented with 20 mM glycerol, which served as the main carbon and energy source. For mutant strains, appropriate antibiotics were added as required at the following concentrations: spectinomycin (50 μg/ml); gentamycin (20 μg/ml); kanamycin (100 μg/ml); and erythromycin (20 μg/ml). Transformation of Synechococcus sp. PCC 7002 was performed as described previously (11).

**Generation of vipp1 Deletion Mutant and a trans-Complemented Strain—**

In agreement with the results of others and despite many attempts, direct deletion of vipp1 was never successful. Thus, a different strategy was employed. First, a PS I-less strain of Synechococcus sp. PCC 7002 was constructed by deleting a part of the gene (bp 287–373 of the coding sequence); the deleted region was replaced with a DNA fragment encoding aacC1, which confers resistance to gentamycin (Fig. 1A). The PCR primers used to amplify the flanking sequence regions for aacC1 were: p5 (5′-CACAGACCGTGTTGCTAAAG-3′) and p6 (5′-GCTTTTGATTTGATTCCCTC-3′). The template DNAs were isolated from Synechococcus sp. PCC 15905, which confers resistance to gentamycin (Fig. 1A).

FIGURE 1. Construction and verification of vipp1 mutant as well as a trans-complemented strain. A, scheme showing the construction of a vipp1 mutant by homologous recombination by using primer set p1 and p2 to amplify the upstream region and primer set p3 and p4 to amplify the downstream region. An antibiotic resistance cassette was ligated into restriction sites added to the appropriate ends of the flanking sequences (see “Experimental Procedures” for other details). B, results of agarose gel electrophoresis of amplicons produced using primers p5 and p6, showing the fully segregated interruption mutant of vipp1. The template DNAs were isolated from the wild type (WT), the trans-complemented vipp1 mutant (Comp), and the vipp1 mutant strain (Δvipp1). The results clearly showed that the wild-type vipp1 and interrupted vipp1:aacC1 alleles had segregated completely in the vipp1 mutant and that the vipp1 gene was still mutated in the Comp strain. Lane M, DNA size markers. C, cultures of equivalent cell density (OD₅₃₀ nm = 1.0) for WT, Comp, and Δvipp1.
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The resulting complemented mutant strain (vipp1:sacC1 pAQ1Ex-PepcBA::ill0617) was repeatedly streaked and grown photoautotrophically in A- medium under standard irradiance conditions (i.e. 250 μmol of photons m-2 s-1).

**Pigment Analysis**—Chlorophyll (Chl) a, carotenoid, and phycobiliprotein (PBP) concentrations were measured as described (15). Pigment concentrations were compared on the basis of equal cell numbers, which were determined from the optical density at 730 nm (OD730 nm : 1.0 OD730 nm = 1.0 ± 0.2 × 108 cells ml-1; see Ref. 15). These measurements were made with cells that had been harvested by centrifugation from cultures grown to late exponential growth phase (OD730 nm = ~0.6 to 0.7 ml-1) and resuspended in 50 mM Tris-HCl, pH 7.0 buffer. Chl a and carotenoids were extracted from cells with 100% methanol, and their concentrations were determined as described (15). To determine relative PBP levels, cells were incubated at 65 °C for 8 min, and a difference spectrum with untreated control cells was recorded as described previously (15).

**Polyacrylamide Gel Electrophoresis and Immunoblotting**—Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed as described (12) on 15% (w/v) polyacrylamide slab gels (30:0.8 acrylamide/bisacrylamide). Equal amounts of cells (100 μl of OD730 nm = 4) were centrifuged, and the cell pellets were collected and resuspended in 40 μl of BugBusterTM protein extraction reagent (Novagen, Madison, WI). Cells were disrupted after 20 min of incubation at room temperature. Aliquots (20 μl) of the whole cell extract were loaded to each lane. Rabbit antibodies against PsA, PsA, and Vipp1 were purchased from Agrisera (Vännäs, Sweden). Immunoblotting was performed as described previously (13).

**Oxygen Evolution Assay**—Whole-chain oxygen evolution as well as respiratory oxygen uptake in wild-type and mutant cells was measured using a Clark-type electrode as described (16). Cells were adjusted to a concentration of 107 cells ml-1; see Ref. 15. These measurements were made with cells that had been harvested by centrifugation from cultures grown to late exponential growth phase (OD730 nm = ~0.6 to 0.7 ml-1) and resuspended in 50 mM Tris-HCl, pH 7.0 buffer. Chl a and carotenoids were extracted from cells with 100% methanol, and their concentrations were determined as described (15). To determine relative PBP levels, cells were incubated at 65 °C for 8 min, and a difference spectrum with untreated control cells was recorded as described previously (15).

**Whole-cell P700 Activity Measurements**—Cells (final OD730 nm = -0.5) were collected and resuspended in 50 mM Tris-HCl, pH 8.3, buffer. The absorbance change at 700 nm was monitored by a model JTS-10 LED pump-probe spectrometer (Bio-Logic). A high power red LED (680 ± 50 nm) provided the actinic illumination. A high power white LED, filtered through a 700-nm interference filter (Edmund Optics, Inc.), provided the measuring pulses.

**Transmission Electron Microscopy**—Thylakoid membranes from wild-type and mutant cells of *Synechoccus* sp. PCC 7002 were visualized by transmission electron microscopy of thin sections as described (17). The ultrathin sections were viewed with a JEM-1200 transmission electron microscope (JEOL Ltd.). Images were captured using TIETZ digital image capture software.

**Lipid Body Detection**—Nile Red staining was performed to detect lipid bodies using a reported method with minor modifications (18). Cells were harvested, washed, and resuspended in 50 mM Tris-HCl, pH 8.0, buffer. Nile Red stock solution (1 μl of a 1 mg ml-1 stock solution in dimethyl sulfoxide) was added to an aliquot of washed cells (100 μl). After staining for 10 min, lipid bodies inside cells were visualized by fluorescence using a Fluoview FV1000 confocal microscope ( Olympus, Center Valley, PA) in scanning mode. The excitation wavelength was 488 nm, and an emission wavelength of 500–600 nm was selected for all experiments.

**Total mRNA Profiling**—Transcriptome profiling was performed as described (10). The vipp1 mutant strain and Comp strain were first adapted to low irradiance (~10 μmol photons m-2 s-1) on medium A+ supplemented with glycerol (20 mM). Cells were reincubated and harvested at OD730 nm = 0.7. Total RNA was then extracted as described (18). The construction of cDNA libraries and sequencing (SOLiD™) were performed in the Genomic Core Facility at Pennsylvania State University. Mapping against the *Synechococcus* sp. PCC 7002 genome was performed using the BWA software package (19). The resulting alignment files were further analyzed with self-developed scripts to extract expression levels for each gene as described previously (10). The RNA sequencing data were deposited in the NCBI Sequence Read Archive under accession number SRP035555.

**Yellow Fluorescent Protein (YFP) Detection**—To determine whether Vipp1 plays a direct regulatory role in the transcription of the psaAB and chlLN operons, promoter regions for psaAB and chlLN were amplified and transcriptionally fused to yfp as well as aphII (conferring kanamycin resistance) separately. The primers used to amplify these promoter regions for psaAB (psaABF and psaABR) and chlLN (chlLNF and chlLNR) are listed in Table 1. The fused constructs were then used to replace open reading frame SYNPPCC7002_A2746 (Fig. 24) in both the vipp1 mutant and the complemented strain by homologous recombination as described (11). Primers used to amplify the upstream and downstream flanking regions of SYNPPCC7002_A2746 are listed in Table 1 (A2746upF, A2746upR, A2746downF, and A2746downR). Transcription profiling analyses under many different conditions showed that ORF SYNPPCC7002_A2746 produces few if any transcripts under most growth conditions (10, 20, 21). Additionally, a deletion mutant of SYNPPCC7002_A2746 has been constructed (Fig. 2B). No detectable growth phenotype was observed for this mutant strain compared with wild type strain, and thus this gene site was used as a neutral site (Fig. 2C). Full segregation of the vipp1 mutant strain and the complemented strain containing the promoter fusions to *yfp* was verified using primer set A2746upF and...
much easier to obtain a fully segregated sp. PCC 7002 and that a null mutant is viable. Because it was believed to be essential in cyanobacteria (6, 9), the results in Fig. 1 showed that the complemented mutant strain accumulated much more Vipp1 protein than the wild type. The complemented strain could grow photoautotrophically (Fig. 1C) and was no longer sensitive to high light conditions. Analysis of thin sections by transmission electron microscopy showed that the thylakoid membranes appeared to have a much simpler organization in the vipp1 mutant, as well as a strain (denoted Comp) in which the resulting vipp1 mutation was complemented in trans, so the Vipp1 levels detected in this experiment can be compared semiquantitatively.

Characterization of the Mutant Strain—The vipp1 mutant strain could not grow photoautotrophically, but this strain could still grow photoheterotrophically under low irradiance conditions (≈10 μmol photons m⁻² s⁻¹) when cells were supplied with 20 mM glycerol. Immunoblotting showed that no Vipp1 protein was detectable in the mutant cells (Fig. 3). Previous studies had shown that a deficiency in Vipp1 led to impairment of the biosynthesis of thylakoid membranes in plants and Synechocystis sp. PCC 6803 (4, 9). Transmission electron microscopy of thin section cells was used to examine the membrane organization in the vipp1 mutant strain. As shown in Fig. 4B, cells of the vipp1 mutant had far fewer thylakoid membranes than the wild type (Fig. 4A). However, some vestigial thylakoid membranes were still present, and this indicated that Vipp1 is not essential for the biogenesis of thylakoid membranes. It should be noted, however, that the thylakoid membranes appeared to be directly connected to the cytoplasmic membrane (see Fig. 4B).

Complementation of the vipp1 Mutation in trans—Because of the multistep procedure employed to construct the vipp1 mutant strain, it was important to demonstrate that the resulting vipp1 mutant could be complemented to rescue a wild-type phenotype. To avoid potential problems arising from homologous recombination of vipp1 alleles, the orthologous vipp1 gene (locus tag, sll0617) from Synechocystis sp. PCC 6803 was used for complementation instead of vipp1 from Synechococcus sp. PCC 7002. Vipp1 from Synechocystis sp. PCC 6803 is 54% identical and 71% similar in sequence to Vipp1 from Synechococcus sp. PCC 7002. A plasmid was constructed in which ORF sll0617 from Synechocystis sp. PCC 6803 was placed under the control of the strong $P_{\text{psaAB}}$ promoter (also from Synechocystis sp. PCC 6803 (14)), and this plasmid was transformed into the vipp1 null mutant. As shown in Fig. 1B, PCR analysis using primer set p5 and p6 (Table 1) showed that the vipp1 gene was still interrupted in the resulting complemented strain, which was denoted as strain “Comp.” Immunoblotting (Fig. 3) showed that the complemented mutant strain accumulated much more Vipp1 protein than the wild type. The complemented strain could grow photoautotrophically (Fig. 1C) and was no longer sensitive to high light conditions. Analysis of thin sections by transmission electron microscopy showed that the thylakoid membranes of the Comp strain cells were indistinguishable from those in wild-type cells (Fig. 4C). These results demonstrate that vipp1 (sll0617) from Synechocystis sp. PCC 6803 can
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fully complement the \textit{vipp1} mutant of \textit{Synechococcus} sp. PCC 7002 and could restore a wild-type phenotype (see additional results, and see “Discussion”).

\textbf{Pigments and Oxygen Evolution Rate—} The \textit{vipp1} mutant was noticeably bluer in color than the wild type (Fig. 1C), so we next analyzed the pigment content and oxygen evolution behavior of the \textit{vipp1} mutant and the Comp strain (Table 2). The Chl \textit{a} content of the \textit{vipp1} mutant was reduced to about 10\% and the total carotenoid content was reduced to about 20\% of the levels in the wild type. The PBP content of the \textit{vipp1} mutant was approximately half that of the wild type (Table 2). The increased PBP content relative to the Chl \textit{a} explains the bluish color of the cells. These values returned to nearly wild-type levels when the \textit{vipp1} mutation was complemented in \textit{trans}. Interestingly, the Chl \textit{a} and carotenoid levels in the \textit{vipp1} mutant were similar to those in a PS I-less mutant, although the PBP content of the PS I-less mutant was essentially the same as that of the wild type (Table 2).

Oxygen evolution rates and respiratory oxygen uptake rates were measured for these strains (Fig. 5). The measurements were either compared on the basis of equal cell number, as determined by the optical density at 730 nm (Fig. 5A), or on the basis of the Chl \textit{a} content (Fig. 5B). As shown in Fig. 5A, the \textit{vipp1} mutant evolved oxygen, but this activity rapidly declined after several min of illumination (data not shown). The initial oxygen evolution rate on the basis of Chl \textit{a} was roughly 2.5-fold higher than that of the wild-type strain but was only about 25\% of the wild-type rate on a per cell basis. These behaviors are very similar to those of a strain lacking PS I (12). When the \textit{vipp1} mutation was complemented in \textit{trans}, the oxygen evolution and respiratory uptake rates for the resulting Comp strain were very similar to those of the wild type. Thus, PS II complexes in the \textit{vipp1} mutant and the PS I-less strain were assembled and were functional. The similar pigment contents and oxygen evolution behaviors of these two strains further suggested that the \textit{vipp1} mutant probably had fewer PS I complexes per cell.

\textit{Low Temperature Fluorescence Emission Spectroscopy—} Fig. 6 shows the low temperature (77 K) fluorescence emission spectra of various \textit{Synechococcus} sp. PCC 7002 strains. When the excitation wavelength was 440 nm to excite Chl \textit{a}, three major emission peaks were observed at 685, 695, and 715 nm for the wild type. The first two peaks principally arise from PS II, whereas the emission peak at 715 nm arises from PS I (13). The fluorescence emission spectrum of the \textit{vipp1} mutant shows no emission peak from PS I and only shows emission peaks associated with PS II. The emission spectrum for the \textit{vipp1} mutant after \textit{trans}-complementation with \textit{sll0617} from \textit{Synechocystis} sp. PCC 6803 was nearly indistinguishable from that of the wild type (Fig. 6). These data strongly implicate Vipp1 in the expression or biogenesis of PS I.

\textit{PS I Activities in Whole Cells—} The low temperature fluorescence emission spectrum of the \textit{vipp1} mutant showed that the PS I content of this strain was severely reduced. To verify that the PS I activity was similarly reduced in the \textit{vipp1} mutant, photobleaching of P700 was directly measured at 700 nm in whole cells with a pump-probe spectrophotometer as described under “Experimental Procedures.” As shown in Fig. 7, photobleaching of P700 occurred when whole cells were illuminated with actinic light, and a slight increase in photobleaching occurred over a 10-s period of illumination. The absorption change at 700 nm was fully reversible when the actinic light was switched off. No photobleaching at 700 nm was detectable for the \textit{vipp1} mutant, but the photobleaching of P700 in the \textit{trans}-complemented strain was similar in magnitude and kinetics to that of the wild type. PS I complexes were isolated from the wild type and from the \textit{trans}-complemented Comp strain. Time-resolved optical spectroscopy on the millisecond time scale showed that the PS I complexes from these two strains had similar extents of photobleaching and lifetimes of charge separation (data not shown). These spectroscopic studies show that

\begin{table}[h]
\centering
\caption{Oligonucleotide primers used in this study.}
\begin{tabular}{ll}
\hline
Name & Sequence (5’−3’) \\
\hline
p1 & CATTGACCTACGAAATACACCAGCAGTGAAGA \\
p2 & GGTGTCCGAAAGCTTTTCTCCGCACTAGC \\
p3 & GAAACCTCTCTTGCTCAAGGAAAAGCACAC \\
p4 & AGACGCTCTTACGTTGACAAGTTCGACGAC \\
p5 & GTCAACTGCACTTCTGTGATTCTGCAGCC \\
p6 & TCAGAATCGAAATACCCGAAAAACCCATAA \\
ExF & GGTGTCTCTATGAGTATTAGTGGACGTTTA \\
ExR & GCACGCAGCATCTACAGATATTGAAACCT \\
pSaABF & ATCTGCGACGCGCGTCTTTGGGTTAAACCT \\
pSaABR & GATGCTTATGAGTACTCTCTCTCTCA \\
chillN & CCGTTCAATCTTTAACCAGACGACGTCG \\
chillNR & CATCGGATCTGAGATCGTCTTCTCCTA \\
A27/46upF & TGCAGCCTTTTTGACCTCCGACCAAAC \\
A27/46upR & ACCATGCTACGACCGAGAGATTTTAA \\
A27/46downF & CCTCGGAAATACCACGACATTGGGAGGAA \\
A27/46downR & CCGCAAAATTTCCTCCGCGGCGTTGA \\
\hline
\end{tabular}
\end{table}
the vipp1 mutant does not assemble functional PS I complexes. Heterologous complementation of vipp1 with ORF sll0617 from Synechocystis sp. PCC 6803 was sufficient to restore normal PS I biogenesis and functionality to the vipp1 mutant of Synechococcus sp. PCC 7002.

### TABLE 2

<table>
<thead>
<tr>
<th>Pigment contents of Synechococcus sp. PCC 7002 WT, Comp, ΔpsaAB, and Δvipp1</th>
</tr>
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<tbody>
<tr>
<td><strong>WT</strong></td>
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<tr>
<td>Chlorophyll a (μg ml⁻¹ OD₇₃₀ nm⁻¹)</td>
</tr>
<tr>
<td>Carotenoids (μg ml⁻¹ OD₇₃₀ nm⁻¹)</td>
</tr>
<tr>
<td>Phycobiliproteins (relative amount)</td>
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**FIGURE 5.** Oxygen evolution and respiration rates for WT, Comp, ΔpsaAB, and Δvipp1, based on equal cell numbers (based on OD₇₃₀ nm) (A) or equal Chl a (B). The vipp1 mutant strain as well as the psaAB mutant strain had much higher oxygen evolution rates than the WT strain when rates were compared on the basis of Chl but much lower oxygen evolution rates when rates were compared on the basis of equal cell numbers. Note that these values were derived from the initial rates of oxygen evolution for the vipp1 and the psaAB mutant strains, because oxygen evolution rates rapidly declined for these two strains that had no PS I activity to drive the reoxidation of the plastoquinone pool. The data shown are averages values for three biological replicates, and the error bars show the standard deviation.

**FIGURE 6.** Low temperature (77 K) fluorescence emission spectra of whole cells of WT, Comp, and Δvipp1. In the Δvipp1 mutant, PS I fluorescence emission at ~715 nm was completely absent, but PSII was still assembled and exhibited normal fluorescence emission at 685 and 695 nm. The excitation wavelength was 440 nm.

**FIGURE 7.** Photobleaching of P700 in whole cells of WT (light gray line), Comp (black line), and Δvipp1 (dark gray line). The PS I activity in the trans-complemented vipp1 mutant strain was almost the same as that of the WT. No P700 photobleaching activity was detected in the vipp1 mutant strain. The actinic light was turned on at 0 s and turned off after 10 s, and absorption difference was measured at 700 nm.

Immunoblotting to Detect PS I Polypeptides—Whole-cell extracts of the wild type, the vipp1 mutant strain, and the trans-complemented vipp1 mutant strain were prepared and subjected to SDS-PAGE, and the resolved proteins were transferred to membrane filters for immunoblotting. Previous
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Studies have shown that PsA, PsB, PsC, PsD, PsE, and PsL are responsible for trimerization of PS I (22), accumulated in the absence of PsA and PsB in the vipp1 mutant (Fig. 8). This observation is consistent with observations made for Synochocystis sp. PCC 6803, for which it was also found that PsL accumulated in membranes independently of the levels of PsA and PsB (23).

Thylakoid Membranes in a psaAB Deletion Mutant—Although previous studies had suggested that Vipp1 was required for thylakoid membrane biogenesis, the results shown above suggested that Vipp1 is required for psaAB expression or the biogenesis of PS I. The presence of vestigial thylakoid membranes in the vipp1 mutant indicated that normal thylakoid biogenesis might require PS I assembly rather than Vipp1. A natural hypothesis arising from these observations is that a PS I-less mutant should produce vestigial thylakoids that closely resemble those in the vipp1 mutant. Fig. 4D shows images of cells of a psaAB deletion mutant of Synochococcus sp. PCC 7002 that is unable to assemble any functional PS I complexes (or subcomplexes (12)). Immunoblotting showed that the PS I-less mutant cells accumulated wild-type levels of Vipp1 (Fig. 3). Like the vipp1 mutant, cells of the psaAB deletion mutant produce vestigial thylakoids that closely resemble those of the vipp1 mutant. Therefore, these results clearly demonstrate that the capacity to produce wild-type thylakoid membranes requires the normal biogenesis of PS I rather than Vipp1.

Detection of Lipid Bodies—Previous studies in plant chloroplasts and Synochocystis sp. PCC 6803 suggested that vipp1 might play a role in membrane biogenesis and might more specifically affect the insertion of membrane lipids (24). However, the results presented here conclusively demonstrate that cyanobacterial Vipp1 plays a role in the expression or biogenesis of PS I. Nile red is a lipid-soluble fluorescent dye that can be used to detect lipids in microorganisms (18). To determine whether lipid bodies were produced in the strains constructed here, Nile red staining was performed to detect lipid bodies. The vipp1 mutant accumulated numerous lipid bodies in the cytoplasm (Fig. 9B), but lipid bodies were not observed in wild type (Fig. 9A). This result excludes the possibility that Vipp1 is involved in the biogenesis of lipid bodies, but it suggests that Vipp1 is directly or indirectly involved in the assembly of lipids into thylakoid membranes. The inability to produce normal amounts of PS I apparently interferes with lipid insertion into the thylakoid membranes, and the lipids apparently then accumulate as lipid bodies in the cytoplasm. Consistent with the results described above, the PS I-less strain also accumulated lipid bodies in the cytoplasm that resembled those in the vipp1 strain (Fig. 9D). When the ability to produce PS I was restored by trans-complementation of the vipp1 mutant, lipids no longer accumulated as lipid bodies in the cytoplasm of the Comp strain (Fig. 9C).

Vipp1 Is Not Required for Transcription of psaAB—In the experiments described above, we showed that PS I is not detectable in cells that lack Vipp1 and that restoration of Vipp1 by trans-complementation reverses all known phenotypic defects associated with the absence of Vipp1. Fig. 10 shows a scatter plot that compares the transcription abundances for each gene in the vipp1 deletion strain in comparison with their abundances in the Comp strain. Four genes, psaAB and chlLN, which occur in two dicistronic operons, showed significantly lower transcript abundances in the vipp1 mutant compared with the trans-complemented strain. These data suggested that Vipp1 might regulate psaAB transcript levels.

To ascertain whether Vipp1 was acting as an activator or repressor of transcription of the psaAB operon, we introduced a promoter fusion of $P_{psaAB}$ to yfp ($P_{psaAB}:yfp$) into a neutral site (SYNC7002_A2746) in the Synochococcus sp. PCC 7002 chromosome in the vipp1 mutant as well as the trans-complemented Comp strain (Fig. 11, A and B). The Yfp fluorescence in these two strains was equal within error (Fig. 11C). Similar results were observed for the $P_{chl\text{L}N}$ fused to ($P_{chl\text{L}N}:yfp$; data
However, the authors of that study concluded that the reduced diploid knockdown strain of decades. However, its function is still unclear. A has been investigated in several organisms over about 2 DISCUSSION

irradiance growth conditions might cause a limitation for Chl than the parental strain because of restoration of PS I. Low levels in the Comp strain. This strain, which is derived from the vipp1 mutant strain can only be grown at very low irradiance levels, it is possible that the Chl LN operon, which encodes two of the three subunits of the light-independent protochlorophyllide reductase (25), is specifically transcribed at higher levels in the Comp strain. This strain, which is derived from the vipp1 mutant, has a much greater demand for Chl a than the parental strain because of restoration of PS I. Low irradiance growth conditions might cause a limitation for Chl a biosynthesis at the level of protochlorophyllide a reduction, which typically is catalyzed by the light-dependent protochlorophyllide a reductase (26). The low light levels might lead to derepression of the light-independent protochlorophyllide a reductase, ChLNBL, in the Comp strain because of an increased demand for Chl a to assemble PS I. This would not occur in the vipp1 mutant because it does not assemble PS I and requires only 10% of the Chl found in the wild type (Table 2).

DISCUSSION

The exact role of Vipp1 has been a longstanding mystery that has been investigated in several organisms over about 2 decades. However, its function is still unclear. A vipp1 merodiploid knockdown strain of Synechocystis sp. PCC 6803 had reduced levels of PS I (27) and fewer thylakoid membranes. However, the authors of that study concluded that the reduction in thylakoid membrane content affected the ratio of PS I and PS II. Other studies suggested that Vipp1 was essential because it played a major role in the biogenesis of thylakoid membranes (4, 24). However, based on the results obtained in this study, precisely the opposite is the case in Synechococcus sp. PCC 7002. Vipp1 is clearly required for the biogenesis of PS I, and the absence of PS I leads to a greatly decreased level of thylakoid membranes in Synechococcus sp. PCC 7002 cells.

In contrast to previous studies in Synechocystis sp. PCC 6803, a fully segregated null mutation was constructed in the vipp1 gene of Synechococcus sp. PCC 7002 in this study, and we were able to complement this mutant heterologously in trans with the Synechocystis sp. PCC6803 vipp1 gene to restore the wild-type phenotype. These experiments clearly demonstrate that the function of Vipp1 is highly conserved, but Vipp1 is clearly not required for viability in the cyanobacterium Synechococcus sp. PCC 7002. Since 1996, the genomes of many cyanobacteria have been sequenced (e.g. see Refs. 28 and 29). Comparative analysis of these genomic data shows that some cyanobacteria, such as certain Prochlorococcus species, lack the vipp1 gene. However, these cyanobacteria clearly still assemble thylakoids and produce functional PS I complexes (30). Thus, Vipp1 is not required for the formation of thylakoid membranes in Prochlorococcus spp.; nor is it essential in all cases for PS I biogenesis. These observations suggest that cyanobacteria must have redundant mechanisms to assemble PS I, probably PS II, and thylakoids. This is almost certainly one of the reasons why it has been so difficult to establish mechanistic details for the biogenesis of PS I and PS II.

It has been reported that reduced expression of Vipp1 in Synechocystis sp. PCC 6803 resulted in a decreased PS I content and an altered PS I/PS II ratio, reduced thylakoid content, and a reduced percentage of trimeric versus monomeric PS I complexes (27). Mutants lacking phosphatidylglycerol (PG) synthase, encoded by the pgsA gene, in Synechocystis sp. PCC6803 are not viable unless PG is added to the growth medium (23). When cells of a pgsA mutant were deprived of PG over many days, a phenotype similar to that for Vipp1 deletion was noted with respect to PS I complexes. Depletion of PG not only led to decreased PS I activity but also caused a depletion of PS I trimers and an increase in PS I monomers. However, PsaL was still inserted into membranes and could reassemble trimeric complexes in the absence of protein synthesis when PG was added back to cells (23). The crystal structure of trimeric PS I complexes from Thermosynechococcus elongatus showed that three PG molecules are tightly associated with each monomeric PS I complex and thus may play a role in PS I biogenesis (2). An A. thaliana mutant strain unable to synthesize PG was no longer able to grow photoautotrophically and had a severe reduction in Chl and thylakoid membranes (31), which could potentially be due at least in part to a loss of functional PS I complexes. Collectively, these results suggest that a relationship exists among Vipp1, PG biosynthesis, and biogenesis of PS I complexes and that collectively Vipp1, PG synthesis, and PS I biogenesis strongly influence thylakoid membrane structure and biogenesis.

Analysis of the trans-complemented vipp1 mutant and the psaAB deletion mutant strains further indicated that Vipp1 is

![Graph showing scatter plot comparing the relative transcript abundances for mRNAs of the vipp1 mutant to those in the trans-complemented vipp1 mutant strain.](image-url)
more likely involved primarily in the biogenesis of PS I complexes rather than thylakoid membrane biogenesis, because PS I complexes and activity are restored to wild-type levels in the trans-complemented vipp1 mutant strain. In contrast, in the vipp1 null mutant, no PS I complexes accumulated, and no PS I activity was detected. Previous studies also suggested that a critical Vipp1 concentration might be required for thylakoid membrane protein complex formation (27). Vipp1 has been reported to form rodlike structures in vivo (32), and it is possible that these Vipp1 structures could assist in the translation, transport, and/or insertion of membrane-associated subunits into thylakoids. Thus, the loss of Vipp1 might interfere with one or more of these processes and therefore interfere with the insertion of the PsaA and PsaB polypeptides into the thylakoid membranes. Although it seems clear that the loss of Vipp1 interferes with one or more of these processes, it certainly is not yet clear at which level Vipp1 acts to interfere with PS I biogenesis. However, the reduced level of psaAB transcripts in vipp1 mutant cells suggests that Vipp1 probably acts directly or indirectly at the level of translation (see below).

In Synechococcus sp. PCC 7002, a psaA deletion mutant lacking PS I had normal transcript levels for vipp1 but greatly reduced thylakoid membrane content, and similarly, the vipp1 mutant that lacks PS I had reduced thylakoid membrane content. Restoration of PS I levels in cells caused thylakoids to return to wild-type levels and overall thylakoid structure, which implies that PS I plays an important role in the formation of structurally normal and functional thylakoid membranes. These observations generally agree with observations that depletion of PS II had only minor effects on thylakoid membrane formation (33) and that intracytoplasmic membranes were much less abundant in a mutant depleted of both PS I and PS II (34). It has been suggested that PS I plays a role in the early steps to form thylakoid membranes and that PS II is then involved in forming highly ordered tubular structures of thylakoid membranes together with PS I (34). A direct interaction of Vipp1 with Albino3.2 protein in Chlamydomonas reinhardtii also implicated Vipp1 in the integration of thylakoid membrane proteins (35). The thylakoid-localized protein, Albino3.2, belongs to the conserved YidC/Oxa1p/Alb3 protein family, and it plays an essential role during the insertion of photosystem reaction center polypeptides (such as PsaA, PsbA (D1), and PsbD (D2)) into the thylakoid membranes (35, 36). Overproduction of Vipp1 occurred when Albino3.2 was depleted in C. reinhardtii (35). This observation suggests that Vipp1 is involved in stabilizing the membrane structure during the Albino3.2-mediated protein insertion into thylakoid membranes and that Vipp1 may deliver photosystem polypeptides to Albino3.2 for insertion into thylakoid membranes. If PsaA and PsaB were cotranslationally inserted into thylakoids during or
after Vipp1 action, this could explain why transcripts for psaAB were reduced in the *vipp1* mutant. Transcript levels for *yidC* were similar in *Synechococcus* sp. PCC 7002 cells in the presence or absence of Vipp1 (see supplemental Table S1). Furthermore, we observed no changes in transcript levels for genes encoding other PS I polypeptides, PS I-specific chaperones (e.g. *rubA*, *ycf3*, *ycf4*), or other general chaperones or proteases. Correspondingly, the transcript levels for all of these components were similar in the wild type, the *vipp1* mutant, the complemented *vipp1* mutant strain, and the *psaAB* deletion strain.

Abundant lipid bodies were detected in the *vipp1* mutant strain, which suggests that Vipp1 is not directly involved in lipid synthesis and accumulation. However, the abnormal localization of lipids in *vipp1* mutant indicates that Vipp1 directly or indirectly affects the insertion of lipids into thylakoid membranes. Previous studies suggested that Vipp1 played a stimulatory role in the cpTat transport system, potentially by enhancing protein binding interactions with lipid-rich regions of thylakoid membranes (37). Interestingly, it appears that lipid synthesis still occurs, but normal lipid insertion into membranes is apparently greatly reduced when PS I complexes are not inserted into the membranes in *Synechococcus* sp. PCC 7002. As observed previously in other organisms, it appears that there is a relationship between membrane biogenesis, lipid insertion, and PS I complex biogenesis and membrane insertion and that Vipp1 is necessary for all of these processes to proceed normally (24).

Nordhues et al. (38) recently studied the role of Vipp1 in *C. reinhardtii* by RNA interference. They found that core complexes for PS I and PS II as well as the cytochrome *b*~6~ complex and ATP synthase were reduced 14–20% in Vipp1-depleted cells, but light-harvesting complex II levels increased by 30%. These authors proposed a highly speculative hypothesis that Vipp1 provides structural lipids for the biogenesis of some protein complexes of the thylakoid membrane. However, the obvious differences in their results in *C. reinhardtii* and those presented here for *Synechococcus* sp. PCC 7002 strongly suggest that Vipp1 may play different roles in prokaryotes and eukaryotes. Alternatively, redundant pathways may exist in cyanobacteria in thylakoid biogenesis or perhaps stress responses. In *Synechocystis* sp. PCC 6803, *vipp1* expression increased under high salt conditions (42), and in *C. reinhardtii*, *vipp1* expression increased under high irradiance (43). These findings suggest that Vipp1 may play a role in responses to these stress conditions, although its expression might also increase if PS I levels increase under these same conditions. However, our results with reporter strains clearly showed that Vipp1 is not directly involved in the transcriptional regulation of either the *psaAB* or *chln* genes of *Synechococcus* sp. PCC 7002. We propose that *psaAB* transcripts decreased because of an effect of Vipp1 on translation or co-translational insertion of these PS I polypeptides into the thylakoid membrane. The decreased transcript levels for *chln* are probably due to the reduced demand for Chl *a* in the Vipp1 mutant, which does not accumulate PS I (Table 1). It is also interesting that the marine symbiont strain UCYN-A, which lacks PS II and cannot fix CO₂, but has retained PS I complexes (44), has also retained two copies of the *vipp1* gene like *Trichodesmium erythraeum*, another nitrogen-fixing marine cyanobacterium.

In conclusion, our results show that Vipp1 is not essential for viability of the cyanobacterium *Synechococcus* sp. PCC 7002 and that it is most likely involved in the biogenesis of PS I, possibly by participating in the insertion of PS I polypeptides into thylakoid membranes. Our results further suggest that normal thylakoid membrane biogenesis is dependent upon assembly of PS I but is not directly dependent on Vipp1. Further studies to elucidate the precise role of Vipp1 in PS I and lipid insertion into membranes will enhance our knowledge of the underlying mechanisms of thylakoid membrane biogenesis and photosynthetic protein assembly.

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REFERENCES

Role of Vipp1 in Thylakoid Membrane Biogenesis

Biochemical Validation of the Glyoxylate Cycle in the Cyanobacterium Chlorogloeopsis fritschii Strain PCC 9212

Shuyi Zhang and Donald A. Bryant

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 Chlorogloeopsis 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 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 CO2 (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 CO2-releasing steps of the TCA cycle. Thus, the glyoxylate cycle enables cells to utilize C2 units (i.e. acetyl-CoA) more efficiently for biomass production. These C2

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.
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 (16, 17). Heliobacteria, green sulfur bacteria, and heliobacteria actually have this cycle. Our studies 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.

In this study, we describe the biochemical validation of the 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 SYNPC7002_A2041 had the highest sequence identity (43%) to the fumarase (shr0018) 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 (SYNPC7002_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 glyoxylate 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) CO₂ in air (standard growth conditions). Low CO₂ 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
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) CO2 in air. Low irradiance or low CO2 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 a 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 a 210-nm UV detector SPD-20A. Different components in the medium were separated on a Supelcosil C610H column (Supelco, Bellefonte, PA), using 4 mM H2SO4 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 standard acetate.

**Cloning, Protein Purification, and Protein Identification**—Open reading frames SYNPC7002_A2041, encoding the putative fumarase of Synechococcus sp. PCC 7002, UYEDRAFT_02681, encoding the putative isocitrate lyase and UYE- DRAFT_02682, encoding the putative malate synthase of Synechococcus sp. PCC 7002—sp. PCC 7002, UYE- DRAFT_02682, encoding the putative malate synthase of Synechococcus sp. PCC 7002, 10 mM sodium acetate was added to the A− medium. Successful, and subsequently, a His tag was introduced into isocitrate lyase and fumarase to catalyse 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 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. Low irradiance or low CO2 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 a 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 a 210-nm UV detector SPD-20A. Different components in the medium were separated on a Supelcosil C610H column (Supelco, Bellefonte, PA), using 4 mM H2SO4 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 standard acetate.

**Cloning, Protein Purification, and Protein Identification**—Open reading frames SYNPC7002_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 pAQA1Ex,PgBA (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 SYNPC7002_A2041 (Table 1). An N-terminal His10 tag was introduced into isocitrate lyase and fumarase to facilitate subsequent protein purification. Initial attempts to add a His10 tag to the N terminus of malate synthase were not successful, and subsequently, a His6 tag was successfully added to the C terminus. The resulting plasmids pAQA1Ex,PgBA, pAQA1Ex,PgBA, pAQA1Ex,PgBA, and pAQA1Ex,PgBA 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 Ni2+-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 MgCl2, 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 MgCl2, 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 MgCl2, 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)
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 methanolysis, double-deionized water (1 ml) was 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 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. 2A 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. C. HPLC analysis showing the formation of malate (peak 1) from fumarate (peak 2) catalyzed by the purified fumarase (SYNPCC7002_A2041). 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.”
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. 3A). 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 \textit{C. fritschii} PCC 9212 could not be overproduced in \textit{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. 3A). 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. 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 glyoxylate 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.”
Glyoxylate Cycle in C. fritschii PCC 9212

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 C4 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. 4A). 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. 4A). As expected, C. fritschii PCC 9212 grew more slowly when cultures were sparged with air (Fig. 4B). 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) CO2. This result shows that acetate can stimulate growth but certainly is not able to supplant CO2 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. 4C).

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. 6A). 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. 6B). 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-CO2 conditions for WT cells (Fig. 6C and D). WT cells exhibited net acetate excretion under these conditions (Fig. 6, C and D), and the same was observed for Synechococcus 7002-glyox under dark conditions.

Acetate assimilation was still observed under low-CO2 conditions in strain Synechococcus 7002-glyox (Fig. 6D), 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 CO2 concentration was crucial for acetate uptake and the acetate uptake rate was...
reduced by almost 50% in the absence of CO₂ (4). These observations confirm that acetate assimilation, the glyoxylate cycle, and CO₂ 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. 7A). BLASTP analysis showed that these two genes also occur in _C. fritschii_ PCC 6912, _Cyanothecaceae_ strains PCC 7822 and 7424, _Pleurocapsa_ minor PCC 7327, _Fischerella_ sp. PCC 9605, _Cyanobacteria_ PCC 7702, _Mastigocoleus testarum_, and _Tolyphothrix botteieli_. 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 (FARLP) 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. 7A). 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. 7B). A similar response was reported in _E. coli_ cells grown in the presence of acetate (35).
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-oxogluutarate, 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 protist, *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 $\beta$-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 $\beta$-oxidation pathway and glyoxylate cycle enzymes were induced in senescing leaves, possibly used for the breakdown of membrane lipids and gluconeogenesis [44].

**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 grown 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.
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.
Glyoxylate Cycle in C. fritschii PCC 9212

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 be mainly 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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Learning new tricks from an old cycle: the TCA cycle in cyanobacteria, algae and plants

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With 2 figures

Abstract: Cyanobacteria, algae, and plants absorb and convert sunlight into chemical potential energy by oxygenic photosynthesis. However, because of the rotation of Earth, sunlight is only available during about one half of the diel cycle. Photosynthetic organisms must therefore store energy-rich compounds during the day to provide the fuel to support growth or maintenance energy production at night. In the presence of oxygen, energy-producing respiration occurs in most organisms, which allows the oxidation of energy-rich substrates for the production of proton-motive force for ATP synthesis and other biochemical work. Together with glycolysis and the oxidative pentose phosphate pathway, the tricarboxylic acid (TCA) cycle is one of the three most important pathways of central carbohydrate metabolism. It has long been accepted that a complete TCA cycle is important if not essential for respiratory energy production in the dark for eukaryotes. However, because biomass production is a more central concern for cyanobacteria, the TCA mostly operates as a branched pathway for the production of two essential precursor metabolites, 2-oxoglutarate and oxaloacetate. It was long believed that cyanobacteria had an incomplete TCA cycle due to the absence of 2-oxoglutarate dehydrogenase (OGDH). However, our recent studies demonstrated that the TCA cycle in most cyanobacteria is completed in manner distinct from the classical TCA cycle through the action of two alternative enzymes, 2-oxoglutarate decarboxylase (2-OGDC) and succinic semialdehyde dehydrogenase (SSADH). This review describes current research progress in understanding the functional similarities and differences among the TCA cycles in cyanobacteria, algae, and plants. Moreover, the possible significance of non-traditional TCA cycles in a broader regulatory and evolutionary context is considered.

Keywords: (missing)

Introduction

Oxygenic chlorophototrophic bacteria (i.e., cyanobacteria) as well as algae and plants use sunlight as the energy source to oxidize water and produce ATP and NADPH for CO₂ fixation. Compared to algae and plants, cyanobacteria have somewhat simpler photosystems (Bryant & Frigaard, 2006; Hohmann-Marriott & Blankenship, 2011), and while all use the reductive pentose phosphate pathway (Calvin-Benson-Bassham cycle) for carbon dioxide fixation, other aspects of their metabolic pathways differ significantly. The tricarboxylic acid (TCA) cycle, also known as the citric acid or Krebs cycle, was mostly established by Sir Hans Adolf Krebs, who actually established a “trinity of cycles” and received a Nobel Prize for his studies on the TCA cycle (Kornberg, 2000). He first elucidated the reactions of the urea/ornithine cycle in 1932, proposed the TCA cycle in 1937, and then completed his trifecta of cycles by describing the glyoxylate cycle in 1957.

The TCA cycle includes key reactions of central carbohydrate metabolism that support ATP synthesis by oxidative phosphorylation by using reducing equivalents from the oxidation of acetyl-CoA (Fernie et al., 2004; Foyer et al., 2011). However, there is accumulating evidence linking the TCA cycle to other pathways, not only in bacteria (Bott, 2007) but also in algae and plants (Araújo et al., 2012). In heterotrophic bacteria, the TCA cycle has two main functions: (a) it oxidizes two-carbon, acetyl-CoA units producing NADH that drives oxidative phosphorylation; and (b) it provides essential precursor metabolites (e.g., oxaloacetate, 2-oxoglutarate (2-OG), and sometimes succinate) that are required for biosynthesis of cellular components, primarily in the biosynthesis of amino acids, porphyrin derivatives (cobalamin, siroheme, heme, chlorophyll, and bilins), and other cellular constituents. Surprisingly, studies have demonstrated that some bacteria have variant TCA cycles (Knoop et al., 2013; McCammon et al., 2003; Singh et al., 2009; Tian et al., 2005; Zhang and Bryant, 2011), and this also occurs...
in algae and plants (Sweetlove et al., 2010; Tielens et al., 2002). These variant TCA cycles could be the evolutionary consequences of metabolic adaptations that focus more on the production of biosynthetic precursors than the classic cycle, which favors the generation of energy over the production of metabolic precursors (Fig. 1). In this review, we discuss the possible regulatory and evolutionary importance of TCA cycle variants that occur in cyanobacteria in relationship to TCA cycle variants that occur in algae and plants. We also examine challenges worthy of future investigation in cyanobacteria, algae, and plants.

**TCA cycle in chlorophototrophic bacteria**

Although chlorophototrophic bacteria (i.e., phototrophic bacteria that synthesize (bacterio)-chlorophylls) have very diverse metabolic lifestyles, their central metabolism is partly dictated by the types of reaction centers they employ. At least three versions of the TCA cycle commonly occur in chlorophototrophic bacteria: the oxidative TCA cycle, the reductive TCA cycle, and the incomplete (branched) TCA cycle. Most chlorophototrophic bacteria are anoxygenic organisms, which use either a homodimeric type-1 (Fe/S-type) or a type-2 (quinone-type) reaction center for the transduction of light into stored chemical potential energy (Bryant & Frigaard, 2006; Hohmann-Marriott & Blankenship, 2011). Bacteria with type-2 reaction centers (e.g., members of the Proteobacteria and filamentous anoxygenic phototrophs from the phylum Chloroflexi) are often facultative phototrophs that can grow under oxic or anoxic conditions; most have an active TCA cycle and many can often perform aerobic respiration (Tang et al., 2011). An interesting recent discovery is a seventh bacterial phylum containing a member, *Gemmatimonas* sp. strain AP64, which synthesizes bacteriochlorophyll a and assembles functional type-2 reaction centers. This bacterium is an aerobic anoxygenic phototroph with a complete TCA cycle (Zeng et al., 2014).

Bacteria that only have homodimeric type-1 reaction centers (e.g., members of the taxa Chlorobi (green sulfur bacteria; GSB); *Firmicutes* (heliobacteria), and *Acidobacteria*) are usually strict anaerobes with specialized, oxygen-sensitive metabolism. An exception is *Chloracidobacterium thermophilum*, which is a microaerophile with a photosynthetic apparatus typical of that found in green sulfur bacteria (Bryant et al., 2007; Garcia Costas et al., 2012; Tank & Bryant, 2014). In GSB, light-driven oxidation of sulfur compounds produces strongly reducing ferredoxins, which are used in the reductive TCA cycle for fixation of CO₂ and biomass production (Evans et al., 1966; Sirevåg, 1995; Tang et al., 2011). A few GSB have a complete oxidative TCA cycle, but *Chlorobaculum tepidum* lacks 2-oxoglutarate dehydrogenase (2-OGDH) and has been shown in some studies to utilize only the incomplete oxidative TCA cycle (from citrate to 2-OG) during mixotrophic growth with acetate (Feng et al., 2010; Tang & Blankenship, 2010; Tang et al., 2011). Heliobacteria can only utilize a limited set of carbon sources, in part due to an incomplete reductive TCA cycle. ATP-dependent citrate lyase and (Si)-citrate synthase activities have not been detected in heliobacteria (Pickett et al., 1994; Tang et al., 2010a). Some more recent studies have indicated that heliobacteria could use the (Re)-citrate synthase to initiate the incomplete oxidative TCA cycle (from citrate to 2-OG) and that carbon flux is mostly carried out through the incomplete oxidative TCA cycle (Tang et al., 2010b). Although (Re)-citrate synthase and (Si)-citrate synthase both catalyze the formation of citrate from oxaloacetate and acetyl-CoA, the difference lies in adding the acetyl group to the “pro-R” (by (Re)-citrate synthase) or “pro-S” (by (Si)-citrate synthase) face of citrate (Tang et al., 2011). Interestingly, the recently discovered acidobacterium, *C. thermophilum*, also seems to lack the gene for 2-OGDH but has a gene for 2-oxoglutarl ferredoxin oxidoreductase (KFOR) (Garcia Costas et al., 2012). Because *C. thermophilum* cannot synthesize branched chain amino acids but has the capacity to degrade them (Garcia Costas et al., 2012), this organism may synthesize 2-oxoglutarate primarily by carboxylating succinate. Finally, it should be noted that two recently described members of the phylum *Chlorobi*, the heterotroph *Logativibacterium album* (Liu et al., 2012a) and the photoheterotroph *Candidatus Thermochlorobacter aerophilum* (Liu et al., 2012b) are microaerophiles that have complete TCA cycles as well as genes for both KFOR and 2-OGDH.

Cyanobacteria are a highly diverse group of oxygenic chlorophototrophic bacteria, which may have evolved as much as 3.5 billion years ago. Once they evolved the capacity for oxygenic photosynthesis, cyanobacteria played a crucial role in creating an oxidizing atmosphere and providing organic carbon by modifying the evolution of other organisms as well (Des Marais, 2000). In the dark under oxic conditions, cyanobacteria perform aerobic respiration, but anaerobic respiration is extremely uncommon in cyanobacteria. Although common among (facultative) anaerobes, no cyanobacterium has been reported to use nitrate as an electron acceptor for anaerobic respiration. However, *Oscillatoria limnetica* has been reported to perform anaerobic respiration using elemental sulfur as the electron acceptor (Oren & Shilo, 1979). Cyanobacteria typically perform fermentative metabolism under anoxic conditions by consuming endogenous carbohydrate reserves such as glycogen (Stal & Moezelaaar, 1997). Interestingly, one group of nitrogen-fixing marine cyanobacteria are proposed to be obligate photoheterotrophs due to the absence of Photosystem II, autotrophic carbon assimilation pathways, and the TCA cycle (Tripp et al., 2010; Zehr et al., 2008). The absence of TCA cycle enzymes for the synthesis of 2-oxoglutarate and oxaloacetate implies that this organism must take up exogenous dicarboxylic acids, or amino acids such as aspartate and glutamate, to provide essential metabolic precursors.
Nearly 50 years ago, two groups independently concluded that cyanobacteria have an incomplete TCA cycle because they lack 2-OGDH, which converts 2-OG to succinyl-CoA (Pearce & Carr, 1967a; Smith et al., 1967). In the intervening years, no gene encoding 2-OGDH has been found in any sequenced cyanobacterial genome (Shih et al., 2013; Wood et al., 2004). Consequently, the observation that most cyanobacteria are obligate photolithoautotrophs was often attributed to their incomplete TCA cycle (Stanier & Cohen-Bazire, 1977; Wood et al., 2004). However, Zhang & Bryant (2011) recently demonstrated that two other enzymes, namely 2-OG decarboxylase (2-OGDC) and succinic semialdehyde (SSA) dehydrogenase (SSADH), can functionally replace 2-OGDH and succinyl-CoA synthetase by converting 2-OG to succinate (Zhang & Bryant, 2011). More specifically, 2-OG is first decarboxylated to SSA by 2-OGDC, and SSA is then oxidized to succinate by SSADH using NADP+ as the electron acceptor. Acting together, these two reactions thus complete the TCA cycle in cyanobacteria (Fig. 1).

Fig. 1. Scheme showing the TCA cycle and variants interconnections with carbon and nitrogen metabolism. Abbreviations: 2-OG, 2-oxoglutarate; 2-OGDC, 2-oxoglutarate decarboxylase; 2-OGDH, 2-oxoglutarate dehydrogenase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; ACL, ATP-dependent citrate lyase; ACO, Aconitase; CS: Citrate synthase; FUM, Fumarase; FRD, Fumarate reductase; GABA, gamma-Aminobutyric acid; GABA-TK, GABA aminotransferase (2-oxoglutarate depened); GABA-TP, GABA aminotransferase (pyruvate depended); GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GOGAT, glutamine:2-oxoglutarate amidotransferase; GS, glutamine synthetase; ICL, Isocitrate lyase; IDH, Isocitrate dehydrogenase; KFOR, 2-oxoglutarate:ferredoxin oxidoreductase; MS, Malate synthase; MDH, Malate dehydrogenase; PDH, Pyruvate dehydrogenase; SCS, Succinyl-CoA synthetase; SDH, Succinic acid dehydrogenase; SSA: succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase. The blue arrows indicate enzymatic reactions (ACL, FRD, KFOR) that are specific to the reverse TCA cycle. The TCA cycle can also operate as a branched pathway depending on the physiological conditions and consumption of 2-OG and oxaloacetate (e.g., modular operation in illuminated plant leaves or in cyanobacteria and other bacteria under many conditions; see main text for details). Although the GABA shut accomplishes the same overall reaction as the 2-OGDC and SSADH, the genes encoding this pathway are only found in a few cyanobacteria. Nitrogen assimilation can include ammonium uptake, nitrate/nitrite reduction, N2 fixation, and/or cleavage of urea by urease. The glyoxylate shunt has been reported to occur in some cyanobacteria (e.g., Anacystis nidulans (Eley, 1988) and Cyanothecae spp. (Bandyopadhyay et al., 2011), but recent studies in Synechocystis sp. PCC 8803 indicate that it does not operate in this cyanobacterium (Knoop et al., 2013).
The genes encoding these two enzymes are organized in an apparent operon in \textit{Synechococcus} sp. PCC 7002 and numerous other cyanobacteria (but not in \textit{Synechocystis} sp. PCC 6803) (Fig. 2). Furthermore, these two genes are highly conserved and, with the exception of \textit{Prochlorococcus} spp. and some marine \textit{Synechococcus} spp. (the alpha cyanobacteria), are found in the majority of cyanobacteria with completely sequenced genomes (Zhang & Bryant, 2011). Although SSADH was correctly identified in most genome annotations, 2-OGDC is a novel thiamine pyrophosphate-dependent decarboxylase that is a member of the acetyl-CoA synthase family and thus is distantly related to IlvB (Fig. 2). 2-OGDH is also a TPP-dependent decarboxylase, and both decarboxylases are distantly related to one of the subunits of pyruvate dehydrogenase (Zhang & Bryant, 2011). It was possible to produce null mutants lacking either or both genes, and a significant growth defect was observed when 2-OGDC was over expressed. Because 2-OG is an essential precursor metabolite for heme, bilin, and chlorophyll biosynthesis, in addition to its role in amino acid and protein biosynthesis (Lancien et al., 2000), it is perhaps understandable that overexpression of 2-OGDC might dramatically lower the intracellular levels of 2-OG and cause a severe, negative impact on cell growth rate. This slower growth rate could also partly be due to the production of SSA, which is likely to be toxic and inhibit cell growth. A surprising and still unexplained result is that mutants lacking either the flavin- or Fe/S-cluster carrying subunits of succinate dehydrogenase exhibited no detectable growth defect, but mutations in the genes encoding either 2-OGDC or SSADH consistently grew slower than wild-type \textit{Synechococcus} sp. PCC 7002 (Zhang & Bryant, 2011). One possible interpretation of this result is that SSA is required to produce a compound that is not essential but has a positive effect on cell growth. An important consequence of these findings is that there is new biochemical intermediate, SSA, in a major pathway in cyanobacteria. For example, SSA can be converted in four steps into 1,4-butanediol (Yim et al., 2011), and potentially other useful compounds could use SSA as a building block.

**The Glyoxylate Shunt in Chlorophototrophic Bacteria**

The glyoxylate shunt is usually correlated with the ability of bacteria to metabolize acetate. All chlorophototrophic members of the Chloroflexi (i.e., \textit{Chloroflexus} spp., \textit{Oscillochloris trichoides} and \textit{Roseiflexus} spp.) have isocitrate lyase and malate synthase. All of these organisms can photoassimilate acetate, and some can grow heterotrophically on acetate using the glyoxylate shunt (Sirevåg, 1995; Zarzicki & Fuchs, 2011). Similarly, most purple sulfur bacteria can photoassimilate acetate and have the enzymes of the glyoxylate shunt, but genes encoding these enzymes appear to be missing in most purple non-sulfur bacteria. Members of the Chlorobi photoassimilate acetate by a different pathway, namely by carboxylation of acetyl-CoA. Finally, heliobacteria and \textit{Chloracidobacterium thermophilum} lack isocitrate lyase and malate synthase.

Until recently, the occurrence of the glyoxylate shunt in cyanobacteria has been controversial, but genome sequencing and improved biochemical analyses seem to provide clarification. Several older studies reported that certain cyanobacteria have isocitrate lyase and/or malate synthase activity (e.g., Eley, 1988; Pearce & Carr, 1967b) or could assimilate acetate (Hoare et al., 1967; Miller and Allen, 1972). However, a recent study showed that the enzymes of the glyoxylate shunt are not detectable in \textit{Synechocystis} sp. PCC 6803 (Knoop et al., 2013). Bandopadhyay et al. (2011) reported that two \textit{Cyanothecaceae} spp. (PCC strains 7424 and 7822) have a dicistronic operon encoding isocitrate lyase and malate synthase. When these genes are used as database queries of other cyanobacterial genomes, neither gene was present in \textit{Synechococcus} sp. PCC 7492/6301 (\textit{Anacystis nidulans}), \textit{Synechocystis} sp. PCC 6803, \textit{Synechococcus} sp. PCC 7002, \textit{Nostoc} sp. PCC 7120, nor other commonly studied cyanobacteria. Only the genomes of three \textit{Chlorogloeopsis} spp., \textit{Pleurocapsa minor} PCC 7327, \textit{Mastigocoleus testarum}, and \textit{Fischeraella} sp. PCC 9605 also contained genes encoding isocitrate lyase and malate synthase. The absence of these two genes in most cyanobacteria implies that they were likely to have been obtained recently by lateral gene transfer. The presence of these two genes in \textit{Chlorogloeopsis} spp. agrees with the demonstrated ability of this organism to assimilate acetate in both the light as well as the dark (Miller & Allen, 1972). Other reports concerning the capacity of cyanobacteria to photoassimilate acetate probably indicate a much more limited capacity for acetate uptake and incorporation and by process(es) that do not employ the glyoxylate shunt.

**Functional involvement of the TCA cycle in cyanobacterial metabolism**

Because at least some cyanobacteria (e.g., \textit{Prochlorococcus} and marine \textit{Synechococcus} spp.) apparently have incomplete TCA cycles (Zhang and Bryant, 2011), the discovery of the TCA cycle variant in other cyanobacteria raises the question, “is there an advantage to the acquisition of these genes in most cyanobacteria?” Given the broad distribution of these two genes, and given the fact that only some cyanobacteria are able to grow heterotrophically in the dark, it is clear that neither an incomplete TCA cycle nor the recently discovered TCA cycle shunt are solely responsible for the obligate photoautotrophic growth exhibited by some cyanobacteria (Meeks, 2011). Instead, the absence of transporters for potential carbon sources or some other limitation resulting from a metabolic control process probably restricts the growth of cyanobacteria in the dark. This also causes one to question whether TCA cycle variants utilize different
Fig. 2. Neighbor-joining phylogenetic tree of 2-OGDC (2-oxoglutarate decarboxylase) and IlvB (acetolactate synthase) homologs in different cyanobacterial species. The tree was generated using 1000 bootstrap resamplings, and bootstrap support values per 100 resamplings are shown for each node when the value was greater than 50%. IlvB from *Escherichia coli* was used as the out-group, and the tree shows that that 2-OGDC is distantly related to the paralogous IlvB family. The arrows to the right of the 2-OGDC portion of the tree show the organization of the genes encoding SSADH (succinic semialdehyde dehydrogenase, orange) and 2-OGDC (green), which are apparently encoded as an operon in many cyanobacteria as shown in the figure but are not colocalized in *Synechocystis* sp. PCC 6803.

**Abbreviations:**

SYN: *Synechocystis* sp. PCC 6803  
Npun: *Nostoc punctiforme* PCC 73102  
ANA: *Anabaena* sp. 90  
SYNPCC7002: *Synechococcus* sp. PCC 7002  
Lepto7376: *Leptolyngbya* sp. PCC 7376  
PCC8801: *Cyanothece* sp. PCC 8801  
NIES39: *Arthrospira platensis* NIES-39  
GVI: *Gloeobacter violaceus* PCC 7421  
Mic7113: *Microcoleus* sp. PCC 7113  
Oscil6304: *Oscillatoria acuminata* PCC 6304  
ECDH1: *Escherichia coli* DH1
regulatory molecules that might efficiently coordinate flux towards specific reactions and thus metabolic intermediates, which might be needed to respond to changes in environmental conditions. 2-OG has important regulatory functions, and its levels are monitored by signaling proteins such as GlnB (P_i) or CcmR (Jiang & Ninfa, 2009; Moorhead & Smith, 2003). The phosphorylation state of GlnB regulates central nitrogen assimilatory processes and is believed to be important in establishing control over carbon/nitrogen metabolism at various systems levels, it may be a good candidate for such control (Forchhammer, 2004; Steinhauser et al., 2012). It is demonstrated that GlnB proteins can act as sensors of the cellular adenylate energy charge (ATP/ADP) and 2-OG level. In response to these metabolic signals, GlnB can regulate many cellular processes, from nutrient transport to gene expression through several protein–protein interactions with GlnB receptor proteins (Forchhammer, 2004; Steinhauser et al., 2012). However, the regulatory mechanisms controlling the TCA cycle are still unclear and need more investigation. The LysR-type transcription factor, CcmR, controls the expression of genes encoding the carbon concentration mechanism of cyanobacteria (Daley et al., 2012). Surface plasmon resonance studies recently showed that 2-OG and NADP\(^+\) function as co-repressors of CcmR, which represses its own transcription along with structural genes encoding high affinity C\(_5\) transporters in Synechocystis sp. PCC 6803 (Daley et al., 2012). This indicates that CcmR is also likely to play an extremely important role in connecting central metabolism to inorganic carbon acquisition for photosynthesis.

Traditional biochemistry and related experimental approaches have provided much valuable knowledge about metabolism and physiology of cyanobacteria with regards to the regulatory mechanisms of the TCA cycle. However, flux balance analysis (FBA) and related methods (e.g., \(^{13}\)C-metabolic flux analysis; \(^{13}\)C-MFA) are comprehensive and powerful tools to investigate the organization of large-scale metabolic networks, can help to decipher predicted from genome analysis, and can unravel cell phenotype in cyanobacteria under different metabolic conditions (Knoop et al., 2013; Vu et al., 2013; Young et al., 2011). Flux balance analysis of phototrophic metabolism in cyanobacteria has shown that the main flux occurs within the Calvin–Benson cycle and that the TCA cycle mostly acts as an incomplete, branched pathway (Knoop et al., 2013; Steuer et al., 2012). This is consistent with the absence of a complete TCA cycle in some cyanobacteria and with the ability to construct single or double mutants lacking 2-OGDH and/or SSADH (Zhang & Bryant, 2011). On the other hand, FBA implies that dark consumption of glycogen in cyanobacteria should include cyclic flux through the TCA cycle, mediated by 2-OGDC and SSADH (Knoop et al., 2013; Steuer et al., 2012).

Some recent FBA models for Synechocystis sp. PCC 6803 also included the glyoxylate shunt to investigate the possible roles of this cycle in cyanobacteria (Fu, 2009; Knoop et al., 2013; Montagud et al., 2010; Shastry & Morgan, 2005). In the glyoxylate shunt, isocitrate lyase cleaves isocitrate to produce succinate and glyoxylate, and malate synthase then converts the glyoxylate into malate (Fig. 1). FBA models suggest that the glyoxylate shunt enzymes would be functional under some growth conditions. The analyses showed that the glyoxylate shunt is not active under photoautotrophic conditions but is predicted to be active under optimal heterotrophic conditions (Shastry & Morgan, 2005), which is consistent with \(^{13}\)C-MFA studies under mixo- and heterotrophic conditions (Yang et al., 2002). However, as noted above, the genes for isocitrate lyase and malate synthase are not present in Synechocystis sp. PCC 6803 and the majority of other cyanobacteria. Although enzyme activities have reportedly been detected in several cyanobacteria, these activities were not identified when more refined methods were employed with Synechocystis sp. PCC 6803 (Knoop et al., 2013). These results suggest two possibilities: (1) alternative enzymes that have not yet been purified and correlated to specific genes could function in acetate metabolism in some cyanobacteria; (2) the previously detected activities were due to non-specific reactions in the biochemical assays employed. Consistent with the latter interpretation, recent isotopic tracing studies indicate that the glyoxylate shunt is not functional and that glyoxylate is mainly used for glycine synthesis (You et al., 2014).

The possible roles of the gamma-aminobutyric acid (GABA) shunt (see Fig. 1) in the heterotrophic metabolism of cyanobacteria have also been investigated. A recent study reported that N-acetylmorphine aminotransferase from Synechocystis sp. PCC 6803 could also function as GABA aminotransferase, which together with glutamate decarboxylase forms the GABA shunt and thus closes the TCA cycle (Xiong et al., 2014). Genes encoding enzymes for the GABA shunt can be found in the genomes of some cyanobacteria (e.g., Synechocystis sp. PCC 6803, Nostoc sp. PCC 7107, Prochlorococcus marinus str. MIT 9303 and Synechococcus sp. RCC307). However, Prochlorococcus marinus str. MIT 9303 and Synechococcus sp. RCC307 lack the 2-OGDC/SSADH bypass, and many other Prochlorococcus and marine Synechococcus species seemingly lack the GABA shunt as well as the 2-OGDC/SSADH bypass. Such organisms either have yet another alternative bypass or must have a branched TCA cycle. Similarly, many other cyanobacteria (e.g., Synechococcus sp. PCC 7002) apparently lack the glutamate decarboxylase needed to complete the GABA shunt. Thus, the GABA shunt seems to be only present in a few cyanobacteria and does not appear to be universal (Xiong et al., 2014). From the point of view of FBA, the GABA shunt is stoichiometrically identical to the recently discovered TCA cycle variant using 2-OGDC and SSADH, and thus both variants should result in identical biomass yields. Interestingly, however, the biomass yield is lower than for the conventional cycle using 2-OGDH under respiratory...
metabolism conditions (Knoop et al., 2013; Shastri and Morgan, 2005). Based on the finding that autotrophic growth was similarly reduced when metabolites were forced through the 2-OGDH complex or the 2-O GDCC/SSADH bypass, but not by forced flux through the GABA shunt, it has been suggested that the GABA shunt may be an evolutionary favorable solution to completing the TCA cycle (Nogales et al., 2012) (Fig. 1). This may also be the reason for the existence of the GABA shunt but not the 2-O GDCC/SSADH bypass in some Prochlorococcus and marine Synechococcus species. Another possible advantage of having the TCA bypass rather than the 2-O GDH complexes may be related to protein synthesis and assembly. 2-O GDH is a highly complex, multi-subunit enzyme compared to 2-O GDH and SSADH as well as the enzymes encoding GABA shunt. Considering the relative unimportance of cyclic flux through the TCA cycle during phototrophic growth because of the products of the light reactions, such a difference in enzyme investment may result in a trade-off between enzymatic efficiency and enzyme synthesis costs (Knoop et al., 2013).

Recent 13C-MFA studies in Synechocystis sp. PCC 6803 showed that the 2-OG was converted to succinate when glutamate was added (You et al., 2014), in agreement with a previous study showing that mutants of Synechocystis sp. PCC 6803 lacking succinate dehydrogenase could still synthesize succinate when 2-oxoglutarate was supplied to the cells (Cooley et al., 2000). However, significant fractions of unlabeled 2-OG, succinate, and malate were observed after unlabeled glutamate was added to 13C-labeled cultures, which may be accomplished by the newly identified TCA bypass through SSA (You et al., 2014). Meanwhile, this study also indicated that the flux from 2-OG through the complete TCA cycle was very small compared to other metabolite fluxes (e.g. fluxes through glycolysis or the Calvin-Benson-Bassham cycle). A low rate of conversion of 2-OG to its downstream metabolites in the TCA cycle was also observed in Synechococcus elongatus PCC 7942 expressing 2-OG permease, in which 2-OG was mainly converted into glutamate and glutamine instead of downstream metabolites of the TCA cycle (Vázquez-Bermúdez et al., 2000). In summary, current evidence suggests that, although a complete TCA cycle exists in many cyanobacteria, there may only be a large flux through this cycle under specific growth conditions. The 2-O GDCC/SSADH bypass and the GABA shunt may mostly serve to regenerate intermediates of the cycle (e.g. SSA under certain conditions may be a useful substrate) or to fine-tune the metabolic balance under certain photomixotrophic conditions. Future research applying systems biology-oriented approaches that integrate data from the transcriptome, proteome, and metabolome may ultimately provide details of how the TCA cycle is regulated in cyanobacteria, as well as show how global metabolic fluxes are controlled under different environmental conditions in organisms that have variant TCA pathways (i.e., cycles vs. branched pathways).

TCA cycle in algae and plants

The main role of mitochondria in algae and plants cells, as in all eukaryotic cells, is the production of ATP, reducing equivalents and metabolic intermediates for use in biosynthesis elsewhere in the cell (Fernie et al., 2004; Nunes-Nesi et al., 2008). The TCA cycle is a central element of mitochondrial respiration linking glycolysis and complete oxidation of acetyl-CoA to the electron transport chain. The TCA cycle is also clearly embedded in a wider metabolic network that allows its activities to contribute to other aspects of metabolism. Although the TCA cycle mainly functions in mitochondria and all enzymes of the TCA cycle apparently occur in mitochondria, many TCA cycle enzymes are also found in other cellular compartments, such as the cytosol (e.g., aconitase, isocitrate dehydrogenase, succinyl-CoA ligase, fumarase, malate dehydrogenase) and peroxisomes (e.g., citrate synthase, isocitrate dehydrogenase, malate dehydrogenase) (Araújo et al., 2012). Whether the TCA cycle is also fully functional in cytosol is still not clear, because 2-OGDH, succinic dehydrogenase and citrate synthase are not located in cytosol (Araújo et al., 2012; Millar et al., 2011). Interestingly, malate dehydrogenase occurs in chloroplasts (Araújo et al., 2012), in which the TCA cycle is not functional due to the absence of all other TCA cycle enzymes. In C4 plants, malate dehydrogenase is an important, light-activated component of the C4 pathway for photosynthesis and CO2 concentration (Can et al., 1999). In C3 plants, the “malate valve” provided by this enzyme is thought to buffer short-term imbalances between light-driven reductant production and consumption (Backhausen et al., 1998).

It has been suggested that the TCA cycle in algae and plants often operates in a modular but not cyclic fashion, especially in plant leaves during daytime when the TCA cycle is mostly open and used to produce 2-OG, which implies that different parts of the cycle may have different metabolic functions depending on the physiological context in which that pathway is operational (Araújo et al., 2012; Sweetlove et al., 2010). In agreement with this idea, studies have shown that the level of accumulation of various organic acids can be extremely variable across species, developmental stages and tissue types in plants (Sweetman et al., 2012). These observations imply that the enzymes involved in the production and interconversion of these metabolic intermediates are tightly controlled. However, even though much progress has been made towards understanding the role of the TCA cycle enzymes in respiration (Nocito et al., 2007; Plaxton & Podestá, 2006), the precise regulation of this cycle, as well as its interactions with photosynthesis and photorespiration remains incomplete (Bauwe et al., 2010; Nocito et al., 2007; Nunes-Nesi et al., 2011). By using isotopic tracing techniques, one study verified that glycolysis and TCA cycle activities are inversely related to the ambient CO2/O2 ratio (Tcherkez et al., 2008). This study also found that high dihydroxyacetone phosphate to glucose-6-phosphate ratios can
lead to a reduction of TCA cycle activity, while TCA cycle activity and glutamate synthesis increased under low CO₂ conditions (Tcherkez et al., 2008).

Some progress has recently been made using systematic reverse genetics experiments to suppress the activity of each enzyme of the TCA cycle, and these studies have confirmed the importance of TCA cycle metabolism in illuminated leaves (Nunes-Nesi et al., 2008). However, these studies have also revealed a surprising complexity in the response. Suppression of some enzymes led to increased photosynthesis (Carrari et al., 2003; Nunes-Nesi et al., 2005), while suppression of others led to decreased photosynthesis (Nunes-Nesi et al., 2007a). While these experiments establish that the enzymatic reactions of the TCA cycle are clearly linked to photosynthetic performance, the mechanisms that underlie the observed responses still need to be fully elucidated. Some other interesting research recently demonstrated that reducing the activity of enzymes of the TCA cycle, such as citrate synthase or succinyl-CoA synthetase, produced relatively small changes in the rate of respiration within photosynthetic tissues (Sienkiewicz-Porzucek et al., 2008; Studart-Guimarães et al., 2007). It was suggested that this modest phenotype may due to compensatory up-regulation of the GABA shunt (see Fig. 1), which is known to be an important but only partly characterized plant pathway, and which is associated with numerous physiological responses (Bouché and Fromm, 2004; Fait et al., 2008). Microarray profiling of transcript levels for genes encoding components of the TCA cycle and GABA shunt have revealed that they are differentially regulated (Fait et al., 2008). It seems reasonable to assume that flux through the GABA shunt increases in response to changing environmental conditions, and the GABA shunt could serve as an adaptive mechanism to maintain the rate of respiration under certain stress situations (Fait et al., 2008).

In algae and plants, the glyoxylate cycle operates in the peroxisome/gloxysosome, providing an effective route to convert C₂-units to C₄-precursors for biosynthesis, which allows growth on fatty acids and C₂-compounds such as acetate and ethanol (Eastmond & Graham, 2001; Kunze et al., 2006). In *Chlamydomonas reinhardtii*, it has been demonstrated that the glyoxylate shunt is required for dark growth on acetate, as well as for efficient growth in the light when acetate is supplied (Plancke et al., 2014). Furthermore, purified isocitrate lyase from *Chlamydomonas reinhardtii* was shown to be inactivated by glutathionylation and reactivated by glutaredoxin, which implies that the glyoxylate shunt may be regulated under specific environmental conditions (Bedhomme et al., 2009). However, the functional significance of these post-translational modifications in response to different growth conditions is not yet well understood. In *Arabidopsis thaliana*, the glyoxylate shunt, together with the β-oxidation of fatty acids, are especially important metabolic pathways in providing carbohydrates and biosynthetic precursors which cannot be generated by photosynthesis during the early stage of seedling establishment (Eastmond & Graham, 2001; Graham, 2008). The β-oxidation pathway and glyoxylate shunt enzymes are induced for the breakdown of membrane lipids and gluconeogenesis in senescing leaves (Chen et al., 2000). However, the coupling of β-oxidation pathway with the glyoxylate cycle is not essential for seed germination in mutants lacking the key enzyme, isocitrate lyase, of the glyoxylate cycle because photosynthesis can compensate for the absence of the glyoxylate cycle during postgerminative growth (Eastmond et al., 2000; Eastmond & Graham, 2001). However, the glyoxylate cycle plays an important role in seedling survival and recovery during prolonged dark conditions that frequently occur in nature (Eastmond et al., 2000). Furthermore, the linkage between the glyoxylate cycle and β-oxidation was only found to occur in aleurone cells but was absent in embryos of germinating barley (Holtman et al., 1994). How the glyoxylate cycle and β-oxidation pathways coordinate interact, and the possible regulatory mechanisms that occur during seedling establishment and postgerminative growth, are still important questions to be answered.

### 2-OG as a key regulatory point

Although studies with cyanobacteria have provided much useful knowledge illustrating the occurrence and functioning of TCA cycle variants in prokaryotes, a few algae and possibly plants, the physiological impact(s) of TCA cycle variants are still not clear. One notable difference is that the GABA shunt and the 2-OGDC/SSADH bypass do not produce guanosine triphosphate by substrate-level phosphorylation. Another possibly important point is that the replacement enzymes in these variants produce NADPH rather than NADH, which implies that the bypass reactions might be regulated by sensing the NADPH:NAD⁺ ratio. It has been suggested that one possible function of TCA cycle variants is associated with responses to low oxygen conditions (Green et al., 2000; Tian et al., 2005). Accordingly, one example of the plasticity of the TCA cycle in response to a low oxygen environment was provided by the metabolic responses to waterlogging in the roots of *Lotus japonicas*, which led to the modular operation of a split TCA cycle (Rocha et al., 2010). Another example of the physiologically diverse uses of the TCA cycle is provided by *Pseudomonas fluorescens*, which is able to generate crucial metabolites such as oxalate, ATP and NADPH, and is able to diminish the synthesis of NADH and CO₂ evolution under aluminum stress (Singh et al., 2009). Under aluminium-stress conditions, expression levels of isocitrate lyase and acetylating glyoxalate dehydrogenase are upregulated, leading to enhanced synthesis of oxalate and NADPH. Furthermore, activities of succinyl-CoA synthetase and oxalate CoA-transferase also increase, which provide an effective route for ATP synthesis. These modifications of the TCA cycle appear to be of crucial
significance in the adaptation to oxidative stress owing to production of increased amounts of the antioxidant NADPH and decreased amounts of NADH (Mailloux et al., 2007). Furthermore, 2-OG has also been shown to be involved in the detoxification of reactive oxygen species (Mailloux et al., 2007). Finally, as noted above, 2-OG also functions as a co-repressor for the LysR-type transcriptional regulator, CcmR, which represses its own transcription as well as that of the structural genes encoding high affinity C₄ transporters in Synechocystis sp. PCC 6803 (Daley et al., 2012). These results all indicate that the newly discovered TCA cycle shunt and the GABA shunt might play important roles in responding to the changes of carbon sources and oxygen concentrations in the environment, possibly by controlling the concentrations of 2-OG and NADPH inside the cell.

In close accordance with the well-established role of 2-OG in many important cellular processes (e.g. ammonia assimilation, regulation of nitrogen and carbon metabolism, precursor metabolite for heme, bilins, and chlorophylls, etc.), several observations show that 2-OG levels in plant cells can reflect the C/N status (Foyer et al., 2011; Lancien et al., 2000; Nunes-Nesi et al., 2010), which implies that 2-OG also plays a signalling role in land plants. Previous results showed that chemical inhibition of the 2-OGDH complex in potato tubers had a clear impact on respiration (Araújo et al., 2008). More pronounced effects were also observed following the inhibition of this enzyme in tomato roots (van der Merwe et al., 2010), which indicates that 2-OG levels are closely correlated with respiratory activities. Furthermore, it has been suggested that the rates of respiration in the light are lower than in the dark in plants. However, indicating that nitrogen metabolism interacts closely with respiration, daytime respiration plays an important role in carbon and nitrogen metabolism and provides at least some of the 2-OG and NADH that are required for nitrogen metabolism (Fig. 1) (Nunes-Nesi et al., 2010; Stitt et al., 2002). Interestingly, some studies indicate that the TCA cycle is almost completely inhibited in illuminated leaves (Tcherkez et al., 2005), while others have found that there is still significant TCA cycle activity in the light (Nunes-Nesi et al., 2007b). Moreover, genetic manipulations of the TCA cycle and mitochondrial electron transport components have produced effects on N metabolism and related processes (Foyer et al., 2011). When illuminated leaves of Brassica napus were incubated with ¹³C- and ¹⁵N-ammonium nitrate, it was found that, while there is considerable incorporation of nitrogen into the newly synthesized glutamate and glutamine, the majority of the carbon in these metabolites is not derived from concurrent CO₂ assimilation (Gauthier et al., 2010). This study showed that remobilization of 2-OG that accumulated during the previous light and dark cycle is probably sufficient to support subsequent glutamate synthesis in the light. Taken together, the results demonstrate that 2-OG serves as a key metabolite for regulating metabolic changes and rechanneling of intermediates between the TCA cycle and nitrogen metabolism.

Finally, in some cyanobacteria the newly discovered TCA cycle bypass provides an additional connection between carbon and nitrogen metabolism through the GABA shunt and SSA, which might serve as a key point for regulation (Fig. 1). Although the results of such metabolic reprogramming have not yet been determined in cyanobacteria, it might be worthwhile to manipulate gene expression levels of the TCA cycle enzymes and investigate the physiological and metabolic changes that occur in cyanobacteria under different stress conditions (e.g. O₂ limitation, carbon limitation, and nitrogen limitation).

Evolutionary relationships and endosymbiosis

It is generally accepted that eukaryotes originated from an ancestral, nucleated heterotroph by the engulfment of free-living bacteria through a process termed endosymbiosis or endocytobiosis (Gray et al., 2001; Martin et al., 2002). This led to organelles with new functionalities for these cells (i.e., mitochondria for respiration and chloroplasts for oxygenic photosynthesis). Mitochondria are generally thought to have arisen from engulfment of an alpha-proteobacterium (Gray et al., 2001), and eukaryotes gained chloroplasts and the capacity for oxygenic photosynthesis through primary endosymbiosis of a cyanobacterium-like cell (Hohmann-Marriott & Blankenship, 2011). Many genes from the cyanobacterial endosymbiont were transferred to the nucleus of the eukaryotic host, leading to the evolution of algae and subsequently plants (Deusch et al., 2008; Reyes-Prieto et al., 2007; Rujan & Martin, 2001). The transferred genes encoded proteins involved in photosynthesis, respiration, and many other metabolic processes, including regulatory functions (Kern et al., 2011; Martin et al., 2002). Although the TCA cycle is typically a mitochondrial pathway in eukaryotes, all of the enzymes of the TCA cycle are encoded in the nucleus in algae and plants (Schnarrenberger & Martin, 2002). A similar situation exists for genes of the Calvin-Benson-Bassham cycle and chloroplasts; nearly all of the genes of this plastid-localized pathway are encoded in the nucleus (Martin & Schnarrenberger, 1997). It has been suggested that prior to the evolution of a molecular machine to import proteins from the host cell cytoplasm into the symbiont, many proteins involved in central metabolic pathways that once belonged to the symbiont could have been transferred to the cytosol after the genes encoding these proteins had been incorporated into the host chromosome (Martin & Miklós, 1998). However, because some enzymes (e.g. succinate dehydrogenase) of the TCA cycle would have been located on the cytoplasmic membrane of the mitochondrial ancestor, a complete transfer of the enzymes in this pathway to the cytosol initially could not occur, and this limitation might have restricted the TCA cycle to mitochondria. In order for these nuclear-encoded proteins to be transported and be functional...
in the symbiont, the genes that were incorporated into the host chromosome must have eventually obtained signal sequences in order to be translocated back to the symbiont. In accordance with this idea, it has been shown that the \( \text{psaE} \) gene, which is located on nuclear genome of \textit{Paulinella chromatophora} (Nakayama & Ishida, 2009), has gained four potential non-AUG translation initiation codons upstream of the previously proposed start codon. One of these non-AUG start codons appears to initiate translation of the associated signal peptide that allows translocation of Psae into the chromatophores (Mackiewicz & Bodyl, 2010; Nakayama & Ishida, 2009).

Detailed phylogenetic analyses indicate that all plant TCA enzymes, except cytosolic aconitase and mitochondrial isocitrate dehydrogenase, are clearly more similar to eubacterial homologs than they are to archaeal homologs (Schnarrenberger & Martin, 2002), and the reductive TCA cycle is a major pathway in many archaea (Schäfer et al., 1989). However, phylogenetic analyses also show that only about half of the enzymes of the TCA cycle in plants can specifically be traced back to an alpha-proteobacterial donor. Origins of the rest of the enzymes in this pathway in plants either have nearly equal similarity to homologs in alpha-proteobacteria and archaea (isocitrate dehydrogenase), or they are more similar to enzymes from eubacterial donors but not specifically to those from an alpha-proteobacterial source (e.g. malate dehydrogenase) (Schnarrenberger & Martin, 2002). Adding to this complexity is the fact that algae and plants contain both mitochondria and chloroplasts. Considering that variants of TCA cycles exist in both cyanobacteria, algae and plants, a question arises as to whether the genes for enzymes of the TCA cycle in cyanobacteria after primary endosymbiosis were transferred to the chromosome of algae and plants and how those would have interacted with the genes that were already present on the chromosome. Alternatively, the TCA cycle genes could simply have been completely lost from the endosymbiotic cyanobacterium during evolution. Some studies imply that the non-alpha-proteobacterial components of the TCA cycle are encoded by genes that were originally present in the ancestral eukaryotic host before the arrival of the mitochondria, and that the products of those genes eventually replaced the products of the genes in the evolving mitochondrion (Gray, 2014). Serving as an example of a very recent primary endosymbiotic event, the recently sequenced genome of the chromatophores in \textit{Paulinella chromatophora} provides tantalizing information. The genes for the TCA cycle are already missing in these chromatophores and presumably have been lost or transferred to the nucleus (Nowack et al., 2008). In the recently sequenced genome of the evolutionarily important Glaucophyte, \textit{Cyanophora paradoxa}, genes encoding 2-OGDH, KFOR, 2-OGDC and SSADH are all missing, although it contains genes for the remaining enzymes in the TCA cycle (Price et al., 2012). This result strongly implies that, like some cyanobacteria, \textit{C. paradoxa} has a branched TCA cycle or that it has some other undescribed TCA cycle variant for the conversion of 2-OG into succinate. Future genome sequencing, phylogenetic analyses of additional cyanobacteria and selected algae, and further biochemical studies, may provide answers to these unresolved questions.

Conclusion

Cyanobacteria, algae and plants are oxygenic photosynthetic organisms that are projected to play a pivotal role in supplying future renewable energy needs, and thus our ability to understand and utilize these organisms efficiently is extremely important. This capability is directly dependent on fundamental studies to understand key metabolic interactions and regulatory processes in these organisms. As such, the recent identification of a complete, non-canonical, TCA cycle in most cyanobacteria corrects a long-held misconception that these organisms have an incomplete TCA cycle due to the absence of 2-OGDH. This discovery provides interesting insights into metabolic and pathway evolution and also provides new opportunities for biotechnological applications through metabolic engineering. Furthermore, this discovery should help to understand carbon–nitrogen balance better in algae and plants.

The vast amounts of rapidly accumulating genome sequence data from prokaryotic and eukaryotic sources will continue to provide opportunities to study the molecular evolution of individual enzymes as well as of complete pathways with multiple enzymatic reactions. Given the subcellular compartmentation inherent in eukaryotes, studies of the transport processes linking organellar metabolic processes to their interactions with nuclear-encoded genes are also of high interest. Additionally, it will be worthwhile to investigate the metabolic rationale for the acquisition and incorporation of new genes/enzymes into the TCA cycle during evolution, because this knowledge could help those attempting to maximize photosynthetic efficiency in engineered cyanobacteria and photosynthetic eukaryotes. Considering the mosaic distribution of TCA cycle variants in cyanobacteria, algae and plants, it will also be interesting to attempt to define the evolutionary events that produced this diversity.

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Reprogramming the glycolytic pathway for increased hydrogen production in cyanobacteria: metabolic engineering of NAD$^+$-dependent GAPDH

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Catabolism of glycogen stored by cyanobacteria occurs during anaerobic auto-fermentation and produces a range of C1–C3 fermentation products and hydrogen via hydrogenase. We investigated both augmenting and rerouting this carbon catabolism by engineering the glycolysis pathway at the NAD$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH-1), its major regulation site at the nexus of two pathways (Oxidative Pentose Phosphate pathway, OPP, and glycolysis/glucoseogenesis). Null (gap1::aphII) and overexpression (gap1$^+$) strains of Synechococcus sp. strain PCC 7002 were constructed in order to produce more NADPH (via rerouting carbon through OPP) and more NADH (via opening the glycolytic bottleneck), respectively. For gap1$^+$:aphII quantitative analyses after four days of dark auto-fermentation showed undiminished glycogen catabolism rate, significant increases of intracellular metabolites in both OPP and upper-glycolysis, decrease in lower-glycolysis intermediates, 5.7-fold increase in NADPH, 2.3-fold increase in hydrogen and 1.25-fold increase in CO$_2$ vs. wild type (WT). These changes demonstrate the expected outcome of redirection of carbon catabolism through the OPP pathway with significant stimulation of OPP product yields. The gap1$^+$ strain exhibits a large 17% increase in accumulation of glycogen during the prior photoautotrophic growth stage (glucoseogenesis), in parallel with a 2-fold increase in the total [NAD$^+$ + NADH] pool, foreshadowing an increased catabolic capacity. Indeed, the rate of glycogen catabolism during subsequent dark auto-fermentation increased significantly (58%) vs. WT, resulting in increases in both NADH (4.0-fold) and NADPH (2.9-fold) pools, and terminal fermentation products, hydrogen (3.0-fold) d-lactate (2.3-fold) and acetate (1.4-fold). The overall energy conversion yield over four days from catabolized glycogen to hydrogen increased from 0.6 mole of hydrogen per mole of glucose (WT) to 1.4 (gap1::aphII) and 1.1 (gap1$^+$) under headspace accumulation conditions (without hydrogen milking). These findings demonstrate the significant potential of metabolic engineering for redirecting carbon pathways for carbohydrate catabolism and hydrogen production in cyanobacteria.

Broader context

Hydrogen, the most abundant element in the universe, exists in trace amounts in its molecular form on Earth’s atmosphere. Hydrogen has a specific enthalpy content (MJ kg$^{-1}$) more than twice that of methane and three times that of coal, and when combusted releases energy and water as the sole products. As a carbon-free, energy-intensive molecule it would be the first choice for renewable fuel if it could be produced economically from water and sunlight. Microbial systems, including some cyanobacteria possessing [NiFe]-hydrogenases, produce and exchange hydrogen efficiently under near reversible conditions via autofermentation of carbohydrates, but typically at low fluxes and low yields. As cyanobacteria produce their own carbohydrates via photosynthesis their potential as solar driven hydrogen producers is being investigated worldwide. Anaerobic catabolism of glycogen produces NAD(P)H – the precursor to hydrogen – through glycolysis (EMP pathway). Here we have applied two metabolic engineering strategies to increase the NAD(P)H levels, first by stimulating glucoseogenesis and glycolysis to produce more NADH, and secondly by rerouting glycogen catabolism through the oxidative pentose phosphate pathway (OPP) to generate even more NADPH. Both strategies work to substantially increase intracellular NAD(P)H levels and improve the hydrogen yield and flux in cyanobacteria, especially when coupled with hydrogen uptake systems.
Introduction

An economically viable, renewable and carbon-neutral production process at any scale is still lacking for molecular hydrogen. Although hydrogen production from water by photosynthetic cyanobacteria and algae is a natural process, it occurs only under certain conditions and suffers from low solar-to-hydrogen conversion yield and rate. Consequently, it is not economically feasible. However, this approach remains promising based on development of transgenic strains. For hydrogen yields and rates of these organisms to be improved significantly, a deeper understanding of their multiple metabolisms and their response to environmental stresses is needed.

Cyanobacteria can survive in a wide range of environmental conditions including extremes of light, salinity, pH, temperature and oxygen. They often face long periods of darkness and anoxia during which they are unable to generate ATP by photophosphorylation or aerobic respiration. Selected strains of cyanobacteria can survive as facultative anaerobes, generating the ATP needed for cell maintenance through auto-fermentative metabolism on glycogen. During auto-fermentative metabolism, the photosynthetically derived storage carbohydrates are catabolized to form a finite range of excreted C1-C3 fermentation products and hydrogen via hydrogenase, the accompanying substrate-level phosphorylation yields ATP for cellular processes.

*Synechococcus* sp. strain PCC 7002 (hereafter, *Synechococcus* 7002), is a unicellular, fast-growing, non-diazotrophic model cyanobacterium that produces hydrogen via a bidirectional NiFe-hydrogenase during dark anoxic conditions. Under these conditions this strain catabolizes intracellular carbohydrates through glycolysis in order to generate ATP needed for cellular maintenance. Through glycolysis 2 ATP and 2 NADH molecules are formed per glucose catabolized (Fig. 1a). As a result, the NADH:NAD^+ ratio increases and ways of regenerating...
NAD$^+$ must exist in order for glycolysis to continue. The reduction of pyruvate to organic acids such as lactate consumes the NADH formed and regenerates NAD$^+$. In addition, when the cell redox poise reaches a threshold level, the bidirectional hydrogenase starts reducing protons to hydrogen further recycling NADH. It was shown that by inactivating the α-lactate dehydrogenase gene (ldhA) of *Synechococcus* 7002, which is the main pathway that consumes NADH during fermentative metabolism, the NADH:NAD$^+$ ratio was increased and consequently the hydrogen yield was increased 5-fold. Similarly, when glycolytic catabolic rates were decreased in a glycolyn-less mutant (glcC::aphII) the NADH:NAD$^+$ ratio decreased and consequently the hydrogen yield decreased 4-fold. These results clearly show that there is a relationship between the redox state of the cell and the fermentative hydrogen yields. Thus, strategies that aim at increasing the intracellular redox poise under dark anaerobic should be pursued.

The oxidative pentose phosphate pathway (OPP) shares the precursor glucose-6-phosphate with glycolysis and converges again with it at the level of glyceraldehyde-3-phosphate (GAP) (Fig. 1a). Catabolism through OPP and lower-glycolysis, despite again with it at the level of glyceraldehyde-3-phosphate (GAP) and greater NADH production. Here we demonstrate that both kinetic bottleneck thus allowing increased glycolytic catabolism and greater NADH production. We pursued two metabolic engineering approaches: (i) deletion of the *gap1* gene and (ii) over-expression of GAPDH-1 in order to relieve the metabolic engineering strategies aimed at enhancing the catabolic rate of OPP and glycolysis yield positive results showing substantially increased pool sizes of NAD(P)H, which in turn led to increased hydrogen production.

**Experimental section**

**Genetic engineering of GAPDH-1**

In order to generate the *gap1* deletion mutant of *Synechococcus* 7002 (*gap1::aphII*), the coding sequence of the *gap1* gene (*SYN-PCC7002_A2697;* http://genome.kazusa.or.jp/cyanobase) was replaced by a kanamycin resistance cassette (*aphII*), using a homologous recombination strategy (Fig. 2a). Transformation and selection were performed as previously described. The complete segregation of alleles was verified by PCR after DNA extraction from wild type and mutant strains (Fig. 2b). The primers used are listed in Table 1.

The *gap1* over-expression strain (designated *gap1*') was made by cloning the *gap1* gene into the pAQ1-based expression system as previously described. The resulting plasmid pAQ1Ex-*PcpcA::gap1* was transformed into the *gap1::aphII* mutant to produce a strain overexpressing GAPDH-1. The presence of *gap1* gene was verified through PCR (Fig. 2c) by using primers positioned within the *gap1* coding sequence. To confirm the expression of GAPDH-1 in the over-expression strain, an RT-PCR reaction was conducted after RNA extraction from WT and *gap1*’ strains (Fig. 2d).

**Strains and culture conditions**

Wild-type *Synechococcus* 7002, the *gap1* mutant and *gap1*’ over-expression strain were grown as described elsewhere. In brief, all three strains were grown photoautotrophically in A medium, supplemented with 2 μM NiCl$_2$ and were sparged with 2% (v/v) CO$_2$ in air. Cells were grown to densities of approximately 10$^6$ cells per mL at 38 °C with a light intensity of 200 μmol photons per m$^2$ s. The strains were then switched to dark anaerobic conditions to induce fermentative metabolism.

**RT-PCR**

Total RNA from each strain was prepared using the High Pure RNA Isolation Kit (Roche Applied Science) by following the protocol recommended by the manufacturer. Total RNA and DNA concentrations were separately determined by using the Quant-iT™ RNA Assay Kit and Quant-iT™ DNA Assay Kit (Invitrogen). Final DNA concentration was controlled to be less than 5% of the RNA concentration. The same amount of RNA (15 ng) was used as template to perform RT-PCR reaction using the Tetro One-Step RT-PCR Kit (Bioline). Control experiments were performed using the same cycle settings but without the reverse transcription step. After PCR, an equal volume of PCR solution was analyzed by electrophoretic agarose gel to compare the differences. For *gap1* expression analyses primer pair RTA2697F and RTA2697R was used, while for *gap2* (*SYN-PCC7002_A0106*) expression analyses primer pair RTA0106F and RTA0106R were used (Table 1).
Fermentative conditions
The cells from 25 mL cultures were harvested by centrifugation when they reached nearly 1.2 OD
730 nm and were washed once with nitrate-free medium A.
Cells were resuspended in 25 mL of nitrate-free medium A. Aliquots of (5 mL) cells were placed in 10 mL vials and were then sealed with crimp-top Te
(on lined rubber stopper and wrapped with aluminium foil to create dark conditions. The headspace was purged for 20 min with argon gas to create anoxic conditions. Four replicates were prepared for each WT and mutant strain for each time point.

Extracellular metabolite analysis (GC and HPLC)
At each timepoint a dark fermentation, hydrogen in the headspace was measured by gas chromatography (GC). The headspace gas was sampled (200 mL) using a gas-tight syringe and analysed with a Perkin Elmer Clarus 680 gas chromatograph equipped with a thermal conductivity detector and argon as the carrier gas. The run parameters were same as reported by Guerra et al.12 At each time point, the spent media was separated from cells by centrifugation and 50 mL aliquots were analysed for lactate, acetate and succinate using an HPLC (Perkin Elmer) equipped with a Rezex ROA column (300/C27.8 mm, Phenomenex) at 45/C14 C and 0.005 N H2SO4 as mobile phase (0.5 mL min/C1 flow rate) with UV detection at 210 nm. Quantification was made using a calibration curve of organic acids in A+/ medium. For each timepoint measurements, four replicates were used and their mean values were considered for statistical comparisons.

Intracellular total carbohydrates analysis (anthrone assay)
Total reducing carbohydrates were assayed at several time-points of fermentation using the anthrone reagent.30 Briefly, cells were boiled in 12 N H2SO4 for 1 h and then the anthrone reagent (0.2 g of anthrone dissolved in 71 mL of concentrated H2SO4 + 29 mL de-ionized water) was added and placed over ice bath. The cells were further boiled for a few more minutes until a clear gradient in color developed in the standards. The cells were immediately placed in an ice bath to stop the reaction. The absorbance was measured at 620–820 nm for both biological samples and standards. The total reducing carbohydrate concentration in the biological samples was derived by comparison to the plotted standard curve. The two-way analysis of variance (ANOVA) was employed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA), to determine the statistical difference of the catabolic rates among the three strains.
Intracellular metabolite pool analysis (LC-MS/MS)

Metabolite extraction and analysis was done as previously described. The cells (1 mL) from the fermentation vials were sampled using a 1 mL syringe and were immediately vacuum-filtered onto a 0.45 μm membrane filter under dark conditions. The membrane filters were quickly inverted into 1.8 mL of 80 : 20 MeOH/H2O (precooled to −20 °C) in clean Petri dishes. After 20 min of incubation at −20 °C, the cell material was scraped off the membrane filters and the solvent with the cells was transferred to microfuge tubes. The solvent was centrifuged at 14,000 × g at 4 °C for 5 min, and the supernatant was removed and stored at −20 °C. The solvent (120 μL) was vacuum-dried (Labconco Centri-Vap Concentrator), and the pellet was resuspended in LC-MS grade water (40 μL) and transferred to LC-MS vials for analysis. The metabolites were analysed by injecting 10 μL of sample into an Agilent 1200 series HPLC coupled to a Model 6490 QQQ mass analyzer equipped with an ion spray source (Agilent Technologies, Waldbronn, Germany). The samples were separated using a XRs 3 C18 column (50 × 2.0 mm, Agilent Technologies) with gradients of 11 mM acetic acid + 10 mM tributylamine in water as solvent-A and methanol as solvent-B with a flow rate of 0.33 mL min⁻¹. The solvent-B was increased from 0 to 40% (0–8 min) and maintained at 40% for 2 min (8–10 min). Further, solvent-B was increased to 90% (10–12.5 min) and maintained for an additional 5.5 min (12.5–18 min), and then switched back to 100% solvent-A at 18.1–20.9 min. The MS was operated in negative ionization mode and the data acquired was analysed using Agilent Mass Hunter software (Build 1.04).

Results and discussion

Catabolic rate of photosynthetically accumulated carbohydrates

In the studies reported here, we have modified the glycolytic pathway of the cyanobacterium Synechococcus 7002 in two ways to increase the intracellular NAD(P)H and further to increase hydrogen production: (i) by creating a gap1 deletion mutant in an attempt to divert carbon catabolism through the OPP pathway; and (ii) by creating a gap1 overexpression strain in an attempt to increase glycolytic catabolism. In both cases the metabolic modification was aimed at increasing intracellular reductant availability during dark anoxic periods in order to increase hydrogen yield via the bidirectional NiFe-hydrogenase.

Because glycolysis involves glucose catabolism and GAPDH-1 is involved in regulating the catabolic rate of glycolysis and potentially OPP, we examined the catabolic rate of carbohydrate consumption during fermentation in the gap1::aphII mutant, the gap1⁺ and WT strains using the anthrone assay. The gap1⁺ strain accumulated the highest amounts of total carbohydrates (61.9 μg of glucose equivalents 10⁸ per cells), followed by WT (52.9 μg of glucose equivalents 10⁸ per cells) and gap1::aphII mutant (41.05 μg of glucose equivalents 10⁸ per cells) after photoautotrophic growth. As shown in Fig. 3, the gap1 overexpression strain produced a 17% increase in glycogen content, significantly above WT (P ≤ 0.001), while the gap1 deletion mutant produced a 22.4% decrease in glycogen content, significantly below WT (P ≤ 0.001). In terms of carbohydrate consumption the gap1⁺ strain had an average catabolic rate of 4.48 ± 0.48 μg of glucose 10⁶ per cells per day. The catabolic rate of the gap1⁺ strain was significantly higher than the other two strains (P ≤ 0.001), determined using two-way ANOVA. The gap1::aphII mutant and WT strain had similar catabolic rates (2.64 ± 0.81 and 2.84 ± 0.58 μg of glucose 10⁶ per cells per day, respectively) with no significant difference (Fig. 3).

Glycolytic metabolite levels in the gap1::aphII and gap1⁺ strains of Synechococcus 7002

In order to understand the impact of the genetic modifications introduced in the glycolytic pathway, the pools of glycolytic metabolites were measured in the WT and mutant strains after 96 h of fermentation using LC-MS/MS (Fig. 4). In our first approach, impairing glycolysis by deletion of the gap1 gene was
expected to reroute carbohydrate catabolism through the OPP pathway under fermentative conditions. When compared to the WT the gap1::aphII mutant accumulated higher levels of upper-glycolytic metabolites such as glucose-6-phosphate (1.4-fold) and GAP (2.3-fold) but decreased amounts of lower-glycolytic metabolites; the 3-phosphoglycerate and phosphoenolpyruvate levels were 0.4 and 0.5-fold lower, respectively. Although the measurement of steady-state metabolite pools is not a direct indication of flux through a pathway, the observations that the gap1::aphII mutant accumulated lower levels of lower-glycolytic metabolites along with higher levels of upper-glycolytic metabolites, OPP metabolites and NADPH compared to the WT strain, support the occurrence of increased carbon catabolism through the OPP in this mutant. These results are consistent with an expected reduced glycolysis rate due to the deletion of the gap1 gene. In spite of the absence of a functional GAPDH-1 in the gap1::aphII mutant, the photoautotrophic growth of the mutant was not different than that of the WT strain, although it accumulated 22.4% less glycogen. Hence, gap1::aphII mutant was expected to have a functional replacement for GAPDH-1. This might be due to a bifunctional role of the mutant was not different from the WT (Fig. 4). This might be due to a bifunctional role of the NADP+-dependent GAPDH (GAPDH-2) encoded by gap2 gene, although it may not be a functional replacement for GAPDH-1. However, this mutant accumulated lower but still readily detectable amounts of lower-glycolytic metabolites compared to the WT (Fig. 4). This might be due to a bifunctional role of the NADP+-dependent GAPDH (GAPDH-2) encoded by gap2 gene, although it may not be as efficient as the GAPDH-1. The pairwise alignment of the amino acid sequences of GAPDH enzymes encoded by gap1 and gap2 of Synechococcus 7002 revealed a 49% identity. GAPDH-2 encoded by gap2 is mainly functional in the Calvin–Benson–Bassham cycle fixing CO₂ and in gluconeogenesis, while GAPDH-1 functions exclusively in glycolysis.\[^{18}\] RT-PCR targeting the gap2 gene in both phototrophic and dark-fermentative conditions showed that the expression levels of gap2 were unchanged in these two conditions and also were unchanged in WT and gap1::aphII mutant (Fig. 5). These unchanged expression levels for gap2 during dark fermentative conditions suggests that GAPDH-2 might be able to function in glycolysis by oxidizing GAP to 1,3-bis-phosphoglycerate, although not as efficiently as GAPDH-1. This finding is in agreement with Valverde et al.\[^{28}\] who showed the glycolytic activity of the gap2 gene product of Synechocystis sp. strain PCC 6803 by functional complementation of an Escherichia coli gap mutant. In contrast, Koksharova et al.\[^{18}\] found that catabolic activity of gap2 gene in Synechocystis sp. PCC 6803 was absent under heterotrophic growth on glucose. They also reported that the gap2 gene product has dual co-substrate specificity for NADH and NADPH, unlike Gap1 which utilized only NAD+/NADH. It is thus possible that, in Synechococcus 7002, GAPDH-2 functionally replaces GAPDH-1 in the gap1 mutant using NADP+ or NAD- as oxidant.

In our second approach we overexpressed the gap1 gene by placing its expression under the control of a very strong cpcBA promoter on the high copy-number plasmid pAQL. We expected to increase the glycolytic catabolic rate leading to the production of increased levels of NADH, which would have to be consumed by forming β-lactate and hydrogen, thus increasing the levels of fermentative end metabolites. The gap1+ strain accumulated increased amounts of lower-glycolytic metabolites compared to the WT: 3-phosphoglycerate increased 2.0-fold, phosphoenol pyruvate increased 2.1-fold, and pyruvate increased 2.6-fold. Interestingly, GAP, the substrate for GAPDH-1, also increased 2.4-fold, and the levels of glucose-1-phosphate and glucose-6-phosphate remained unchanged. This outcome on steady-state pool sizes suggests partial alleviation of the glycolytic bottleneck to downstream steps past pyruvate. Taken together with the 17% higher yield of photoautotrophic accumulation of glycogen and its higher anaerobic catabolic rate observed in the gap1+ mutant (Fig. 3), these results are consistent with increased pool sizes in both gluconeogenesis and glycolysis. Additionally, gap1+ exhibits no spillover of carbon into intermediates of the OPP pathway (Fig. 6a), while also accumulating two-fold more total glycolytic pyridine nucleotides, [NADH]+[NAD+] (Fig. 6b). Both outcomes are consistent with a greater mass flux through glycolysis. However, the observed two-fold higher redox poise [NADH]/[NAD+] (Fig. 6c) is predicted to cause allosteric down regulation of GAPDH specific activity which acts opposite to the greater mass flux and thus reacts to retain the glycolytic bottleneck at GAPDH. Evidence for allosteric regulation of GAPDH by the NADH/NAD+ ratio was reported by Garrigues et al. in Lactococcus lactis, as the major regulating factor inhibiting GAPDH and leading to higher accumulation of GAP and greater formation of lactate via LDH.\[^{21}\] The Michaelis constant (Kₘ) for NAD+ reduction by GAPDH (the glycolytic isoform) determined from in vitro assays was found to vary among different organisms in the range from 50 to 258 μM concentration (Table 2). Based on these reported values for Kₘ we expect allosteric downregulation of GAPDH in the gap1+ overexpression strain, thus accounting for the 2.4-fold higher accumulation of GAP in the present study. A plumbing analogy is that even though the pipe diameter gets larger a tighter relative constriction at the regulatory valve can counteract that flux.

### OPP pathway metabolite levels in the gap1 deletion and gap1+ overexpression strains of Synechococcus 7002

The intracellular levels of OPP metabolites were quantified in the engineered GAPDH-1 and WT strains after 96 h of fermentation using LC-MS/MS. In comparison to the WT, the gap1::aphII mutant generally accumulated increased amounts of ribulose-5-phosphate (1.8-fold, P ≤ 0.001), xylulose-5-phosphate

![Fig. 5](image_url)
(1.4-fold, \( P \leq 0.01 \)), 6-phosphogluconate (1.3-fold, \( P \leq 0.1 \)) and erythrose-4-phosphate (1.1-fold, \( P \leq 0.2 \)) (Fig. 6a). Although the magnitude of the changes are small, these results consistently suggest that the gap1::aphII mutant rerouted some of its carbohydrate catabolism through OPP pathway, which is further supported by the increased pool of GAP (2.3-fold, \( P \leq 0.01 \)) (Fig. 4), the intermediate at which the OPP and glycolytic pathways converge. In addition, the gap1::aphII mutant evolved 25% more CO\(_2\) compared to WT, measured in the headspace of fermentative vials (Fig. 7e). Because the OPP pathway yields 3 moles of CO\(_2\) per mole of glucose while glycolysis yields none, this outcome shows that the gap1 deletion mutant rerouted some of its carbon catabolism through the OPP pathway. The 25% higher CO\(_2\) evolution by the gap1::aphII mutant is significantly lower than the theoretical (3-fold) yield of CO\(_2\) expected from the OPP pathway when similar amounts of glycogen are catabolized compared to WT. The experimental difference refers to residual CO\(_2\) only in the headspace and is therefore a lower limit that excludes dissolved CO\(_2\) in the medium. Furthermore, the re-fixation of dissolved CO\(_2\) under dark anaerobic conditions is possible and may account for this lower estimate.

By contrast in the gap1\(^+\) strain, generally lower or similar levels of OPP metabolites were observed in comparison to the WT; xylulose-5-phosphate decreased (0.5-fold, \( P \leq 0.001 \)), 6-phosphogluconate decreased (0.7-fold, \( P \leq 0.2 \)), while slightly increased amounts of ribulose-5-phosphate (1.2-fold, \( P \leq 0.01 \)) were observed. The levels of erythrose-4-phosphate were nearly the same in both gap1\(^+\) and WT strains (Fig. 6a). The gap1\(^+\) overexpression strain yielded 1.1 mole hydrogen per mole glucose, compared to 0.6 mole hydrogen per mole glucose by WT, and 1.4 mole hydrogen per mole glucose by the gap1::aphII mutant. This 1.9-fold boost mirrors the increase in fermentative products (lactate and acetate; Fig. 7a and b). By contrast, a much larger increase in hydrogen is expected if the additional glucose catabolic flux came through the OPP pathway, owing to the 3-fold larger theoretical yield of NADPH via OPP (7 NAD(P) H/glucose; Fig. 1a). These results suggest that the gap1\(^+\) strain

**Table 2** The \( K_m \) values for glycolytic GAPDH with NAD\(^+\) specificity among different organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>( K_m / \mu M ) (NAD(^+))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis sp. PCC6803</td>
<td>50–100</td>
<td>Koksharova et al. (1998)(^{18})</td>
</tr>
<tr>
<td>Trypanosoma cruzi</td>
<td>258 ± 150</td>
<td>Cardoso et al. (2008)(^{12})</td>
</tr>
<tr>
<td>Bacillus steatorrhophilus</td>
<td>100</td>
<td>Clermont et al. (1993)(^{33})</td>
</tr>
<tr>
<td>Trypanosoma brucei</td>
<td>200</td>
<td>Ngantchou et al. (2010)(^{34})</td>
</tr>
</tbody>
</table>
did not use the OPP pathway for glucose catabolism in a manner significantly different from WT.

**Intracellular concentrations of pyridine nucleotides and their cofactor pairs (NADH/NAD⁺ and NADPH/NADP⁺) levels in the gap1::aphII and gap1⁺ strains of Synechococcus 7002**

A main aim of this study was to increase the levels of NADPH and NADH and, in turn, to increase the hydrogen production via bidirectional NiFe-hydrogenase. In order to measure the intracellular levels of NADPH and NADH during fermentation in both of these strains and the WT we used LC-MS/MS. The gap1::aphII mutant was predicted to reroute some glucose-6-phosphate to the OPP pathway and thereby to enhance the NADPH availability in the cells (Fig. 1a). Indeed, the gap1::aphII mutant accumulated larger amounts of NADPH (5.7-fold) compared to the WT (Fig. 6b). This increased size of the total pool and higher relative accumulation of NADPH by the gap1::aphII mutant is consistent with the substantially increased amounts of OPP pathway metabolites observed in this mutant (Fig. 6a) and the 25% increase in CO₂ excretion to headspace (Fig. 7e). The gap1::aphII mutant yielded 2.3-fold more hydrogen compared to WT, also consistent with the increase in reduced pyridine nucleotides via OPP. When measuring steady-state metabolite pools it can be difficult to discriminate between increased metabolic flux into a metabolite vs. lower flux out of a metabolite. For the GAPDH knockout mutant the higher accumulation of OPP metabolites and GAP during dark fermentative conditions is due to increased flux through OPP and cannot be caused by backup at GAP that slows flux through OPP. We conclude this because the mutant has undiminished glycogen catabolic rate vs. WT with 25% more CO₂ excreted, while accumulating higher steady-state levels of OPP metabolites and GAP, and 5.7-fold higher NADPH, which can only come from flux through the OPP pathway.

These results suggest NADPH as a plausible donor of electrons to NiFe-hydrogenase or by NADH produced by the activity of the transhydrogenase from the increased amount of NADPH. It is currently thought that the bidirectional NiFe-hydrogenase uses only NADH as electron donor for reduction of protons, and to date, it has not been shown that the bidirectional hydrogenase is capable of using NADPH in vivo. Both NADH and NADPH served as electron donors for bidirectional NiFe-hydrogenase in vitro. In fact, hydrogenase-I from Pyrococcus furiosus was shown to use NADPH as an electron donor.

The gap1::aphII mutant accumulated nearly the same amount of NADH as WT (0.9-fold, Fig. 6b), but was expected to have a lower NADH content than the WT. This could be the result of transhydrogenase rebalancing the cellular availability of NADPH and NADH (Fig. 1c). A transhydrogenase gene (SYNPC7002_A984; A985; A986) that transfers electrons from NADPH into NADH is present in the genome, but clear proof of its activity during dark-anoxic fermentation is still elusive. Although, the transcription levels of all three subunits (pntA, pntB and pntC) had higher expression levels under dark-oxic conditions, only pntA had higher expression level under dark-oxic condition compared to standard growth conditions.

The generation of deletion and overexpression mutants of the transhydrogenase gene in a gap1::aphII mutant background would clarify this important question and that is currently being pursued in our lab.

In contrast to gap1::aphII mutant, the gap1⁺ strain was anticipated to increase the catabolic rate of glycolysis, which could yield increased amounts of NADH in cells. The gap1⁺ strain accumulated higher amounts of both NADH and NADPH (4.0-fold and 2.9-fold, respectively) compared to WT (Fig. 6b). The increased levels of NADH in the gap1⁺ strain are in agreement with the increased amounts of lower-glycolytic pathway metabolites (Fig. 4) and the higher catabolic rate of glycogen. The ratios of reduced and oxidized pyridine nucleotides (NADPH/NAD⁺ and NADH/NAD⁺) are often better reporters of the redox state of the cell than the concentrations of NADPH or NADH alone. In comparison to the WT, the NADPH/NAD⁺ value in the gap1::aphII mutant increased 6.7-fold while the NADH/NAD⁺ ratio was unchanged (Fig. 6c). In the gap1⁺ strain, both cofactor pairs NADH/NAD⁺ and NADPH/NAD⁺ increased by 2.0 and 2.9-fold, respectively, compared to WT (Fig. 6c). This demonstrates that the gap1::aphII and gap1⁺ strains successfully created more reducing intracellular environments and larger pools of reductant that can potentially be used for hydrogen production.

Analysis of gap1⁺ strain after fermentation showed increased amounts of lower-glycolytic metabolites, 4-fold higher NADH levels along with a higher carbohydrate catabolic rate, in comparison to those of WT. As a consequence of the higher intracellular NADH availability, the yields of hydrogen and v-lactate were increased 3.6-fold and 2.3-fold, respectively. Similarly, increased NADPH production was observed in E. coli upon complementing the gapA deletion through overexpression of the NAD⁺-dependent GAPDH gene (gapC) from C. acetobutylicum, and an increase in the rate and yield of ethanol production from v-xylene by increasing the NADPH availability by the overexpression of a NAD⁺-dependent GAPDH in S. cerevisiae was observed. Our findings on the gap1 overexpression strain support previous suggestions that GAPDH-H is the choke point of the glycolytic pathway under fermentative conditions, and overexpressing gap1 is an effective strategy for relieving this limiting step of glycolysis under these conditions. Although the gap1⁺ strain showed similar levels of OPP metabolites to WT, (Fig. 6a), it accumulated 2.9-fold higher amounts of NADPH (Fig. 6b). This higher amount of NADPH might be due to ferredoxin:NADP⁺ oxidoreductase oxidizing the reduced ferredoxin, which is supported by 1.5-fold higher acetate excretion by gap1⁺ strain. Another possibility is that transhydrogenase oxidizes highly available NADH to NAD⁺ while further reducing the NADP⁺ to NADPH. Our results also indicated that the gap1⁺ strain with 4-fold higher NADH availability produced the highest amounts of v-lactate and hydrogen compared to the WT. It is thus possible that by introducing the gap1⁺ construct in the ldhA mutant, which was previously shown to produce 5-fold higher hydrogen due to a higher NADH availability, could lead to still greater hydrogen yield. This hypothesis is now being actively pursued in our laboratory in Synechococcus 7002.
Pyruvate conversion to lactate, acetate and succinate

In *Synechococcus* 7002 during fermentative conditions a large fraction of the pyruvate produced by glycolysis is reduced to β-lactate by β-lactate dehydrogenase (LdhA). Pyruvate can also be converted to acetate, while succinate and alanine are other minor excreted fermentation end-products. The reduction of pyruvate to β-lactate, succinate and alanine involves NADH consumption while pyruvate oxidation to acetate yields reduced ferredoxin that can then be used to reduce NADP⁺ to NADPH via Fd:NADP⁺ oxidoreductase. In order to assess the effects of the *gap1* genetic modifications on the yields of end-fermentation metabolites, the spent fermentation media of the *gap1*::aphII, *gap1*+ and WT strains were analyzed by HPLC. The *gap1*::aphII mutant had no significant difference in β-lactate excretion, and it had lower acetate excretion (0.46-fold on day 4) than WT cells (Fig. 7a and b). The *gap1*+ strain excreted significantly higher amounts of both lactate (2.3-fold on day 4) and acetate (1.5-fold on day 4) compared to WT on all the time-points analyzed (Fig. 7a and b). Both mutant strains excreted similar amounts of succinate compared to WT cells (Fig. 7c). These results are inconsistent with higher β-lactate productivity in the case for which higher levels of glycolytic NADH was present (*gap1*+), while higher levels of NADPH via OPP do not seem to affect β-lactate yields.

Increased hydrogen production by metabolically engineered *GAPDH-1* strains of *Synechococcus* 7002

We examined the hydrogen producing capacity of the *gap1*::aphII and *gap1*+ strains of *Synechococcus* 7002 in response to their modified reductant availability by analyzing the headspace of fermentation vials. Both *gap1*::aphII and *gap1*+ strains showed significantly increased amounts of hydrogen compared to WT at all the three points of fermentation that were sampled. The increased yields of hydrogen correlated with the increased levels of NADPH in these mutants measured using LC-MS/MS (Fig. 6b). The WT strain produced hydrogen at a rate of 0.9 ± 0.08 nmol 10⁰ per cells per day. The *gap1*+ strain, with a 4.0-fold higher NADH and a 2.9-fold higher NADPH than WT, produced 3.0-fold more hydrogen, with a rate of 2.7 ± 0.09 nmol 10⁶ per cells per day, compared to the WT strain. The *gap1*::aphII mutant, with 5.7-fold higher NADPH and 0.9-fold NADH than WT, produced 2.3-fold more hydrogen at a rate of 2.1 ± 0.08 nmol 10⁶ per cells per day (Fig. 7d).

So far, in *Synechococcus* 7002, the *gap1*::aphII mutant presented here yielded the highest energy conversion of 1.4 mole of hydrogen per mole of glucose, compared to 1.2, 1.1 and 0.6 mole of hydrogen per mole of glucose in the *ldhA* knockout, ⁶ *gap1*+ over expression and WT strains respectively. Though the energy conversion yield of the *gap1*::aphII mutant is highest among *Synechococcus* 7002 strains, it is lower compared to wild strains that rely more on fermentation for survival, notably *Gloeocapsa alpicola* CALU 743 (3.96) and *Arthrospira maxima* CS-328 (0.9-2.1).³ Furthermore, using an approach that relies on selective hydrogen removal (“hydrogen milking”) resulted in an increase in the energy conversion yield to 4.7 mole of hydrogen per mole of glucose for *Arthrospira maxima* CS-328.¹¹ Thus we are considering this hydrogen milking approach in our future studies of transgenic strains of *Synechococcus* 7002, to further increase the energy conversion yield of hydrogen, where the NiFe-hydrogenase functions exclusively unidirectional towards hydrogen production.

Conclusions

Here we pursued two metabolic engineering strategies to increase the carbon catabolism through OPP or glycolytic pathways with the final objective of increasing the yields of hydrogen produced under fermentative conditions. By deleting the *gap1* gene coding for the GAPDH-1, we reduced the contribution of glycolysis and increased the contribution of the OPP pathway to fermentative metabolism. This resulted in higher levels of NADPH and consequently more hydrogen produced by the bidirectional NiFe-hydrogenase either directly or indirectly by NADH formation by transhydrogenase. By overexpressing the *gap1* gene, we increased the rate of glycolysis, which supports previous suggestions that GAPDH-1 is the bottleneck of glycolytic catabolism during dark anoxic conditions. As a consequence of relieving this bottleneck, NAD(P)H levels increased and as a consequence, hydrogen yields also increased.

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Honors and Awards
Chinese Government Award for Outstanding Self-financed Students Abroad (2014)
16th International Congress on Photosynthesis Student Scholarship (2013)
NSF Travel Grant for 11th Cyanobacteria Workshop (2013)
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The Pennsylvania State University Paul Berg Prize in Molecular Biology (2012)