CYANOBACTERIA: THE GREEN *E. COLI*

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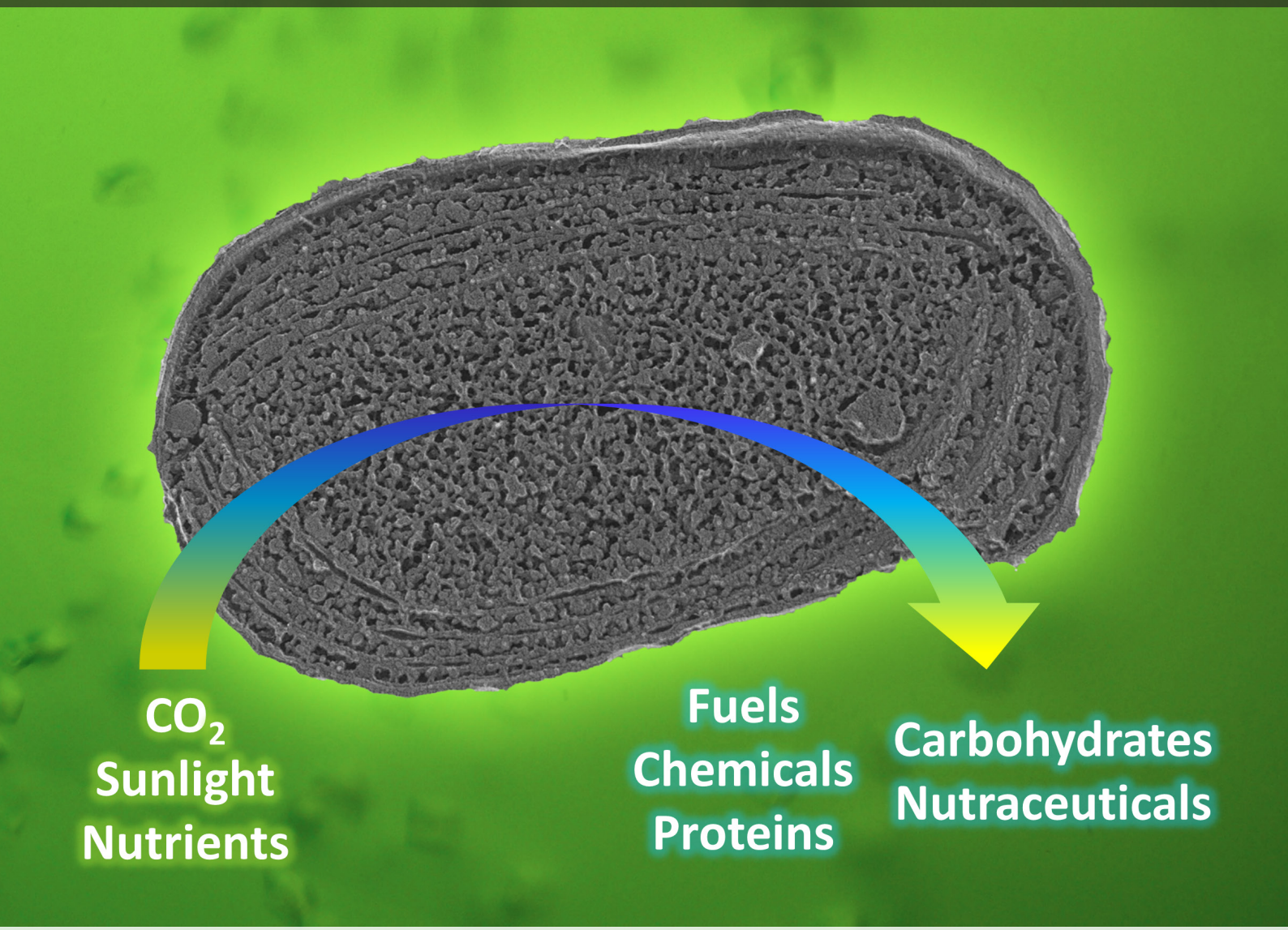

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CYANOBACTERIA: THE GREEN *E. COLI*

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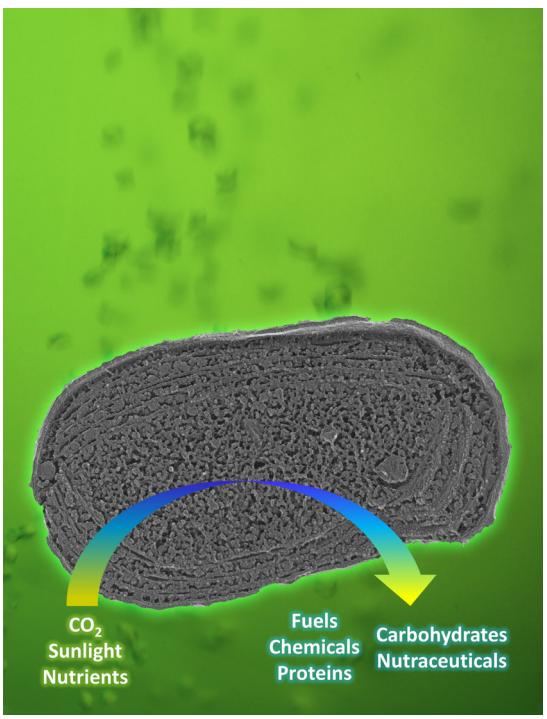

Quick-freeze deep-etch electron micrograph (QFDEEM) of the cyanobacterium *Synechococcus* sp. PCC 7002, a host for genetic modification and biotechnology applications. Photo credit: Anne Ruffing (Sandia National Laboratories) and Ursula Goodenough (Washington University in St. Louis)

As the world struggles to reduce its dependence on fossil fuels and curb greenhouse gas emissions, industrial biotechnology is also "going green." *Escherichia coli* has long been used as a model Gram-negative bacterium, not only for fundamental research but also for industrial applications. Recently, however, cyanobacteria have emerged as candidate chassis for the production of commodity fuels and chemicals, utilizing CO_2 and sunlight as the main nutrient requirements. In addition to their potential for reducing greenhouse gas emissions and lowering production costs, cyanobacteria have naturally efficient pathways for the production of metabolites such as carotenoids, which are of importance in the nutraceutical industry. The unique metabolic and regulatory pathways present in cyanobacteria present new challenges for metabolic engineers and synthetic biologists. Moreover, their requirement for light and the dynamic regulatory mechanisms of the diurnal cycle further complicate the development and application of cyanobacteria for industrial applications. Consequently, significant advancements in cyanobacterial engineering and strain development are necessary for the development of a "green *E. coli*."

This Research Topic will focus on cyanobacteria as organisms of emerging industrial relevance, including research focused on the development of genetic tools for cyanobacteria, the investigation of new cyanobacterial strains, the construction of novel cyanobacterial strains via genetic engineering, the application of "omics" tools to advance the understanding of engineered cyanobacteria, and the development of computational models for cyanobacterial strain development.

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EDITORIAL
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Editorial: Cyanobacteria: The Green *E. coli*

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Keywords: cyanobacteria, green *E. coli*, cyanobacterial metabolites, cyanobacterial biofuels, cyanobacterial products, genetic modification of cyanobacteria, genetic engineering of cyanobacteria

The Editorial on the Research Topic

Cyanobacteria: The Green *E. coli*

As the world struggles to reduce its dependence on fossil fuels and curb greenhouse gas emissions, industrial biotechnology is also "going green." *Escherichia coli* has long been used as a model Gram-negative bacterium, not only for fundamental research but also for industrial applications. Recently, however, cyanobacteria have emerged as candidate chassis for the production of commodity fuels and chemicals, using CO_2 and sunlight as their main sources of carbon and energy (Ducat et al., 2011; Machado and Atsumi, 2012). In addition to their potential for reducing greenhouse gas emissions and lowering production costs, cyanobacteria have naturally efficient pathways for the production of carbohydrates and proteins as well as metabolites such as vitamins and carotenoids, which are of importance in the nutraceutical industry (Wang et al., 2014). The unique metabolic and regulatory pathways present in cyanobacteria present new challenges for metabolic engineers and synthetic biologists. Moreover, their requirement for light and the dynamic regulatory mechanisms of the diurnal cycle further complicate the development and application of cyanobacteria for industrial applications. Consequently, significant advancements in cyanobacterial engineering and strain development are necessary for the development of a truly "green *E. coli*."

This Research Topic highlights cyanobacteria as organisms of emerging industrial relevance and discusses unique challenges posed by these photosynthetic hosts. Original Research articles in this issue demonstrate the production of energy-dense biofuels in engineered cyanobacterial hosts, including alkanes (Klahn et al.), free fatty acids (Ruffing; Work et al.), and terpenes (Davies et al.). The use of cyanobacteria for the production of plant secondary metabolites is reviewed by Xue and He. In addition to the production of metabolites, Jensen and Leister present their Opinion article on exploiting cyanobacteria for modifying photosynthesis, allowing for more rapid modification and characterization compared to plant hosts. Collectively, these articles provide a broad overview of the potential applications enabled by cyanobacterial hosts, yet this is certainly not an exhaustive list. Cyanobacteria have been successfully engineered in other applications including biosensing, bioremediation, protein production, and hydrogen production (Ruffing, 2011), and diverse, new applications for cyanobacteria are most certainly under development.

A common challenge in engineering cyanobacteria is often the paucity of characterized genetic tools available for cyanobacterial hosts. Oftentimes, genetic tools developed for *E. coli* may be applied in a cyanobacterial host, yet the function of these *E. coli* elements can vary greatly when expressed in a cyanobacterium (Markley et al., 2015). The synthetic constructs may also be more effective when integrated with the cyanobacterial metabolism, requiring the development of new tools that interface with the circadian rhythm of cyanobacteria and the diurnal nature of photosynthetic metabolism. This Research Topic includes a review by Camsund and Lindblad of transcriptional tools and design principles for engineering transcriptional systems in cyanobacteria. Further, Branco dos Santos et al.

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present the compelling perspective that mitigation of carbon emissions is more likely to succeed if cyanobacteria are viewed as cell factories for bioproducts, similar to *E. coli*, rather than solely as a means for CO_2 capture.

In addition to genetic tool availability for cyanobacteria, other unique challenges result from the use of these photosynthetic hosts in biotechnology applications. Metabolite production, particularly biofuel synthesis, may have toxic or detrimental effects on the physiology of the cyanobacterial host, as described in an Original Research article by Pei et al., analyzing the effects of exogenous biofuels on the protein network in *Synechocystis* sp. PCC 6803. The photosynthetic constraints of light sensing and light harvesting are discussed in a Perspective by Montgomery. In his Hypothesis and Theory article, Burnap analyzes systems-level constraints of autotrophic productivity in cyanobacteria and argues that intracellular crowding imposes limitations on the allocation of proteomic resources. An interesting case-in-point may be the recent rediscovery of a fast-growing cyanobacterium, with a generation time of ~2 h that apparently does so in part by not devoting resources to glycogen synthesis during early stages of growth (Yu et al., 2015). Lastly, Jones provides an important Perspective on a largely ignored problem in cyanobacterial engineering, namely that of genetic instability. As illustrated by the articles in this Research Topic, the successes and challenges of developing cyanobacteria as cell factories make this an exciting time for the green *E. coli*.

AUTHOR CONTRIBUTIONS

Dr. AR and Prof. TK served as co-editors for the Research Topic: Cyanobacteria: The Green *E. coli*. Dr. AR conceived of the idea for the research topic. Both Dr. AR and Prof. TK served as editors for the manuscripts in this Research Topic and contributed to writing the introductory editorial.

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Cyanobacteria as an experimental platform for modifying bacterial and plant photosynthesis

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One of the fascinating characteristics of photosynthesis is its capacity for repair, self-renewal, and energy storage within chemical bonds. Given the evolutionary history of plant photosynthesis and the patchwork nature of many of its components, it is safe to assume that the light reactions of plant photosynthesis can be improved by genetic engineering (Leister, 2012). The evolutionary precursor of chloroplasts was a microorganism whose biochemistry was very similar to that of present-day cyanobacteria. Many cyanobacterial species are easy to manipulate genetically and grow robustly in liquid cultures that can be easily scaled up into photobioreactors. Therefore, cyanobacteria such as *Synechocystis* sp. PCC 6803 (hereafter "*Synechocystis*") have widely been used for decades as model systems to study the principles of photosynthesis (**Table 1**). Indeed, genetic engineering based on homologous recombination is well-established in *Synechocystis*. Moreover, new genetic engineering toolkits, including marker-less gene deletion and replacement strategies needing only a single transformation step (Viola et al., 2014) and novel approaches for chromosomal integration and expression of synthetic gene operons (Bentley et al., 2014), allow for large-scale replacement and/or integration of dozens of genes in reasonable time frames. This makes *Synechocystis* a very attractive basis for the experimental modification of important processes like photosynthesis, and it also suggests innovative ways of improving modules of related eukaryotic pathways, among them the combination of cyanobacterial and eukaryotic elements using the tools of synthetic biology.

IMPROVING THE PHOTOSYNTHETIC LIGHT REACTIONS IN CYANOBACTERIA

In plants, the activity of the Calvin cycle (in particular the RuBisCO-mediated carbon fixation step) is considered to represent the major brake on photosynthetic efficiency under saturating irradiance and limiting CO_2 concentrations (Quick et al., 1991; Stitt et al., 1991; Furbank et al., 1996). Autotrophic growth of *Synechocystis*, on the other hand, is constrained by the rate of phosphoglycerate reduction, owing to limitations on the ATP/NADPH supply from the light reactions (Marcus et al., 2011). In fact, cyanobacteria cannot absorb all incoming sunlight due to light reflection, dissipation, and shading effects. In some cases, significant numbers of the photons absorbed by the antennae are not used for energy conversion due to dissipation mechanisms. It has therefore been proposed that uneven light distribution could be avoided by using cell cultures with smaller antenna sizes packed in high-density cell cultures, thus allowing good light penetration into the inner parts of the reactor. Proof of principle for this concept has been obtained in the green alga *Chlamydomonas reinhardtii* (Beckmann et al., 2009), but antenna truncations in *Synechocystis* have so far failed to enhance biomass production (Page et al., 2012). Indeed, increased truncations of phycobilisomes were associated with reductions in photoautotrophic productivity, which were attributed to marked decrease in the PSI:PSII ratio (Collins et al., 2012).

A radically different approach to altering the light-harvesting capability of cyanobacteria and extending the range of wavelengths absorbed involves the introduction into cyanobacteria of the light-harvesting complex II (LHCII) of land plants. In principle, this should be a straightforward exercise, as the complex has a simple structure, containing in its minimal version essentially only one type of Lhcb polypeptide together with chlorophylls (Chl) a and b. Although *Synechocystis* strains that produce large amounts of Chl b in addition to the naturally occurring Chl a have been generated (Xu et al., 2001), the expression of stable Lhcb proteins presents a problem, possibly because they do not fold correctly and are quickly degraded (He et al., 1999). Thus, inefficient light-harvesting remains the principal barrier to high-efficiency *Synechocystis* biomass growth.

IMPROVING THE PHOTOSYNTHETIC LIGHT REACTIONS OF PLANTS IN CYANOBACTERIA

The gain in photosynthetic efficiency, obtainable when, for instance, photosystems (PS) require less repair and photoprotection, should be significant. It is clear that crop plants and even model plants like *Arabidopsis thaliana* or *Physcomitrella patens* are the systems least suited for testing such approaches, given their long life cycle and inaccessibility to efficient (prokaryote-type) genetic engineering technologies (**Table 1**). Therefore, redesigning plant PS will require novel model organisms in which such concepts can be implemented, tested, and reiteratively improved.

Table 1 | Characteristics of current model systems for photosynthesis.

Organism	Type of photo-synthesis[a]	Homologous recombination	Life cycle	Shot-gun complementation	Heterotrophic propagation
Synechocystis	Prokaryotic	Yes	<1 day	Yes	Yes
Chlamydomonas	Eukaryotic	No	<1 day	No	Yes
Physcomitrella	Eukaryotic	Yes	Several weeks	No	Restricted[b]
Arabidopsis	Eukaryotic	No	Several months	No	Restricted[b]

[a]*This distinction refers to the presence of phycobilisomes ("prokaryotic") or Lhc proteins ("eukaryotic") and associated regulatory differences.*

[b]*Refers to non-photoautotrophic mutants.*

Cyanobacteria, particularly *Synechocystis*, will play an important role in such attempts because of its superior genetic tractability. Thus, the long-term goal is to introduce elements of plant photosynthesis into model cyanobacteria like *Synechocystis* and optimize their effects by genetic engineering. Consequently, chimeric PS employing, for instance, plant cores and antenna complexes from algae could combine features from the whole range of diversity available in eukaryotes, while allowing their impacts to be tested and their properties to be optimized in a prokaryote. Besides the technical advantages of this strategy, it has the added attraction of delegating most of the required work with genetically modified organisms (GMOs) to *Synechocystis*. Reducing the transgenic work done directly in plants might also improve the acceptability of the approach to a public, which has proven to be, at best, skeptical of GMOs.

IMPROVING CO_2 FIXATION

Cyanobacteria, like plants and algae, use the Calvin cycle for assimilation of CO_2. The first step in CO_2 assimilation is the carboxylase reaction catalyzed by RuBisCO, which results in the production of two molecules of 3-phosphoglycerate; one of these is recycled to regenerate ribulose-1,5-bisphosphate (RuBP), whereas the other is converted to biosynthesis of sugars, terpenoids, and fatty acids (Melis, 2013). However, RuBisCO can also react with molecular O_2 in a process called photorespiration. This oxygenase reaction produces one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate, which acts as an inhibitor of enzymes involved in photosynthetic carbon fixation. Therefore, photorespiration reduces the overall efficiency and output of photosynthesis, since there is a net loss of both CO_2 and nitrogen.

Of the four distinct forms of RuBisCO (Andersson and Backlund, 2008; Tabita et al., 2008), form I is the most widespread, being found in plants, algae, and cyanobacteria. The cyanobacterial version comprises eight small (RbcS) and eight large (RbcL) subunits. Not surprisingly, RuBisCO is widely conserved across species, but some of its natural variants are slightly more effective than others. For instance, heterologous expression of RuBisCO from the purple-sulfur bacterium *Allochromatium vinosum* in *Synechococcus elongatus* sp. PCC 7942 increased CO_2 assimilation by almost 50% (Iwaki et al., 2006). Therefore, metagenomic analysis of natural RuBisCO diversity may identify superior enzymes to be engineered into a cyanobacterial host for detailed characterization and platform improvement.

Besides its catalytic subunits RbcL and RbcS, RuBisCO seems to need the molecular chaperone RbcX for proper folding. In some cyanobacteria, the *rbcX* gene colocalizes with the genes encoding RbcL and RbcS in the chromosome. However, to what extent this chaperone is actually needed is still unclear, and the folding/assembly process needs further investigation (for a recent review, see Rosgaard et al., 2012). In plants, activation of RuBisCO by RuBisCO activase is essential for catalysis; however, evidence of a requirement for RuBisCO activase for optimal function of cyanobacterial RuBisCO is lacking (Rosgaard et al., 2012).

Although RuBisCO is the major enzyme responsible for carbon fixation, cyanobacteria possess an additional assimilation mechanism that accounts for nearly 25% of CO_2 fixation (Yang et al., 2002). Phosphoenolpyruvate carboxylase (PEPC) catalyzes the reaction that fixes HCO_3^- on phosphoenolpyruvate (PEP) to form oxaloacetate and inorganic phosphate in the presence of Mg^{2+} (O'Leary, 1982). This enzyme is widely distributed in all plants and many bacteria. Attempts to improve plant CO_2 fixation by expression of a cyanobacterial PEPC with diminished sensitivity to feedback inhibition have been unsuccessful; the resulting transgenic plants even showed decreased fitness (Chen et al., 2004).

In the cytosol of cyanobacteria, RuBisCO is found in proteinaceous microcompartments known as carboxysomes (Kerfeld et al., 2010). A carboxysome consists of a shell assembled from roughly 800 protein hexamers, forming the 20 facets of an icosahedron, and 12 pentamers that form its corners (Heinhorst et al., 2006). The carboxysome encapsulates RuBisCO complexes and plays a central role in a mechanism that concentrates inorganic carbon providing enough CO_2 for the enzyme to favor the carboxylase reaction. In the cytosol, carbonic anhydrases convert CO_2 to HCO_3^-, thereby trapping the inorganic carbon species inside the cells. The carboxysome is rather impermeable to O_2, but it readily takes up HCO_3^- (Price et al., 2008). Inside the carboxysome, specialized carbonic anhydrases catalyze the release of CO_2 from the incoming HCO_3^-. The number of carboxysomes and the expression levels of carboxysome genes increase significantly when cyanobacterial cells are limited for CO_2 (Heinhorst et al., 2006). Carboxysomes can potentially be exploited as synthetic compartments, similar to eukaryotic organelles, to rationally organize pathways or networks within a spatially distinct subsystem (Kerfeld et al., 2010).

The terpenoid and fatty acid biosynthetic pathways receive only about 5 and

10% of the photosynthetically fixed carbon, respectively, and this allocation is constitutive but stringently regulated (Melis, 2013). If photosynthetic organisms are to be used as a platform for pathways devoted to the biosynthesis of terpenoid- or fatty acid-derived products, this product-to-biomass carbon portioning must be increased significantly.

SYNTHETIC BIOLOGY

The aim of synthetic biology is to engineer biological systems by designing and constructing novel modules to perform new functions for useful purposes. "Building blocks" (i.e., genes, enzymes, pathways, or regulatory circuits) in synthetic biology are thought of as modular, well-characterized biological parts that can be predictably combined to yield novel and complex cell-based systems following engineering principles (Endy, 2005). In this context, the photosynthetic complexes (PS I and II) in the thylakoids of cyanobacteria can be regarded as building blocks, which can be integrated into novel biosynthetic pathways. Ideally, the biosynthetic pathway should be located in the thylakoids or at least in close proximity to the photosynthetic electron transfer chain, allowing the biosynthetic enzymes to tap directly into photosynthetic electron transport and energy generation, and even draw on carbon skeletons derived from CO_2 fixation. Recently, an entire cytochrome P450-dependent pathway has been relocated to the thylakoids of tobacco chloroplasts and shown to be driven directly by the reducing power generated by photosynthesis in a light-dependent manner (Zygadlo Nielsen et al., 2013; Lassen et al., 2014). This demonstrates the potential of transferring pathways for structurally complex chemicals to the chloroplast and using photosynthesis to drive the P450s with water as the primary electron donor.

Synthetic biology in cyanobacteria still lags behind conventional species such as *E. coli* and yeast in terms of molecular tools, defined parts, and product yields. Some progress has been made in redirecting photosynthetically fixed carbon toward commercially interesting compounds. The C_5 molecule isoprene is a volatile hydrocarbon that can be used as fuel and as a platform-chemical for production of synthetic rubber and high-value compounds. For photosynthetic generation of isoprene in cyanobacteria, the isoprene synthase gene from the plant *Pueraria montana* (kudzu) has been successfully expressed in *Synechocystis* and isoprene was indeed produced (Lindberg et al., 2010). However, drastic metabolic engineering will be required to redirect carbon partitioning away from the dominant carbohydrate biosynthesis toward terpenoid biosynthesis. In fact, heterologous expression of the isoprene synthase in combination with the introduction of a non-native mevalonic acid pathway for increased carbon flux toward isopentenyl-diphosphate (IPP) and dimethylallyl-diphosphate (DMAPP) precursors of isoprene resulted in a 2.5-fold improvement in isoprene yield (Bentley et al., 2014).

Tightly regulated and inducible protein expression is an important prerequisite for product yield and predictability in synthetic biology approaches. In this context, riboswitches are attracting increasing interest. Riboswitches are functional non-coding RNA molecules that play a crucial role in gene regulation at the transcriptional or post-transcriptional level in many bacteria (Roth and Breaker, 2009). In general, the sensing domain (aptamer) of riboswitches is combined with a regulating domain. The regulating domain can comprise several types of expression platforms to control gene expression. For instance, direct binding of a specific ligand to the aptamer domain can be used to attenuate transcription termination or translation initiation (Roth and Breaker, 2009). Recently, a theophylline-dependent riboswitch was established as a strict and inducible protein expression system in *S. elongatus* PCC 7942 (Nakahira et al., 2013). Three theophylline riboswitches were tested, and the best one exhibited clear on/off regulation of protein expression. In the ON state, protein expression levels were up to 190-fold higher than in the absence of the activator. Moreover, it was possible to fine-tune the level of protein expression by using a defined range of theophylline concentrations.

CONCLUSION

Cyanobacteria are receiving increasing interest as experimental scaffolds for the modification of their endogenous photosynthetic machineries, as well as the integration and engineering of modules of plant photosynthesis. Therefore, we believe that cyanobacteria will be extensively used by many plant biologists as additional model system in future analyses. Indeed, for the identification of the entire set of components necessary for photosynthesis only cyanobacteria are suitable as experimental platforms. If this is achieved, the next goal is to transfer this photosynthetic module to other (non-photosynthetic) organisms like *E. coli*. Moreover, cyanobacteria are attractive as a "green" platform for synthetic biology to produce high-value compounds, chemical feedstocks, or even fuels.

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Synechocystis: not just a plug-bug for CO_2, but a green *E. coli*

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Following multiple reports warning for threats posed by raising levels of atmospheric CO_2, it is of paramount importance that human society rapidly evolves to be sustainable. Processes relying on photosynthetic microorganisms, converting CO_2 and water into compounds of interest, fueled by light, are very pertinent, particularly if not directly competing for arable land. Here, we identify specific research questions that remain to be targeted to exploit the full potential of cyanobacterial cell factories. We argue that this approach will be more likely to be successful if organisms such as *Synechocystis* are not perceived as mere chassis for CO_2 fixation, but rather considered as the "green" *E. coli*.

Keywords: *Synechocystis*, photosynthesis, systems biology, sustainability, genetic engineering

INTRODUCTION

Atmospheric levels of CO_2 have steadily increased since the late nineteenth century accelerating to an unprecedented rate from the mid-twentieth century onward. Multiple experts have warned for the consequences posed by the latter. Their tone of urgency has been increasing as early predictions materialize. Signs of consensus seem to be emerging among decision makers, elevating the relevance of environmental sustainability on the political agenda (IPCC, 2014).

Sustainability requires maintenance of chemical balances of human activities, harmonizing them with the existing natural processes (Dhillon and von Wuehlisch, 2013). Human societies are mostly fueled by the oxidation of carbon-containing fossilized deposits, leading to CO_2 release. One of the efforts to reduce our dependency on fossil fuels focused on exploiting microorganisms to transform plant-derived sugars into bulk chemicals and/or biofuel. Although important toward balancing CO_2 fluxes, this approach presents several drawbacks. To name but a few, (i) it requires large fractions of arable land, which exerts further pressure on natural resources; (ii) it competes with human food supply; (iii) it leads to a huge amount of biowaste, implying sunlight being used inefficiently. Considerable research efforts have been placed on minimizing these shortcomings. However, advancements still fall short of what is necessary for fully sustainable economies with the current living standards.

The usage of oxygenic photosynthetic prokaryotes as cell factories to directly convert CO_2 and water into compounds of interest fueled by light and having oxygen as the by-product, presents itself as a very promising alternative. This approach would potentially resolve most of the issues highlighted above, reducing substantially the need of soil and water and leading to less waste. Here, while emphasizing the inherent advantages of this approach, we will expose the areas that require improvement in order to make this a truly economically feasible alternative to current production methods. Focus will be placed on the scientific and technological issues that need further attention to transform lab-scale "proof-of-concept" experiments into full-blown industrial processes.

CYANOBACTERIAL PHOTOSYNTHETIC MACHINERY

If one wants to engineer a phototrophic organism into a catalyst for the reaction:

$$CO_2 + H_2O \rightarrow \text{product}(\text{e.g.,}C_2H_6O) + O_2$$

thereby bypassing biomass formation, one has the choice of using plants (green) algae or cyanobacteria. As genetic accessibility and photosynthetic efficiency are highest in the latter, they are the organisms of choice. This particularly holds for the production of small compounds (e.g., ethanol, butanol) that accumulate extracellularly, not requiring intracellular storage capacity. The situation may be different, for instance, in the production of pharmaceutical proteins, for which green algae may be more profitable because of their larger cytoplasm (Wijffels et al., 2013).

Ultimately, all sustainable processes in which CO_2 is fixed via oxygenic photosynthesis rely on its efficiency and capacity. As there are differences in this process between pro- and eukaryotic microorganisms, we will discuss those here, pointing out some key advantages of the cyanobacterial photosynthetic machinery, along with key related aspects that remain to be fully elucidated.

FUNDAMENTAL ADVANTAGES

Of the three clades of oxyphototrophs, cyanobacteria have the highest photosynthetic efficiency. It has been shown that in competition experiments between a cyanobacterium and algae, cyanobacteria out-compete the others (Mur et al., 1977). Additionally, where the highest energetic efficiency of C3 and C4 plants has been estimated to be 4.5 and 6% of the incoming solar power, respectively, cyanobacteria can perform significantly better (Janssen et al., 2000). Indirectly, the same conclusion can be drawn from measuring the light intensity at which phototropic microorganisms transit from oxygen consumption to oxygen production (compensation point), also because of their lower maintenance requirements.

Cyanobacteria (along with red algae) contain phycobilisomes, making them more robust in the efficient absorption of polychromatic visible and far-red irradiation. These antennae are suitable targets to engineer increased photosynthetic performance. This can be directed not only at the size, composition, and color of these phycobilisomes (important during scale-up; see further below) but also at the role of the orange carotenoid protein (OCP). The latter is the key component in a mechanism that causes exciton dissipation directly into heat, when cyanobacteria are subjected to over-saturating light intensities.

This OCP-mediated quenching mechanism is one of many regulatory mechanisms that modulate photosynthetic efficiency in oxyphototrophs [see Cardol et al. (2011), Hellingwerf et al. (1994)]. The list includes the zeaxanthin cycle, PSI trimerization, various forms of cyclic electron transfer (see further below), state transitions, circadian regulation of photosystem expression, and chromatic adaptation. However, recently, it has been emphasized that each oxyphototroph only makes use of a limited number of these. For instance, the PS-II repair mechanism in which damaged D1 polypeptides are replaced, rather than replacing the entire photosystem, is absent in *Synechocystis*. Instead, this organism protects itself via a set of flavodiiron proteins that divert electrons from the linear Z-scheme into a cyclic pathway, thereby preventing over-reduction of the cells, when either too much light or too little CO_2 is available (Bersanini et al., 2014).

The presence of an extensive set of CO_2/HCO_3^{2-} uptake systems in cyanobacteria, assures that their oxygenic photosynthesis is not plagued by "photorespiration," the dissipative process in which the rubisco enzyme mistakes an oxygen molecule for CO_2. This is another important factor that contributes to their high efficiency of photosynthesis. The small remaining rate of "photorespiration" may have a key function in amino acid biosynthesis in the organism (Bauwe et al., 2012).

KEY QUESTIONS THAT REMAIN TO BE TARGETED

Unlike cyanobacteria, algae are compartmentalized. This has a significant impact on the tools available for synthetic biology, with clear advantages for cyanobacteria. Nevertheless, each compartment catalyzes a characteristic part of the metabolism of eukaryotes, each with its specific regulation. Although often a disadvantage, this can sometimes be exploited. Engineering fatty acid biosynthesis for increased biodiesel production (Weselake et al., 2008) is an example of this successful exploitation of a compartmentalized cell.

The redox state of the plastoquinone pool in the thylakoid membranes has been implied as the regulatory parameter in many key processes in oxyphototrophs (Allen, 2005). In chloroplasts, the balance of electron transfer from PS-II to PSI straightforwardly determines this redox state. In cyanobacteria, there are additional pathways that are important (**Figure 1**), for instance, the supply of electrons by the main dehydrogenase (NDH-1) and withdrawal by the respiratory oxidases (Schuurmans et al., 2014). Consequently, it is much more complex to understand the control of the PQH_2/PQ redox state in cyanobacteria.

This redox state has often been derived from pulse-amplitude modulated fluorescence (PAM) measurements. We have recently reported that PAM is fraught with artifacts when applied to *Synechocystis* (Schuurmans et al., 2014), and that the redox state is strictly controlled around its midpoint potential (except when cells are leaving lag-phase). The PAM signals most likely reflect the redox state of the primary quinone of PS-II (Q_A). Among the artifacts is a significant contribution of phycobilisome fluorescence to the PAM signals (Schuurmans et al., 2014). Proper interpretation of these PAM signals is important because many aspects of photosynthesis can be derived from them.

One characteristic that is derived from PAM measurements is the "rate of cyclic electron flow" (Fan et al., 2007), i.e., the rate at which electrons follow a cyclic path around PSI via an electron transfer reaction between NDH-1 and plastoquinone. While consuming part of the light energy, this will lead to production of ATP only (i.e., without coupled NADPH formation), and therefore, alter the ratio of synthesis of these high-free-energy intermediates (Hellingwerf and Konings, 1985). In some situations, as under salt stress, the cell uses this process to its advantage. They initiate high rates of cyclic electron flow to generate extra ATP to drive transport reactions (van Thor et al., 2000). Moreover, cells are much more complex than depicted in many textbook representations, as multiple forms of cyclic electron transfer can occur, with rates that are strongly dependent on the physiological state of the cell (**Figure 1**). As each of these has its own specific proton:photon stoichiometry, it is nearly impossible to estimate the ratio of production of NADPH and ATP *in vivo*. It will be very important for a better understanding of the energetics and metabolism of cyanobacteria to find ways to measure this ratio.

Next to NADPH, also NADH is an important reduced cofactor in cyanobacterial cells. The redox potential of the latter is often reported to be higher than of the $NADPH/NADP^+$ couple, although few solid data are available on this. Many (engineered) metabolic pathways require reducing equivalents. In cyanobacteria, the preferred cofactor would be NADPH, considering the driving force of the reaction, but many high-capacity (catabolic) reductases are NADH-specific. Redox cofactor matching is therefore important in design of product formation by cyanobacteria.

SYNTHETIC BIOLOGY TOOLBOX

REQUIREMENTS SPECIFIC TO PHOTOSYNTHETIC ORGANISMS

Synechocystis is a great chassis to engineer the production of compounds directly from CO_2. This requires modification of its metabolic network, by removal and/or addition of components via genetic modification. This network contains many anabolic pathways, which ensures that the carbon fixed is efficiently channeled

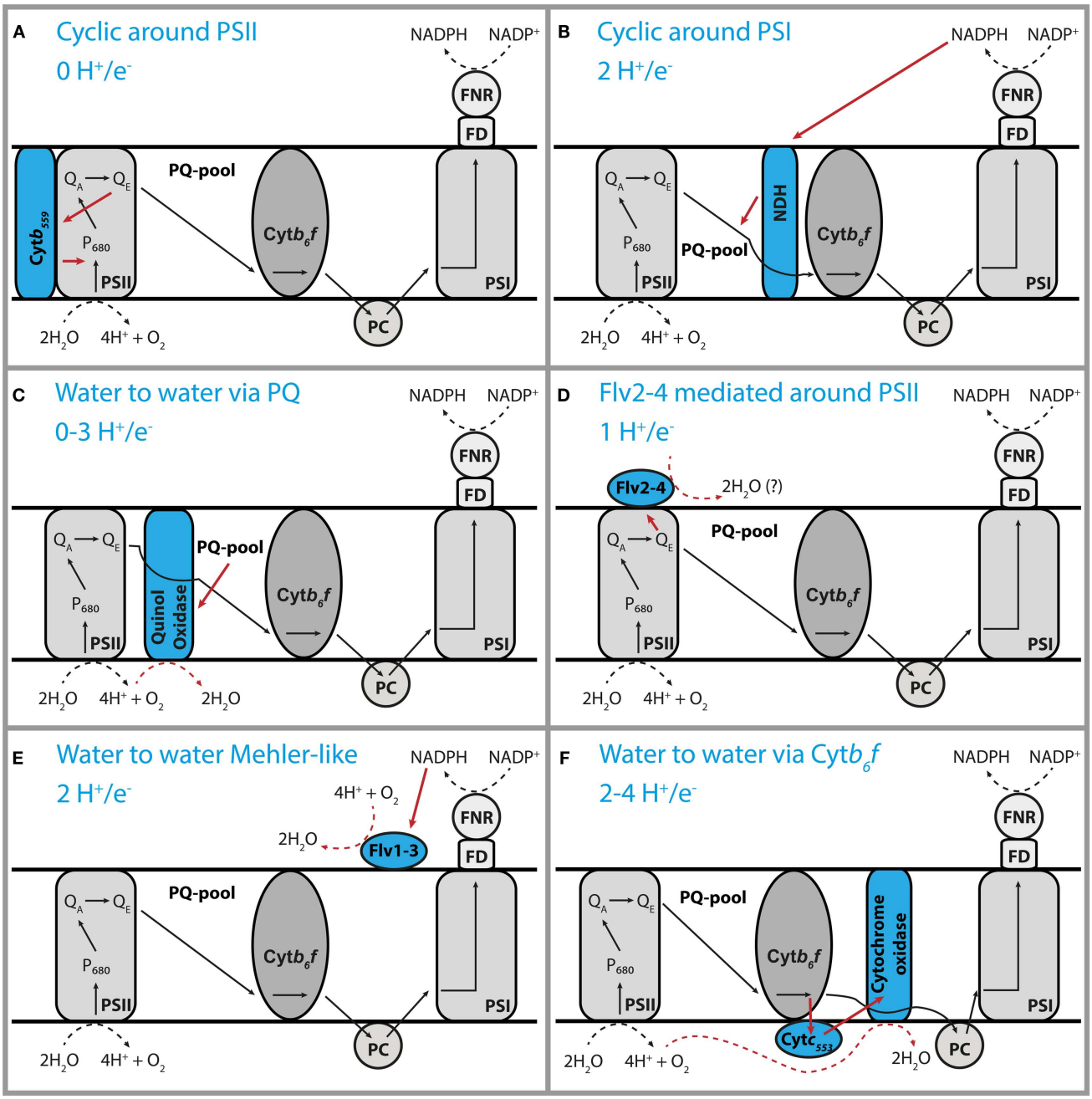

FIGURE 1 | Inventory of pathways for cyclic electron flow in cyanobacteria. Schematic representation of six different forms of cyclic electron transfer that may occur in cyanobacteria **(A–F)**. The tentative H^+/e^- stoichiometry of each form of cyclic e^- transfer is indicated. The electron acceptor of the Flv2–4 protein has not been characterized yet. This cyclic transfer of electrons may make the NADPH and ATP output of the photosynthesis machinery different from the 3/2 textbook ratio.

into biomass. Engineered pathways tap at convenient hub metabolites, often with detrimental effects on growth (Nogales et al., 2012; Savakis et al., 2013). For this reason, it is crucial to design synthetic circuits that are stabilized by expressing the necessary enzymatic machinery and/or regulatory switches at just the right moment. Considering the available technology for large-scale cultivation, the improvement of induction systems responsive at high-cell densities would be most useful, reducing tension between anabolic and product formation pathways.

STATE-OF-THE-ART

Some cyanobacteria are naturally transformable, making them very attractive photosynthetic production hosts. Contrary, their polyploidy (Griese et al., 2011) requires additional efforts to ensure

full segregation after mutagenesis and presents additional challenges during the generation of gene disruption libraries (Tyo et al., 2009). Here, we will focus on the state-of-the-art methods to genetically modify these organisms and regulate their gene expression.

Genetic modifications

Markerless gene knock-out/in strategies for cyanobacteria are attractive because of the limited number of resistance cassettes available. Several methods have been successfully applied (**Table 1**). Among these, the *sacB*-sucrose method is widely used, but its application is limited to glucose-tolerant strains, while the *mazF*-nickel method can circumvent this. The *acsA*-acrylate method was developed for *Synechococcus* PCC7002, but may also be useful for other cyanobacteria. However, it has the drawback of possibly generating undesired mutations. The *upp*-5-fluorouracil method works according to a similar principle, such that *upp* or *acsA* need to be inactivated first in order to make cells sensitive to 5-fluorouracil or insensitive to acrylate, respectively. The *upp*-5-fluorouracil method is considered more efficient because 5-fluorouracil can inhibit cells that contain even a single copy of *upp*. In addition to these counter-selection methods, the Flp–FRT (recombinase and recognition site, respectively) based method also works, provided the Flp plasmid can be lost quickly. Another strategy consists of using essential-cyanobacterial gene complementation for plasmid maintenance, like *recA* (Akiyama et al., 2011), but this strategy is limited by plasmid maintenance. Recent reports characterize cyanobacterial CRISPR–Cas systems (Hein et al., 2013; Kopfmann and Hess, 2013; Scholz et al., 2013). Probably, mutants obtained using the latter will be reported soon. Use of TALEN systems can be expected soon after (Montague et al., 2014).

Table 1 | Markerless gene knock-out and knock-in methods for cyanobacteria.

Methods	Strains tested	Reference
sacB-sucrose	*Synechocystis* sp. PCC6803 (only glucose-tolerant strain)	Lea-Smith et al. (2014), Viola et al. (2014)
mazF-nickel	*Synechocystis* sp. PCC6803	Cheah et al. (2012)
acsA-acrylate	*Synechococcus* sp. PCC7002	Begemann et al. (2013)
upp-5-Fluorouracil	*Synechococcus elongatus* PCC7942 *Synechocystis* sp. PCC6803 *Synechococcus* sp. PCC7002	Aikens and Turner (2013), Xu and Green (2012)
Flp–FRT	*Synechococcus elongatus* PCC7942 *Synechocystis* sp. PCC6803	Tan et al. (2013)

Regulation of gene expression

Expression can be regulated either through inducible promoters or other inducible elements, such as riboswitches. Riboregulators may include two parts: a cis-repressed mRNA, which forms a stem–loop structure that occludes the ribosomal binding site and prevents target gene translation, and a trans-activating RNA. A set of riboswitches has been developed for *Synechocystis*, which achieves 13-fold expression differences (Abe et al., 2014).

A large number of systems inducible by metal ions, metabolites, and macronutrients have already been characterized (Berla et al., 2013). One remaining challenge is that inducers, because of leakiness or limited dynamic range, cannot sufficiently control expression. Research on the inducing mechanism might present a possible solution to these problems. For example, through a single mutation in the core domain of the *lac* repressor in *E. coli* (Gatti-Lafranconi et al., 2013) or the use of a DNA-looping promoter in *Synechocystis* (Camsund et al., 2014), a wider dynamic range and decreased leakiness was reported for LacI-repressed promoters. Alternatively, with degradation tags, mRNA, and hence protein-lifetime can be modulated (Huang et al., 2010).

MATHEMATICAL MODELING

LESSONS LEARNT FROM METABOLIC ENGINEERING OF FERMENTATIVE ORGANISMS

Systems biology approaches, i.e., the iterative combination of experimental data with theoretical modeling aiming at a systems level understanding, have been gaining pertinence in metabolic engineering (Teusink and Smid, 2006). The importance of computational biology extends beyond helping us deal with large-data sets. Although simplistically portrayed as such, pathways engineered for the production of compounds of interest are never "plug'n'play." They always have repercussions to the host (Branco dos Santos et al., 2013), which therefore should not be perceived as "plug-bugs." This is an important message from the first biotechnological processes for fermentative organisms – engineering cyanobacteria is more likely to succeed if the whole host and its interactions with the environment and neighboring cells are considered.

MAKING (USEFUL) PREDICTIONS OF PHENOTYPIC RESPONSES

Models useful for cyanobacteria are very diverse and should be chosen depending on their purpose. Whether mathematical descriptions should contain mechanistic detail or remain phenomenological depends on the data available. It is important to consider whether a model will be probed to predict the outcome of perturbations to a given state, or rather to simulate steady-state conditions. The boundaries of any model should be stipulated, accepting that all models are only valid under certain (preferably explicit) assumptions.

Mechanistic vs. phenomenological

As illustrated above, certain aspects of the photosynthetic machinery of cyanobacteria still remain elusive. The dynamics of this central process will undoubtedly affect all other rates in a cell, production rate of target compounds included. Does this mean that efforts to model these organisms are futile? Not in our perspective – with the right data one can approximate energy conversion during photosynthesis, generating useful predictions that help understand other processes for which mechanisms are known. The level of mechanistic detail included in a model should be carefully pondered. Too much leads to parameter uncertainty while too little results in predictions that provide little insight. There is one particular modeling strategy that would be interesting to see applied to cyanobacteria – whole cell economy. Understanding the cost/benefit relationship between having different modes available in different environments can shed light on how and why specific

pathways are regulated and what trade-offs cells face (Molenaar et al., 2009).

Dynamic vs. steady-state models

Besides the purpose of the model itself, whether or not to include time as an explicit dimension in a model depends mostly on how much kinetic information is available (Santos et al., 2011). Stoichiometric models of cyanobacterial metabolism outnumber kinetic ones. At least three factors are behind this: (i) models of these organisms are often used to simulate "steady-state" fluxes toward biomass and products, given a fixed input of light and nutrients (Nogales et al., 2012); (ii) the kinetics of key reactions remain elusive, most notably cyclic electron transfer; (iii) there is a lack of standardized data sets that would allow accurate estimation of parameters. Nevertheless, to make kinetic models of these organisms is very pertinent, both for basic science and various applications (Young et al., 2011). These allow a better understanding of how cells respond to a perturbation, and carry flux through different routes.

Model size matters

Models should not be a complete description of all processes in a cell, but rather a description of those necessary to fulfill its purpose. Most cyanobacteria have extended anabolic versatility compared to fermentative organisms, resulting in few auxotrophies. Expression of genes encoding enzymes involved in anabolic pathways varies depending on external conditions (Mitschke et al., 2011). When simulating growth is the purpose, which usually is for cyanobacteria, it is unsurprising that genome-scale models are most popular (Feist and Palsson, 2010). However, by lumping reactions, insightful smaller models can be created (Kelk et al., 2012). These might eventually allow predicting dynamic behavior with reduced uncertainty.

FROM PROOF-OF-CONCEPT TO INDUSTRIAL SCALE

Biotechnological applications with photosynthetic prokaryotes amount to a few M€ annually (http://www.algaeindustrymagazine.com/). Though this is the modest amount if compared to oil-producing eukaryotic algae and hetero-fermentative microorganisms, for reasons already discussed, it is expected (and urgently needed) that this value will gradually increase during the next decade. So, how to make proof-of-principle studies blossom into large-scale sustainable industrial processes?

CHALLENGES AND OPPORTUNITIES DURING THE SCALE-UP

Compared to fermentative organisms, which have been exploited by humans for at least over 8 millennia (Salque et al., 2013), the rational usage of photosynthetic prokaryotes is in its infancy [a notable exception being their food and feed applications (Habib et al., 2008)]. The resulting lack of empirical knowledge hampers their economic attractiveness. Albeit issues such as preventing contaminations and improving downstream processing can be improved, arguably, the major limitation remains light supply. This issue is being addressed by attempting to improve light penetration into photobioreactors, e.g., through antenna-size reduction (Kirst et al., 2014; Lea-Smith et al., 2014). This can also be achieved by higher carbon partitioning toward product rather than biomass, if clever production hosts are engineered (Liu et al., 2011).

FINAL REMARKS

The development of truly sustainable biotechnological processes relying on photosynthetic prokaryotes is a promising solution against the major environmental challenge faced by humanity. Increased understanding of the physiology of these organisms has the highest priority. This can be rapidly achieved if research approaches successfully used in heterotrophic microorganisms are increasingly applied to prokaryotic oxyphototrophs. This will be more successful if organisms such as *Synechocystis* are not perceived as mere chassis for CO_2 fixation, but rather treated as "green" *E. coli*. The input of many research efforts will allow for a much more complete understanding, ultimately, resulting in the development of improved sustainable cell factories.

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Genetic instability in cyanobacteria – an elephant in the room?

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Many research groups are interested in engineering the metabolism of cyanobacteria with the objective to convert solar energy, CO_2, and water (perhaps also N_2) into commercially valuable products. Toward this objective, many challenges stand in the way before sustainable production can be realized. One of these challenges, potentially, is genetic instability. Although only a handful of reports of this phenomenon are available in the scientific literature, it does appear to be a real issue that so far has not been studied much in cyanobacteria. With this brief perspective, I wish to raise the awareness of this potential issue and hope to inspire future studies on the topic as I believe it will make an important contribution to enabling sustainable large-scale biotechnology in the future using aquatic photobiological microorganisms.

Keywords: cyanobacteria, genetic instability, metabolic engineering, biotechnology, mutation, recA

In our laboratory, we are like many others interested in engineering the metabolism of cyanobacteria with the objective to convert solar energy, CO_2, and water (perhaps also N_2) into end-products of commercial value. In comparison to *Escherichia coli*, engineering cyanobacteria can be a slow experience. Imagine if after several rounds of painfully slow construction, transformation, selection, and verification, that one of the modifications is undone. Or, consider crafting an ideal biotechnological strain that is cultivated at a large scale and finding out mid-way through a commercial run that productivity suddenly and unexpectedly drops. It may be caused by one or few nucleotide mutations or a complete loss of a varyingly sized fragment, both resulting in either a loss of gene expression or the functionality of the RNA or protein it encodes. For the purpose of this perspective, we refer to these unwanted changes as "genetic instability" and ignore other potential reasons for loss of productivity such as contamination with other biological species that outcompete or even consume the biotechnological catalyst (Wang et al., 2013).

Our unpublished observations and personal communication with colleagues suggest that such instances of genetic instability are commonly observed. Chances are it will not be reported, nor will the nature of the "instability" be investigated. That is often the fate of negative results; few people are willing to spend resources in chasing up loose ends, which are unlikely to enhance or result in a publication. Instead, they will try it again or try something different, hoping it will work the next time. If such genetic instability is indeed common, yet seldom reported, it is worthy of being called an elephant in the room, or at least a baby elephant; important, obvious, yet largely ignored.

ADAPTIVE EVOLUTION AS A RESPONSE TO ENVIRONMENTAL STRESS

Cyanobacteria have evolved over a long period of time to be successful in their native dynamic environments. Following the "capture" of some of these strains from their natural environment and maintenance under artificial conditions that are likely to be different (i.e., laboratories), it can be asked whether such strains have evolved as a response to this change in their environment. An answer to this may come from examining articles where cyanobacteria have been re-sequenced or sub-strains originating from a single ancestor have been sequenced (Kanesaki et al., 2012; Trautmann et al., 2012). The history of cultivation and exposure to stress is likely to remain unknown in these cases, although it can be assumed that routine laboratory maintenance presents an environment that differs from that in nature (Ikeuchi and Tabata, 2001).

Four sub-strains of *Synechocystis* sp. PCC 6803 (GT-Kazusa, PCC-N, PCC-P, and PCC-M) that originated from the same original isolate [Isolated in 1968; GT-Kazusa was first sequenced in 1996 (Kaneko et al., 1996)] have been maintained in different laboratories. When all four strains recently were (re-)sequenced (Kanesaki et al., 2012; Trautmann et al., 2012), genetic differences were indeed identified. The changes [45 in total, mainly single nucleotide polymorphism (SNPs, i.e., exchange of nucleotides), six 1 bp deletions or insertions, four larger deletions (12–154 bp) and the remainder mobile elements] are visually mapped out in Figure 3 in the work of Trautmann et al. (2012). The impact of those genetic changes on functionality are difficult to connect without further study except where concrete changes in the phenotype (e.g., phototaxis, glucose-uptake, and clumping) have been observed in strains where the amino acid sequence of known proteins have been modified. Interestingly, some of the genes that were affected are known to be regulators associated with stress responses, including *pmgA* (Takahashi et al., 2008), *hik10* (Shoumskaya et al., 2005), *hik25*, and *hik33* (Mikami et al., 2002).

GENETIC "INSTABILITY" IN CYANOBACTERIA IN RESPONSE TO METABOLIC ENGINEERING

In *E. coli*, metabolic engineering has under some conditions been shown to result in genetic instability that at least in part correlates with the level of protein expression and also presence of

repeat elements (Tyo et al., 2009; Sleight and Sauro, 2013). Like with *E. coli*, cyanobacterial strains are in many cases engineered successfully and instability is not observed, e.g., ethanol (Dienst et al., 2014) and isoprene (Bentley et al., 2014). However, a handful of reports of genetic instability in engineered cyanobacteria exist. Takahama et al. (2003) introduced an expression-construct for an enzyme called ethylene forming enzyme (*efe*) into *Synechococcus elongatus* PCC 7942. Although strains that survived the selective pressure were able to synthesize ethylene, they also exhibited a yellow–green appearance. Healthy green–blue variants soon appeared from colonies streaked out from the original transformed culture; however, such strains contained a truncated *efe* gene and did not produce ethylene. The phenomenon was reproducible as serial dilution consistently resulted in the transformation of a population of "unhealthy" ethylene-producers into a population of predominantly healthy strains incapable of ethylene formation. Also the mechanism of disruption was reproducible, with repetitive stretches in the *efe* gene disrupted in each examined case. Altogether, this suggested that expression of the *efe* gene product had a negative effect on the growth of *S. elongatus* PCC 7942 and that repetitive gene hot-spots were modified with a higher frequency than average. We were curious to understand this further and constructed a plasmid expression system for *efe* in *Synechocystis* sp. PCC 6803 with the hope of enabling a model system for genetic instability in cyanobacteria. In contrast to what was observed in *S. elongatus* PCC 7942, however, we observed no sign of instability in *Synechocystis* sp. PCC 6803 even with strains maintained as liquid cultures for more than 6 months (Guerrero et al., 2012).

Other examples also exist. Jacobsen and Frigaard (2014) constructed a mannitol-producing *Synechococcus* sp. PCC 7002 strain in which one particular construct variation consistently exhibited a loss of productivity and an inability to achieve complete segregation. Further analysis revealed a single base frame-shift resulting in the loss of mannitol productivity, and restoration of growth, reminiscent of the observations with *efe* (Takahama et al., 2003). Similarly, Angermayr et al. (2012) found that one slowly growing *Synechocystis* sp. PCC 6803 strain, that was engineered to produce lactic acid, appeared to revert to the wild-type growth rate. Analysis of one of the introduced parts (*sth*, which encodes a soluble NADPH:NADH transhydrogenase) in strains with improved growth revealed a duplication event causing the formation of premature stop codons. In another example, a single nucleotide mutation in *atoB*, one of several genes introduced to *Synechococcus* sp. PCC 7942 in order to produce isopropanol, was repeatedly found. This caused a change in one amino acid residue, which resulted in a reduction but not elimination of enzyme (and pathway) functionality (Kusakabe et al., 2013). Genetic instability was also observed in an earlier study where an electron-transfer protein (PetE) was over-expressed in the same species (Geerts et al., 1995). After the introduction of a second antibiotic selection marker in a strategic position, however, stable expression was observed following selection with the new antibiotic. Subsequently, the construct was stably maintained for over a year even in the absence of antibiotics, suggesting that the construct only was unstable up until the point at which complete segregation had been achieved.

RecA

The observations of genetic instability in cyanobacteria mentioned above seem to relate to structural (i.e., changes in genetic sequence) rather than segregational instability (characterized by loss of entire modifications, typically observed with plasmids). In *E. coli*, these biotechnologically deleterious incidents have been found to be attenuated by the deletion of *recA* (Tyo et al., 2009), encoding a key enzyme for DNA recombination. In early studies, however, the complete elimination of the closest homolog to *E. coli* RecA (sll0569, 77% amino acid similarity in *Synechocystis* sp. PCC 6803; see also **Table 1**) in cyanobacteria was not possible despite repeated attempts, suggesting that RecA is very important in photosynthetic prokaryotes (Murphy et al., 1990). Minda et al. (2005), however, were subsequently able to obtain a fully segregated deletion mutant of *recA* in *Synechocystis* sp. PCC 6803 by carrying out the selection procedure under initially dark and thereafter low-light conditions. The *recA* mutant was highly sensitive to UV-light but displayed a similar rate of growth as the wild-type strain under low-light conditions. They concluded that RecA was essential under conditions where DNA was damaged and that high light in earlier studies had prevented the complete elimination of *recA* as a result of such damage. As it is likely challenging to fully control the quantity of light emanating from sunshine under outdoor large-scale conditions, the deletion of *recA* may therefore not be applicable to aquatic photosynthetic biotechnology in order to enhance genetic stability.

Several other genes with a role in both DNA repair and mutation have also been identified in *E. coli* (**Table 1**). The complement of genes in this small selection is not fully represented in model cyanobacteria species, particularly genes associated with the "*SOS*" response. It is of course possible that proteins with the corresponding functionality in *E. coli* exist in cyanobacteria, yet are not identified by simple BLAST.

IS GENETIC "INSTABILITY" INDUCIBLE IN CYANOBACTERIA?

It is in *E. coli* at least (Fijalkowska et al., 1997; McKenzie et al., 2000). The SOS response is an induced mechanism for both rapid repair of DNA (Kuzminov, 1999) and adaptive evolution (McKenzie et al., 2000). The latter is characterized by the promotion of mutations, rather than prevention, in order to achieve offspring that have adapted to the new growth-limiting conditions (McKenzie et al., 2000) and therefore may survive better. Examples of these conditional changes include the switch from an assimilable to a non-assimilable (by the wild-type) substrate or exposure to antibiotic agents (Michel, 2005). The mutator activity associated with the adaptive SOS response related mutations requires constitutive RecA activity that is induced by stalling DNA replication or binding of RecA to single-stranded DNA (McKenzie et al., 2000), though the mutation does not necessarily occur exclusively at the site of the stall (Fijalkowska et al., 1997). The link between the induction of a general SOS response and changes in environmental or physiological growth conditions, the original reason for adaptive evolution, however, is less clear.

LexA is the first main control element for the induced mutagenic SOS response in *E. coli*. Although a homolog of *lexA* exists in cyanobacteria (**Table 1**), it does not appear to have a role in DNA repair, at least in *Synechocystis* sp. PCC 6803 (Patterson-Fortin

Table 1 | A selection of genes associated with both the production and avoidance of genetic mutations in *E. coli* and their homologs in cyanobacteria.

E. coli	6803	7942	7002	7120
METHYL-DIRECTED DNA REPAIR BY mutHLS COMPLEX				
mutL	slr1199	synpcc7942_1780	SYNPCC7002_A0649	alr3055
mutS	sll1165	synpcc7942_2247	SYNPCC7002_A0178	alr3638
mutH	–	–	–	–
RECOMBINATIONAL REPAIR				
recA	sll0569	synpcc7942_0348	SYNPCC7002_A0426	all3272
recJ	sll1354	synpcc7942_1886	SYNPCC7002_A0980	alr5127
recF	sll1277	–	SYNPCC7002_A2277	all3374
SOS RESPONSE				
LexA	sll1626	Synpcc7942_1549	SYNPCC7002_A1849	alr4908
dinB (polIV)	–	–	–	alr7254
polB (polII)	–	–	–	–
umuCD (polV)	sll5122-23	synpcc7942_1549-50	–	–
uvrA	slr1844	Synpcc7942_0848	SYNPCC7002_A1468	alr3716
RuvA	sll0876	Synpcc7942_2298	SYNPCC7002_A1270	all0375
RpoS	sll0184 (sigC)	Synpcc7942_1557	SYNPCC7002_A0364	alr4249

The presence or absence of a corresponding homologous E. coli gene in cyanobacteria was determined by BLASTP using KEGG with a probability cut-off with an E-value of $2e^{-10}$ and at least 50% amino acid sequence similarity as compared to the full-length translation product of the corresponding E. coli gene. The minus sign (–) indicates that a gene satisfying the above criteria was not found. 6803, Synechocystis sp. PCC 6803; 7942, Synechococcus elongatus PCC 7942; 7002, Synechococcus sp. PCC 7002; 7120, Anabaena sp. PCC 7120.

et al., 2006). As the LexA-regulon is essential for induced mutation in *E. coli*, it would be interesting to understand how this mechanism is managed in cyanobacteria, and whether it is realized or not, in the absence of a DNA repair associated LexA. Interestingly, a computational analysis across a large number of cyanobacterial genome sequences suggests that LexA has a similar role to that in *E. coli* in most cyanobacteria, though not in *Synechocystis* sp. PCC 6803 (Li et al., 2010), the most common model species used for cyanobacteria research.

The sigma factor RpoS (Sigma 38), a regulator of a generalized stress response (Battesti et al., 2011), is a second control element for DNA mutation in *E. coli*. Here, the role of SOS in RpoS-regulated DNA mutation is solely to upregulate the expression of DinB (Galhardo et al., 2009), an error-prone DNA polymerase, which does not have any close homologs in model cyanobacteria (**Table 1**). The closest homolog of RpoS in *Synechocystis* sp. PCC 6803 is SigC, though SigC displays slightly greater amino acid sequence similarity to *E. coli* RpoD (Sigma 70). As the two *E. coli* sigma factors, RpoS and RpoD, have considerably different roles, yet highly similar sequences, it is challenging to elucidate what role any homologs may have in other species.

In conclusion, the search for genes involved in the repair and/or mutation of DNA in cyanobacteria, that are homologous to those in *E. coli*, is not straightforward. It is possible that all components needed to implement effective mutation (or relax the quality of repair) of DNA under particular conditions may be present in cyanobacteria, only we do not yet know any or all components that constitute the required system. Clearly, some components will differ. For example, LexA has a completely different role in cyanobacteria than in *E. coli*, and there does not appear to be a trace of some of the error-prone DNA polymerases. The external environment under which cyanobacteria evolved is also likely to be markedly different from the environments that *E. coli* evolved in.

RESPONSE OF GENES WITH A ROLE IN DNA REPAIR AND MUTATION TO STRESS

If DNA mutations are inducible, one possibility is that genes with a role in DNA repair and mutation (**Table 1**) respond to conditions in which there is environmental stress or a metabolic burden. In recent omics studies, the whole-system response of cyanobacteria has been evaluated with strains that have been exposed to potential fuel production targets (Liu et al., 2012; Qiao et al., 2012; Tian et al., 2013; Zhu et al., 2013). Notably, in all studied cases, the exposure to the solvent had a measurable negative effect on growth, and the relative quantity of several hundred transcripts and/or proteins was modified. Of those genes listed in **Table 1** (genes associated with DNA repair or mutation), only two were affected by any of the treatments: The accumulation of UvrA (slr1844) was enhanced by ethanol exposure (Qiao et al., 2012) and the accumulation of RecF (sll1277) was enhanced by hexane exposure (Liu et al., 2012).

In contrast, Dienst et al. (2014) analyzed the transcriptomic response of *Synechocystis* sp. PCC 6803 to the introduction of an ethanol pathway. Surprisingly, even though the presence of the ethanol pathway resulted in a marked negative effect on growth, only three mRNAs displayed a clear quantitative difference, none of which is known to be associated with genetic modifications or stress. This result is surprising on two levels: Firstly, there was no reported loss of productivity (an indicator of genetic instability) over presumed repeat experiments, despite a marked negative effect on growth and cultivation for 18 days. Secondly, there was no immediate (i.e., not evolved) transcriptional response to either the pathway or reduced growth. This contrasts markedly to the

above-mentioned studies where solvents (including ethanol) were added externally to the medium. Was the negative effect on growth in the Dienst study not sufficiently strong? The ethanol concentration that the engineered strains reached [0.6% (v/v)] was several-fold lower than in studies where it was added externally, and in previous studies, there was no measurable change in the growth rate at this concentration (Kämäräinen et al., 2012). The observed negative growth effect may therefore at least in part be caused by an effect of the metabolic engineering (e.g., antibiotic resistance, heterologous protein over-expression, metabolic interference, intermediate toxicity), rather than as a direct consequence of the continuously increasing concentration of ethanol. Still, if genetic instability only exploits differential fitness to allow mutations to propagate, the cause of poor fitness (by engineering or environmental manipulation) should not matter.

ALTERNATIVE SOLUTIONS

Most initial engineering tools in cyanobacteria did not allow for user-controlled regulated protein expression, and such non-silenceable systems were used in all the reports of genetic instability in cyanobacteria described above, with one exception (Geerts et al., 1995). Recently, there has been progress toward the development of regulatable promoters for engineering cyanobacteria that are capable of user-controlled regulation of protein expression over a wide dynamic range. Although a perfect system is arguably not yet in sight, several promising alternatives are at least available (Guerrero et al., 2012; Huang and Lindblad, 2013). Whether their use may limit genetic "instability" or not is an interesting question that warrants further investigation.

CONCLUSION

The limited amount of available information makes it difficult to assess how great an issue genetic instability really is toward the aim of developing aquatic photobiological biotechnology. Several other issues also exist including excessively expensive cultivation infrastructure and culture crashes as a result of biological contamination/invasion. In the absence of a *recA*-based solution, how can we prevent genetic instability? Currently, there are no other solutions in the literature that are obvious. At first, however, it would be good to at least confirm on an analytical level that genetic instability is indeed an issue and if so to determine whether or not it is inducible in cyanobacteria. It would be advisable to extend such studies to more than one model cyanobacteria species as they appear to be quite different in this respect, see (Guerrero et al., 2012) and **Table 1**. Thereafter, the development of a model system that is not excessively unstable, yet also not too stable, would pave the way for both targeted and non-targeted screens for factors that are essential for the process. This may then lead on to possible solutions to minimize the negative impact of genetic instability in cyanobacteria for both fundamental and applied sciences, and thereby contribute toward the development of economically sustainable aquatic photo biotechnology using engineered biology in a hopefully not too distant future.

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The regulation of light sensing and light-harvesting impacts the use of cyanobacteria as biotechnology platforms

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Light is harvested in cyanobacteria by chlorophyll-containing photosystems embedded in the thylakoid membranes and phycobilisomes (PBSs), photosystem-associated light-harvesting antennae. Light absorbed by the PBSs and photosystems can be converted to chemical energy through photosynthesis. Photosynthetically fixed carbon pools, which are constrained by photosynthetic light capture versus the dissipation of excess light absorbed, determine the available organismal energy budget. The molecular bases of the environmental regulation of photosynthesis, photoprotection, and photomorphogenesis are still being elucidated in cyanobacteria. Thus, the potential impacts of these phenomena on the efficacy of developing cyanobacteria as robust biotechnological platforms require additional attention. Current advances and persisting needs for developing cyanobacterial production platforms that are related to light sensing and harvesting include the development of tools to balance the utilization of absorbed photons for conversion to chemical energy and biomass versus light dissipation in photoprotective mechanisms. Such tools can be used to direct energy to more effectively support the production of desired bioproducts from sunlight.

Keywords: biotechnology, cyanobacteria, light signaling, photoinhibition, photosynthesis, phycobilisomes, synthetic biology, systems biology

INTRODUCTION

The use of photosynthetic organisms as biotechnological platforms for the production of bioproducts has gained significant interest in recent years. These organisms have the potential to combine the functions of photocatalyst and production platform in a single organism, thereby potentially truncating the process from harvesting solar energy to the production and isolation of important bioproducts, including biofuels (Lindberg et al., 2010; Melis, 2012; Oliver and Atsumi, 2014). The general potential of cyanobacteria as biofactories or for biotechnological applications has been addressed in recent reviews (Lindblad et al., 2012; Oliver and Atsumi, 2014).

Cyanobacteria and algae have been described as better photosynthetic platforms than land plants due to high photosynthetic rates and a greater potential for the diversion of photosynthetically fixed carbon to the production of target molecules (Melis, 2009). This higher potential is primarily due to there being no need to divert energy to support non-photosynthetic tissues, which can comprise a significant proportion of the organism in the case of plants (Melis, 2012). Although still somewhat early in their development as biotechnology chassis, some developmental or engineering modifications have been tested for improving the use of cyanobacteria as effective production platforms. Increasing the partitioning of photosynthetically produced carbon to pathways of interest can increase overall yield of the production of target molecules in engineered cyanobacteria. Cyanobacteria can be engineered to couple the expression of genes that increase flux through pathways which provide key substrates or precursors to engineered pathways to further promote the production of target molecules (Bentley et al., 2014; Halfmann et al., 2014; Kiyota et al., 2014). Alternatively, knocking out natural carbon sinks or storage products, such as glycogen synthesis, can result in a greater proportion of carbon partitioning to non-native products (Li et al., 2014; van der Woude et al., 2014).

Oxygenic photosynthesis has been proposed to be theoretically limited to an 8–10% efficiency of solar-to-biomass energy conversion (Melis, 2009). This theoretical limit can be attained under low light conditions, but drops to 2–3% or even lower for some species in full sunlight (Melis, 2012). Sub-optimal light, light-harvesting complexes that are not in balance with the external environment, or photoinhibitory mechanisms associated with excess light absorption are among the factors that can contribution to this limitation. One mechanism for dissipating excess light is non-photochemical quenching (NPQ), which is the dissipation of excess energy as heat (Niyogi and Truong, 2013). NPQ is the primary mechanism that appears to limit solar-to-biomass conversion efficiency (Melis, 2012). This observation suggests NPQ regulation as a potential target for improving the development of cyanobacteria as bioproduction strains. Other targets for improving biotechnological or industrial applications using these organisms have included assessing the impact of source–sink relations on productivity. Prior studies indicate that engineered cyanobacterial strains which have an additional sink for carbon, either through incorporation in particular products or through export of carbon-based compounds from the cell, respond by exhibiting increased rates of carbon dioxide assimilation and increased rates

of photosynthesis (Ducat et al., 2012; Ungerer et al., 2012; Oliver et al., 2013; Bentley et al., 2014; Halfmann et al., 2014).

The ability to photosynthesize and adapt to variable environments are the primary reasons that cyanobacteria exhibit great potential for bioengineering and biotechnological applications. However, some potential damaging impacts of light or light-dependent utilization of energy for acclimation responses must be balanced to maximize the efficacy of cyanobacteria as production chassis. The content and number of phycobilisomes (PBSs), i.e., accessory photosynthetic light-harvesting complexes, and core photosystems have to be balanced to maximize light absorption for the conversion of light energy to chemical energy in the form of photosynthate, while also minimizing the absorption of excess light. Excess photoexcitation can lead to photoinhibition and phototoxicity and thereby limit the production of target molecules. The utilization of light in photomorphogenic or light-dependent growth and developmental responses, for which the fitness implications are still not well defined, can be quite costly energetically and thus could impact organismal productivity. Thus, the impact of light on additional phenotypes requires attention as these light-dependent processes can contribute to or draw away energy available for the improvement of solar-to-biomass conversion efficiencies or conversion of photons to chemical energy used to support production of desired compounds.

LIGHT IMPACTS ON CULTURE GROWTH AND PRODUCTIVITY

The importance of light both in providing energy to fuel the growth of cyanobacterial and algal strains and the bioproduction of target molecules, as well as the potential damaging effects of excess light has been addressed, primarily from the perspective of minimizing shading and maximizing productive exposure of all cells in a culture to light (Scott et al., 2010). However, the impact of light on cultures can be quite complex in that light can initiate acclimation responses in addition to primary photochemistry. Acclimatory light responses can either increase or decrease overall cellular productivity (**Figure 1**).

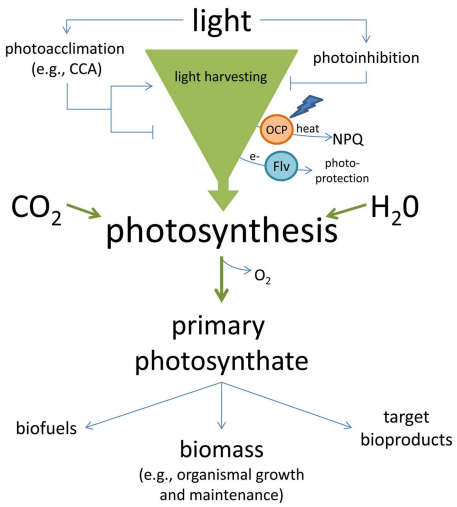

FIGURE 1 | Light-harvesting in cyanobacteria and potential costs and/or contributions to solar energy conversion. Light harvested in cyanobacteria can be used with carbon dioxide (CO_2) and water (H_2O) for photosynthesis. When excess energy is absorbed, it can result in damage to the photosystems and photoinhibition, or energy can be dissipated as heat by proteins such the orange carotenoid protein (OCP) or electrons can be transferred to alternative acceptors such as flavodiiron proteins (Flv) during photoprotection. In response to the environment, acclimation responses which tune the size and pigment composition of photosystems to maximize light absorption, or alter the size and composition of light-harvesting complexes to reduce or increase light absorption, can modify the organismal potential for light-harvesting. Light energy that is used for the reactions of photosynthesis can be converted to chemical energy (primary photosynthate) that supports growth and maintenance, including biomass production, or can be diverted and used for the production of target bioproducts and/or biofuels.

LIGHT-REGULATED TRANSCRIPTIONAL AND CELLULAR RESPONSES IMPACT ENERGY PRODUCTION CAPACITY IN CYANOBACTERIA

Light impacts cyanobacterial photosynthetic complex composition and cellular physiology

The acclimation response, historically known as complementary chromatic adaptation (CCA), is one process by which cyanobacterial cells use a transcriptional response to produce PBSs that are spectrally tuned to maximally absorb the prevalent wavelengths of light in the ambient environment (Gutu and Kehoe, 2012). CCA also drives light-dependent alterations of cellular or filament morphology (Bennett and Bogorad, 1973; Bordowitz and Montgomery, 2008). CCA, thus, through tuning light absorption to the external environment maximizes the potential for light energy to chemical energy conversion. Notably, CCA itself is under the regulation of a light-sensitive photosensory protein, i.e., RcaE, which controls the transcription of PBS-encoding genes (Kehoe and Grossman, 1996; Terauchi et al., 2004) and light-dependent morphological changes (Bordowitz and Montgomery, 2008; Singh and Montgomery, 2014). A mismatch between PBS protein composition with light available results in an initial loss of photosynthetic efficiency until the protein composition is recalibrated with the predominant wavelengths of light available (Campbell, 1996).

A significant contribution of energy to altering other aspects of growth and development occurs in response to changes in the external light, including morphological phenotypes. Changes in the prevalent wavelengths and intensity of light can lead to CCA-associated shifts between spherical and rod-shaped cells (Bennett and Bogorad, 1973; Bordowitz and Montgomery, 2008; Pattanaik et al., 2012; Walters et al., 2013). Distinct wavelengths have also been shown to induce cellular differentiation in some cyanobacteria from vegetative cells to other cell types including motile hormogonia (Damerval et al., 1991) or spore-like akinetes (Thompson et al., 2009). These morphological changes have largely unknown impacts on efficiency and solar energy conversion. Whereas, the ability of cells to modulate photosynthetic pigment composition is beneficial to long term culture productivity, the energetic costs or benefits of photo-dependent morphological control is less well understood. It has been suggested, however, that the morphological effects may be related to controlling cell volume and the related capacity for thylakoid membrane content, which drives PBS quantity primarily for adaptation to low light conditions (Montgomery, 2008; Pattanaik et al., 2012; Walters et al., 2013). Thus, the morphological alterations may regulate photosynthetic membrane

capacity and thereby contribute to optimizing photosynthetic efficiency in response to changes in the external environment.

Similar to the benefits of regulating PBS protein or pigment composition, distinct photosystem (PS) proteins may also be used under different environmental conditions. Such alterations occur as a part of acclimation responses that are generally associated with fitness advantages under particular conditions, which include differences in light quality and/or intensity (Vinyard et al., 2013, 2014). In addition to protein substitutions or modifications, the overall antenna size may be modulated to alter cellular productivity and/or for cellular photoprotection. There are noted correlations of photosynthetic light-harvesting complex size and number with photoacclimation or photoprotection responses (Jodłowska and Latała, 2013). Diel (Jacquet et al., 2001; Poulin et al., 2014) and circadian cycles (Cervený and Nedbal, 2009) can also impact chlorophyll levels, which can be directly related to potential productivity and/or photoinhibition. Such acclimation responses suggest strong effects of growth conditions on the potential productivity of strains. These acclimation responses can either increase light-harvesting for photosynthesis or alternatively can promote photoprotection, which limits damage while often simultaneously reducing photons harvested for the photochemical reactions of photosynthesis (**Figure 1**).

The production of different versions of light-absorbing photosynthetic proteins or modulations of antenna size, in addition to other light-dependent changes in growth and development or photomorphogenesis, require energy that is spent during the acclimation responses – energy that is no longer available for the production of target molecules or products. To address this potential non-productive use of energy during acclimation, some analyses have been conducted to assess the benefits of truncating antennae size on supporting an overall increase in photosynthetic productivity for dense mass culturing. Truncated antennae promote light penetration and absorption for photosynthesis, while simultaneously reducing the potential for photoinhibition (reviewed by Melis, 2009).

Light impacts cyanobacterial transcription through the regulation of sigma factors

Sigma (sig) factors that impact cyanobacterial gene transcription are themselves regulated by environmental factors (Imamura and Asayama, 2009). For example, Sig B levels increase in response to stress, including under heat shock, in response to nitrogen starvation and in darkness (for review see Imamura and Asayama, 2009). Analysis of a Δ*sigB* mutant in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) indicated that SigB serves as a negative regulator of the expression of a number of photosynthesis-related genes, among others (Foster et al., 2007). Thus, SigB accumulation under stress conditions likely contributes to downregulation of photosynthetic capacity, and thus could have a negative impact on photosynthetic capacity when overexpressed. Some cyanobacterial sigma factors also have been reported to be involved in the circadian regulation of rhythmic gene expression (Tsinoremas et al., 1996; Nair et al., 2002). The accumulation of others is regulated by light, including SigB, SigD, and SigE (Imamura et al., 2003a; Tuominen et al., 2003). These light-induced sigma factors have been shown to impact the transcription of light-induced PBS and PS genes (Imamura et al., 2003a; Summerfield and Sherman, 2007; Yoshimura et al., 2007; Pollari et al., 2009). SigB and SigD have also been shown to have a role in cellular adaptation of *Synechocystis* to high light conditions (Imamura et al., 2003b; Pollari et al., 2008, 2009, 2011), as has a SigD homolog in *Synechococcus elongatus* PCC 7942 (Seki et al., 2007). Recent studies with *Synechocystis* strains in which several *sig* genes were deleted highlighted an additional role of Sig proteins in modulating light use efficiency in response to moderate changes in light intensity (Tyystjärvi et al., 2013). Several sigma factors are, thus, directly linked to regulation of photosynthetic potential and the capacity for phototoxicity.

The correlation of several sigma factors with the regulation of photosynthesis-related genes and light use efficiency suggests that modulation of these genes could impact the development of cyanobacterial strains as production platforms. In this regard, *sigB* overexpression has been shown to improve the use of *Synechocystis* as a platform for producing the biofuel butanol, as well as for improving tolerance to non-optimal temperature and reducing reactive oxygen species (ROS) generation (Kaczmarzyk et al., 2014). By comparison, *sigE* overexpression negatively impacts the expression of PS genes and results in reduced photosynthetic efficiency in nutrient-replete growth (Osanai et al., 2013). Thus, the regulation of sigma factors *in vivo* has complex outcomes. Apart from photosynthesis-related genes, there may well be other non-photosynthetic genes whose expression is modulated by environmental regulation of sigma factors that could be vitally important for maximizing solar energy conversion and the development of cyanobacteria as biotechnological platforms. The utilization of systems biological approaches, including transcriptomics and proteomic analyses, to explore such connections could prove vitally important for further development of cyanobacterial production systems that have defined means for tuning gene expression over a wide range of conditions to promote robust and sustainable production of value target molecules.

PHOTONS THAT ARE NOT CONVERTED TO CHEMICAL ENERGY LIMIT PRODUCTION AND CAN CAUSE CELLULAR DAMAGE

Unless regulated, excess or variable light can lead to photoinhibition and the potential generation of damaging compounds. Phototoxicity is induced by high light, fluctuating light, and nutrient deficiencies that can impact the abundance and composition of light-harvesting complexes. An imbalance between light-harvesting complexes and the quality and/or quantity of available light can result in the generation of ROS, which can lead to nucleic acid and protein damage and lipid peroxidation (reviewed by Blokhina et al., 2003). There are several known physiological mechanisms for dealing with potential phototoxicity, including photoinhibition and regulating the amount of light transferred to the photosystems through dissipating excess absorbed light energy. Photoinhibition results from a reduction in photosynthetic efficiency due to light-induced damage of photosystems (PSs), primarily PSII (Tyystjärvi, 2008). Excess light is predominantly dissipated through defined mechanisms such as NPQ or loss of excess energy as heat or fluorescence (Niyogi and Truong, 2013) (**Figure 1**). Dissipatory regulation of light primarily occurs through state transitions and protein-mediated photoprotective mechanisms.

Dissipation of excess light through state transitions

State transitions regulate the amount of energy transferred from PBSs to the photosystems through physical movement and/or disruption of the association of the PBS with PSI or PSII at the thylakoid membrane surface (Joshua and Mullineaux, 2004). State transitions, which are defined as State 1 or 2, allow a rapid mechanism for regulating light absorption by the photosystems. In State 1, energy from PBSs is preferentially transferred to PSII; however, when light absorption exceeds the capacity of PSII excitation, a transition to State 2 occurs during which energy from PBSs is funneled to PSI (Mullineaux and Emlyn-Jones, 2005). Traditionally, lateral mobility or diffusion on the thylakoid membrane surface of PBSs between PSI and PSII reaction centers has been described as a mechanism for state transitions in cyanobacteria. Recently, a cyanobacterial megacomplex, which contains a PBS complex together with both PSI and PSII has been described (Liu et al., 2013). The megacomplex is still proposed to function to transfer energy to either PSI or PSII likely through distinct mechanisms (Liu et al., 2013). If the occurrence of this megacomplex is widespread in cyanobacteria, a revision of exactly how state transitions occur may be necessary as the need for lateral diffusion of PBS for state transitions may not be required. The coordination or control of PBS energy transfer to PSII vs. PSI is one significant point of regulation of excitation energy transfer and its conversion to chemical energy. Thus, the regulation of state transitions has the potential to drive the balance of photosynthetic energy transfer or energy dissipation for fine-tuning the conversion of chemical energy to biomass.

Photoprotective mechanisms used to protect against excess light absorption

One mechanism for the dissipation of excess absorbed light energy depends on the function of the orange carotenoid protein (OCP) and its accessory protein fluorescence recovery protein (FRP). OCP and FRP work together to regulate energy flow from the PBS to PS in a NPQ of energy (Kirilovsky and Kerfeld, 2012, 2013). OCP is a carotenoid-bound photoactive protein that is activated by blue light (**Figure 1**). Photoactivated OCP binds to the PBS core and converts absorbed light energy to heat, thereby diverting energy transfer to the photosystems (Kirilovsky and Kerfeld, 2012, 2013). FRP releases activated OCP from the PBS and converts it back to its ground state, thereby resetting the system (Kirilovsky and Kerfeld, 2013). OCP has also been recently shown to have an independent role in protecting cells from strong light-induced single oxygen production, which is distinct from its role in binding to PBSs (Sedoud et al., 2014).

Another cyanobacterial photoprotective mechanism to divert potential overexcitation of the PSs involves the flavodiiron (Flv) proteins. These proteins function in removing electrons from the electron transport chain and transferring them to alternative electron acceptors (for review see Mullineaux, 2014) (**Figure 1**). The expression of *flv* genes is regulated by environmental factors including increased light intensity (Zhang et al., 2009; Bersanini et al., 2014) and fluctuating light (Allahverdiyeva et al., 2013). A greater understanding of the regulation of OCP and Flv systems may allow targeted induction of these systems to support the efficacy of cyanobacteria as biotechnological chassis.

IMPLICATIONS OF LIGHT-ASSOCIATED GROWTH RESPONSES ON THE USE OF CYANOBACTERIA AS PRODUCTION STRAINS

CURRENT LIMITATIONS ASSOCIATED WITH LIGHT RESPONSES IN CYANOBACTERIAL PRODUCTION STRAINS

Increasing carbon sink strength and/or adding additional carbon sinks to alter source–sink relationships and potentially relieve sink-related feedback inhibition of photosynthesis have been attempted to improve productivity in cyanobacterial strains. Current tools for increasing the production of soluble sugars in cyanobacteria include subjecting cells to stresses such as high salt concentrations in growth media (Ducat et al., 2012; Du et al., 2013; Hays and Ducat, 2014). However, such conditions often lead to reduced accumulation of photosynthetic pigments and associated reductions in growth (e.g., Kanesaki et al., 2002; Marin et al., 2004; Bhadauriya et al., 2007; Singh and Montgomery, 2013). For cells subjected to stress, exposure to excess light can lead to the production and accumulation of damaging ROS. Therefore, subjecting cells to stress as a means to induce the accumulation of soluble carbohydrates has significant drawbacks. Nitrogen-limitation of *Synechococcus* sp. PCC 7002 has been used to increase the carbon-to-nitrogen ratio and glycogen content to improve its utility as a biomass feedstock (Möllers et al., 2014). Nitrogen starvation of cyanobacteria leads to degradation of PBS complexes (Paone and Stevens, 1981; Stevens et al., 1981; Salomon et al., 2013). Under these conditions, cyanobacteria exhibit a reduction in growth and increased potential for the induction of photodamage similar to salt stress. Ultimately, the molecular bases of stress-related carbon allocation responses such as salt-induced soluble sugar production are often not well understood (Melis, 2013). Thus, knowledge-based attempts to genetically manipulate cyanobacterial strains to support increased production of target products are not possible to a significant degree.

BENEFICIAL LIGHT-ASSOCIATED PROPERTIES OF PRODUCTION STRAINS

Production strains ideally will limit energy contributed to acclimation responses, particularly due to transient changes in the external environment, to maximize photosynthetic efficiencies – unless such an acclimation response is absolutely critical for survival. One method for avoiding the absorption of excess light that must be dissipated by NPQ and/or reducing the shading of cells in dense cultures or in benthic environments has been to isolate strains with smaller light-harvesting systems or truncated light-harvesting antenna (TLA) mutants (Kirst and Melis, 2014). These strains have been proposed to allow deeper penetration of light into cultures that should be associated with increased culture productivity (Melis et al., 1999; Melis, 2009; Kirst and Melis, 2014; Lea-Smith et al., 2014). Several methods have been used to identify or generate TLA mutants (Kirst and Melis, 2014); however, there has not been a straightforward association of such mutants with improvements in productivity (Blankenship and Chen, 2013). Recent studies indicate that interactions between light intensity and carbon availability may impact the degree of productivity for TLA mutants compared to wild-type cultures (Lea-Smith et al., 2014).

One suggested alternative to the creation of TLA mutants is to use cells that can absorb light in an expanded region of the visible spectrum to increase the total light that is used to drive photosynthesis and thereby overcome "shading effects" (Blankenship and Chen, 2013). One such possibility is to grow cells with distinct chlorophyll pigments that absorb distinct wavelengths of light in mixed culture to increase photon use efficiency (Scott et al., 2010). Beside the common chlorophyll *a* that is found in cyanobacteria, there are less common red-shifted chlorophylls. At least two red-shifted chlorophyll molecules, i.e., chlorophyll *d* and *f*, have been described (Swingley et al., 2008; Chen et al., 2010). Once the biosynthetic pathways of these red-shifted molecules are better understood, their synthesis may be introduced into a chlorophyll *a* containing strain using synthetic biology approaches to expand the range of visible light absorption supporting photosynthesis in a single organism. Another potential alternative is to grow organisms with distinct pigment compositions of the auxiliary PBSs or those that can adapt their PBSs to the external light conditions in mixed cultures. Alternate approaches require additional knowledge about the mechanisms used by cells to regulate antenna composition, size, and modulation *in vivo*.

CONCLUSION

The increasing number of sequenced cyanobacterial strains should facilitate an increased understanding of regulatory and physiological mechanisms used by these organisms to adapt to variable environments. This knowledge may serve as bases for improved engineering and biotechnological adaptation in cyanobacteria (Jin et al., 2014). Although light sensing and light-harvesting have been the focus of the discussion here, carbon dioxide capture, fixation, and recycling are also targets or points of interest for maximizing the biotechnology applications for cyanobacteria (e.g., Ducat and Silver, 2012; Rosgaard et al., 2012). These areas and others will be equally benefited, and perhaps synergistically deployed, based on continued insights into the basic mechanisms and impacts of environmental variation on a range of cyanobacteria and the development of appropriate tools to improve adaptation of these strains as broadly applicable production platforms.

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Systems and photosystems: cellular limits of autotrophic productivity in cyanobacteria

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Recent advances in the modeling of microbial growth and metabolism have shown that growth rate critically depends upon the optimal allocation of finite proteomic resources among different cellular functions and that modeling growth rates becomes more realistic with the explicit accounting for the costs of macromolecular synthesis, most importantly, protein expression. The "proteomic constraint" is considered together with its application to understanding photosynthetic microbial growth. The central hypothesis is that physical limits of cellular space (and corresponding solvation capacity) in conjunction with cell surface-to-volume ratios represent the underlying constraints on the maximal rate of autotrophic microbial growth. The limitation of cellular space thus constrains the size the total complement of macromolecules, dissolved ions, and metabolites. To a first approximation, the upper limit in the cellular amount of the total proteome is bounded this space limit. This predicts that adaptation to osmotic stress will result in lower maximal growth rates due to decreased cellular concentrations of core metabolic proteins necessary for cell growth owing the accumulation of compatible osmolytes, as surmised previously. The finite capacity of membrane and cytoplasmic space also leads to the hypothesis that the species-specific differences in maximal growth rates likely reflect differences in the allocation of space to niche-specific proteins with the corresponding diminution of space devoted to other functions including proteins of core autotrophic metabolism, which drive cell reproduction. An optimization model for autotrophic microbial growth, the autotrophic replicator model, was developed based upon previous work investigating heterotrophic growth. The present model describes autotrophic growth in terms of the allocation protein resources among core functional groups including the photosynthetic electron transport chain, light-harvesting antennae, and the ribosome groups.

Keywords: cyanobacteria, growth rate, molecular crowding, optimization, photosynthesis, ribosomes

HOW FAST CAN CYANOBACTERIA GROW?

There is a very wide range of maximal growth rates observed among cyanobacterial strains (Carr and Whitton, 1982). Fast growing model cyanobacterial strains can be grown with doubling times in the range of 3–6 h under optimal conditions (Binder and Chisholm, 1990; Nomura et al., 2006; Kim et al., 2011; Ludwig and Bryant, 2011). On the other hand, many cyanobacterial strains have doubling times on the order of once per day (Carr and Whitton, 1982). Moreover, even the fastest growing cyanobacteria are still much slower growing than many heterotrophic bacteria and yeasts. Furthermore, the factors accounting for the diversity of maximal rates of cyanobacterial growth remain poorly understood and it appears that autotrophic growth tends to be slower than heterotrophic growth, which can be as short as ~10 min doubling times (Labbe and Huang, 1995). This is important because researchers often include "fast growth" among the criteria in choosing a cyanobacterial strain for engineering. Since the fastest rate of growth in heterotrophs occurs in "rich" media containing abundant amino acids and cofactors and since the main macromolecular investment in cell growth is the synthesis of proteins, then a reasonable hypothesis is that autotrophic metabolism in cyanobacteria results in slower maximal growth because of the necessity for the synthesis of the amino acids and all other cell components from CO_2. However, assuming it is the burden of synthesizing amino acids, then the question arises whether this is because of the energetic cost of making amino acids, such as ATP consumed per amino acid and "opportunity costs" of not using ATP for other cell functions that contribute to cell reproduction. A bioinformatics analysis using codon bias as an indicator for expression rates found that less expensive amino acids are preferably utilized for highly expressed protein (Akashi and Gojobori, 2002). However, energetically more expensive amino acids also tend to require more biosynthetic steps, and consequently a greater number of enzymes. Moreover, a more exhaustive analysis found that amino acid utilization rates for protein were only weakly correlated, if at all, with the bioenergetic costs of their synthesis (Barton et al., 2010). Or is it something else, such as greater cellular space devoted to the corresponding biosynthetic enzymes? As discussed below, recent theoretical and experimental studies point to the latter and suggest that the ultimate speed limit relates to the physical constraints of packing all necessary molecular machinery, small molecules, and ions into the confined space (cytoplasmic

and membrane) of the cell yet have small enough cell dimensions to allow sufficient nutrient exchange (**Figure 1**). Analysis of the physical state of cytoplasmic water in *E. coli* under different osmotic conditions indicates that macromolecular crowding limits growth rate, probably through decreasing the "kinetics of some biopolymer diffusion processes" (Cayley and Record, 2003). Accordingly, intracellular crowding appears to be the main constraint to growth and sets the upper bound on the size of the proteome. Evidence for this includes the analysis of the impact of protein overexpression on maximal growth rates (Scott et al., 2010; Scott and Hwa, 2011; You et al., 2013) and the effects of crowding on diffusion within the bacterial cell (Klumpp et al., 2013; Soh et al., 2013; Parry et al., 2014). Crowding also would affect the size to the metabolome, primarily because of the limited amount of free water, as discussed below. Based on these considerations, the total size of the proteome is likely bounded by intracellular crowding constraints and, assuming a relatively fixed amount of dissolved ions and metabolites[1], the allocation of proteomic resources becomes a "zero sum game." This is one of the key points for this discussion since we are trying to understand the physical basis optimal proteomic allocation strategies. As discussed below, the results of modeling studies and the consideration of "overflow" metabolism is best explained by a limitation in the total amount of protein that can be crowed into a cell, yet remain soluble and diffusionally mobile. From that perspective, it could be that the autotrophic lifestyle requires a comparatively large investment into photosynthetic and other anabolic proteins. This would include the large investment into extensive internal membranes for photosynthetic machinery as well as the need for large amounts of the catalytically inefficient carbon-fixing enzyme, Rubisco. Correspondingly, this comparatively greater investment would come at the expense of the macromolecular machinery dedicated to cell duplication including ribosomes, initiation factors, cell division proteins, and all the metabolic precursors required for the duplication. Conversely, it might be expected that very fast growing bacteria (Labbe and Huang, 1995) have a greater investment in the machinery for cell duplication and a streamlined metabolic capacity that constituted by a minimal set of core enzymes, transporters, and metabolites for the provision of precursors to support the operation of the cell duplication machinery.

[1]As far as the author is aware, information on possible changes in the total size of the metabolome as a function of different conditions is not readily available.

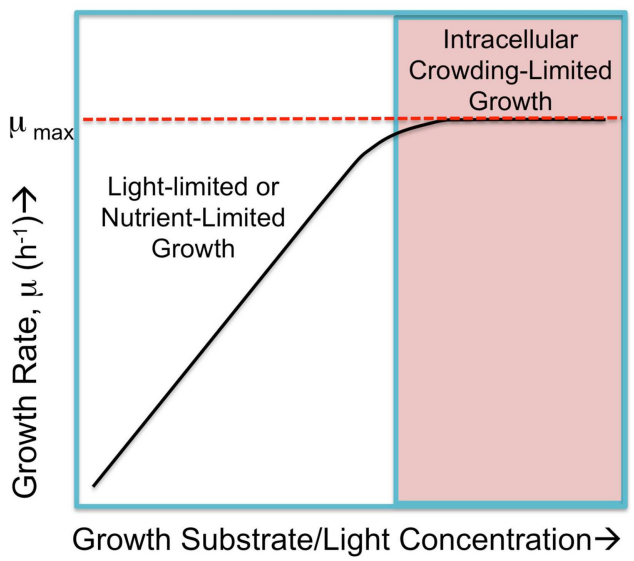

FIGURE 1 | Hypothetical cyanobacterial growth rate, μ, in response to substrate abundance falls into two regimes. Submaximal rates of growth occur when either the light intensity or nutrient availability is limited and growth rates increase in proportion to increases in the limiting commodity, as shown in the left portion of the hypothetical graph. When these are not limiting, the growth rate is the saturated, maximal rate, μ_{max}. This maximal rate is hypothetically limited by physical constraints such as packing all necessary molecular machinery, small molecules, and ions into the confined space (cytoplasmic and membrane) of the cell yet have small enough cell dimensions to allow sufficient nutrient exchange. Cells approach a maximal rate where internal factors, referred to as "intracellular crowding-limited" dominate. Figure adapted from O'Brien et al. (2013), but the "macromolecular expression (ME)-limited" or "proteome-limited" growth rate is here considered as limited intracellular crowding as discussed in the text.

ENGINEERING AND MODELING CYANOBACTERIA

Because of their comparative cellular simplicity and ease of genetic manipulation, cyanobacteria are the object of numerous biotechnological efforts for metabolic engineering for the renewable production of biofuels and high-value products. Compared with algae and plants, cyanobacteria are easier to genetically modify and are amenable to organism-wide metabolic modeling, which are attributes that lend themselves to synthetic biology approaches [for current review see Berla et al. (2013)]. Cyanobacteria have thus become the targets of biofuel production that includes the engineered production of ethanol (Deng and Coleman, 1999), butanol (Lan and Liao, 2011), isoprene (Lindberg et al., 2010), ethylene (Ungerer et al., 2012), sugars (Ducat et al., 2012), and lactate (Angermayr et al., 2012). Besides biofuel production chemical feedstock, high-value compounds, and "nutraceutical" products are envisioned and important progress has been made (Xue et al., 2014).

In parallel with this progress in genetic engineering of desired physiological characteristics into cyanobacteria, systematic modeling approaches for understanding the productivity of genetically modified cyanobacteria have been developed. To gain insight on the physiological characteristics of the strains targeted for engineering studies, researchers have developed large-scale models of metabolic networks based upon annotated gene content that has been deduced from genomic sequences for a number of both heterotrophic and autotrophic bacteria [technical approaches reviewed in Covert et al. (2001), Feist et al. (2009), Steuer et al. (2012)]. Such models can include hundreds and thousands of enzyme-catalyzed reactions based upon the predicted gene content of an organism. The term "metabolic network re-construction" is used to describe the process of developing such models. The successful application of this approach is time-consuming and requires, among other things, careful manual evaluation of algorithmically assigned gene annotations and strategies for "filling in" predicted enzymes when the corresponding genes are missing from

the genomic analysis. However, the rewards for developing a robust metabolic re-construction appear to be large (Knoop et al., 2010, 2013; Nogales et al., 2012). When combined with linear programming methods, the network models can be used for predicting, *in silico*, the metabolic flux patterns under different assumed environmental conditions using linear programing methods termed flux balance analysis (FBA). Of the possible computational approaches, constraint-based FBA has already proven useful and appropriate for the predictive analysis of the production of engineered bioproducts (Nogales et al., 2013). FBA has been used for the theoretical evaluation of photosynthesis in cyanobacteria under different trophic conditions including photoautotrophic, photoheterotrophic, and heterotrophic growth (Knoop et al., 2010, 2013; Nogales et al., 2012). Recently, the theoretical yields of different excreted products such as butanol and sucrose from engineered cyanobacteria have been analyzed and the computational models give predicted values that are close to experimentally observed yields (Nogales et al., 2013). One of the conclusions for that analysis is that autotrophic metabolism in cyanobacteria is relatively inflexible with respect to genetic engineering and that the network properties of autotrophic metabolism place basic limitations on the yields of engineered products.

There are also important experimental applications for reconstructed metabolic networks. This includes metabolic flux analysis (MFA), which uses pulse-labeling of cells with substrate atoms tagged using stable isotopes. This approach has been applied in cyanobacteria to analyze metabolic fluxes under autotrophic conditions (Young et al., 2011). Again, metabolic network models are used, but they are used as input to fit the experimental labeling patterns (Young, 2014). Thus, metabolic network re-construction is of utility from both the experimental and theoretical perspectives and allows insight into the details and global features of microbial growth and metabolism.

COURSE-GRAINED MODELS OF MICROBIAL METABOLISM

Besides the detailed and metabolically realistic FBA models mentioned above, simpler "course-grained" modeling approaches to understanding growth and global features of microbial growth have been developed. Of these, the models of heterotrophic microbial growth (Molenaar et al., 2009; Scott et al., 2010; Scott and Hwa, 2011) have lent themselves to comparison to the corresponding FBA models. In some respects, the simpler alternatives for heterotrophic microbial growth and metabolism have produced explanatory results that were not sufficiently addressed the early FBA models[2]. Molenaar et al. observed, for example, which so-called "overflow metabolism" of microbial growth that was not adequately accounted for in the earlier implementations of FBA (Molenaar et al., 2009). Overflow metabolism is a form of metabolic energy spilling involving the excretion of energy-rich precursors by heterotrophic microbes under certain trophic conditions (discussed below). The course-grained models of Molenaar et al. explicitly take into account the limitation of a finite proteome and frame the problem in terms of a self-replicator cell growth model that optimizes the allocation of finite proteomic resources to maximize growth rates (Molenaar et al., 2009). These models optimize the allocation of cellular protein resources in a small set of general cellular functions such as transport of substrate into the cell, conversion of substrate into metabolic precursors for protein and lipid synthesis, and though overly simplistic, they were able to reach some key conclusions that are consonant with more sophisticated FBA approaches (O'Brien et al., 2013). Both the course grained and FBA modeling are most realistic when maximal growth rates are constrained by "proteomics costs" and the inclusion of this constraint allows for an accounting for certain counter-intuitive physiologic behaviors such as overflow metabolism.

Another course-grained modeling approach, which from the Hwa group, also considers the size of the proteome as a fundamental limiting factor in heterotrophic microbial growth (Scott et al., 2010) (**Figure 2**). These models combine the empirical growth rates with an analysis that derives from the long-standing observation that microbial growth rates are linearly proportional to the cellular content of ribosomes (Schaechter et al., 1958). Using this approach, the authors derived a set of quantitative relationships between growth rate, gene expression, and cellular composition

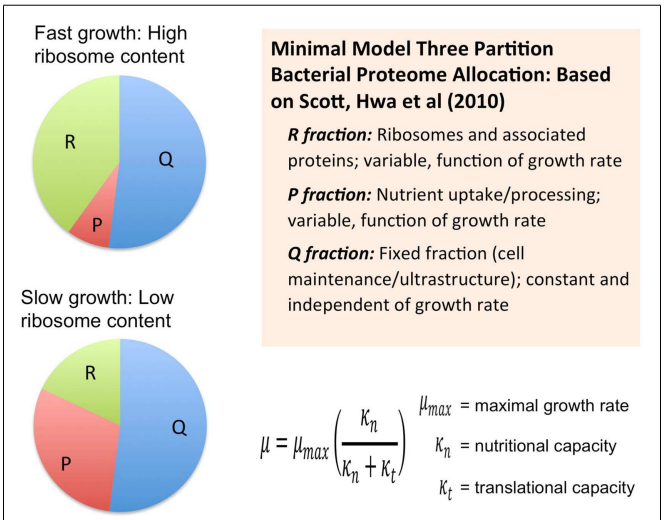

FIGURE 2 | Allocation of the proteome among different sectors as defined by the analysis of growth in the development of a phenomenological theory regarding its control (Scott et al., 2010; Scott and Hwa, 2011). Bacterial proteome consists of a fixed fraction, Q, whose proportion is constant and largely unaffected by the growth rate of the cells and may contain proteins for cell maintenance and ultrastructure. The remainder of the proteome minimally is partitioned into two additional fractions, R and P, which represent ribosome affiliated proteins and nutrient uptake and processing proteins, respectively. The R and the P fractions are observed to reciprocally change their proportions as a function of cell growth rate with the R fraction reaching its largest magnitude under fast growth conditions. This coincides with the observation that ribosomes are more abundant in faster growing cells compared to more slowly growing cells. This same theory was used to derive a Monod-like relationship of observed growth rate in relationship to nutrient availability as reflected by parameter κ_n, along with the translational capacity represented by the parameter κ_t.

[2]However, this situation has dramatically changed with the development of FBA metabolic models that have been extended to include gene expression and proteomic constraints, which correspondingly gives predictions for conditions where overflow metabolism occurs (O'Brien et al., 2013).

in terms of allocation of proteomic resources toward different functional classes of protein (Scott et al., 2010; Scott and Hwa, 2011). The minimal model for heterotrophic bacteria is comprised of a finite proteome consisting of three sectors: a fixed core sector designated "Q" that, for a given species is invariant in proportion of the total proteome, a ribosome sector, "R" of variable size consisting of all ribosomal proteins and their affiliates, and a "P" sector, also variable in size, associated with nutrient uptake. The fixed Q sector might consist of proteins involved and cell maintenance metabolism and the biogenesis of ultrastructure. The sizes of the different sectors were determined experimentally, for example, by manipulation of the growth rate under different nutritional regimes or with different inhibitors. The Q sector was experimentally found to be ~55% of the total proteome for *E. coli* and was not found to change its size across a variety of trophic conditions suggesting that it is, indeed, fixed in proportion of the total proteome. The size of the R sector is variable and directly proportional to the growth rate in accord with the linear relationship between ribosome content and growth rate. Interestingly, the course-grained replicator models of Molenaar mentioned earlier have the linear ribosome-growth rate relation built into them. The third sector, "P" is also variable in size and expands at the expense of the R sector. The P sector exerts a positive effect on growth by providing nutrients that ultimately feed precursors into the R sector. In effect, the P sector "feeds" the R sector with precursors for protein synthesis. However, optimal growth requires a balanced proteomic allocation of the P and R sectors. Increases in the P sector, due, for example, to the necessity of increased nutrient uptake capacity by synthesizing more transporters, occurs at the expense of the R sector and correspondingly results in a lower rate of growth than if less of the proteome were invested in the P sector and more in the R sector. Additional sectors can be elaborated by subdivision of the three sectors of the minimal model and it was shown how the expression of unnecessary protein tends to reduce growth rates due to the reduction in the sizes of the R and P sectors. Extension of this approach has provided new explanatory insight into the role of cyclic AMP in coordinating catabolic and anabolic responses to shifting nutrient regimes (You et al., 2013). In each case, the models necessarily constrain the size or cost of the proteome in order to obtain the far reaching conclusions regarding the way proteomics resource allocation govern maximal growth rates in microbes and how the different functional modules are regulated to achieve this. It is also important to note that each of these cases involves an experimental approach to provide estimates of the sizes of the sectors.

While these approaches have not been applied to cyanobacteria, there is already a rich and advanced set of modeling efforts for algae and cyanobacteria that are oriented toward autotrophic productivity in natural marine and aquatic environments [c.f. Ross and Geider (2009)]. Although, there is likely a huge opportunity to find the parallels between these successful modeling approaches for autotrophs and the FBA and course-grained models discussed above, the main objective of this communication is to discuss the hypothesis that the proteomic constraints that have proven necessary for successful modeling of heterotrophic microbial growth are also of fundamental significance in explaining cyanobacterial growth and productivity.

OVERFLOW METABOLISM IS A DISSIPATIVE PROCESS THAT REFLECTS THE PROTEOMIC CONSTRAINTS OF MICROBIAL GROWTH

It is useful to consider one of the puzzles of microbial physiology: the seeming wastefulness in the phenomenon of metabolic spilling. In heterotrophic microbes, metabolic spilling involves the release of incompletely catabolized energy-rich metabolites and has physiological roles that extend beyond biochemical considerations such as redox balancing. Instead, it appears necessary for maximal growth under certain trophic conditions despite the apparent wastefulness of the process. When heterotrophic cells have an excess of a carbon/energy source they may metabolize this compound using less efficient pathways (e.g., lower ATP yield per substrate) with the consequent wasteful release of energy-rich compounds such as ethanol, acetate, and lactate despite the fact that environmental and physiological conditions would allow for more energy-efficient utilization of the carbon source. For example, the well-known Pasteur effect, which involves metabolic switching between efficient and less efficient pathways. Here, yeast cells are observed to switch between less energy-efficient fermentation and more efficient aerobic metabolism by depending upon the availability of oxygen. However, what is less discussed are the circumstances where the Pasteur mechanism is over-ridden and fermentative metabolism occurs even under aerobic conditions – i.e., overflow metabolism occurs. This situation, known as the Crabtree effect in yeasts (van Dijken et al., 1993), occurs under conditions of excess substrate carbon and is characterized by the operation of fermentation pathways even when oxygen as a terminal electron acceptor is present, potentially allowing the more efficient utilization of substrate. This phenomenon also occurs in bacteria and tumor cells under conditions of heterotrophic substrate excess and, interestingly, under conditions of nitrogen limitation. Although metabolically wasteful, utilization of low efficiency enzymes appears to allow for faster growth under these regimes. Apparently, the wasteful metabolism reflects a trade-off that also rewards the minimization of costs for the catabolic enzymes, with the cost of the enzymes being either the relative costs of synthesis of the enzymes or the relative costs of cytoplasmic space occupancy by the enzymes of limited cytosolic space (see next section). These costs are summarized by the phrase "proteomic limitation" as modeled in advanced FBA models that take account macromolecular expression (O'Brien et al., 2013) or in course-grained models that explicitly account for the allocation of proteomic resources (Molenaar et al., 2009; Scott et al., 2010; Scott and Hwa, 2011).

Overflow metabolism in autotrophs is less studied than in heterotrophs, but may also play important physiological roles. Though not metabolic energy spilling *per se*, all organisms capable of oxygenic photosynthesis, including cyanobacteria, have the ability to dissipate excess light energy in the form of non-photochemical quenching (NPQ) (Niyogi and Truong, 2013). This involves the dissipation of excitation energy captured by the light-harvesting antennae by regulated mechanisms that convert the excitation energy into heat under conditions where the energy cannot be productively utilized by the photosynthetic reaction centers. Thus, NPQ is a form of energy spilling. Although excitation energy spilling is widely discussed in the area of photosynthesis,

the concept of metabolic energy spilling in photosynthetic organisms is less thoroughly considered. In principal, metabolic energy spilling could occur as the release of compounds from the cell or the action of futile cycles that dissipate, for example, excess reductant produced by the light reactions. A physiologically significant example is a futile cycle associated with the CO_2-concentration mechanism (CCM) of cyanobacteria. The CCM is a metabolic process that not only satisfies the requirement of the inorganic carbon supply by energetically acquiring bicarbonate, but it also dissipates excess energy under high light conditions by spilling of large amounts of acquired bicarbonate by release into the environment (Tchernov et al., 2003). Therefore, in aquatic systems, light may produce a net increase in the amount of bicarbonate dissolved in the media rather than the expected decrease due to uptake by the carbon fixation activity by the Calvin–Basham–Benson (CBB) cycle. Because the CCM is powered by the products of the photosynthetic electron transport (PSET) chain, ATP and NADPH, the excretion of bicarbonate provides an escape valve when the electron transport chain is more active than CO_2-fixation by the CBB cycle, as under conditions of excessive light. Metabolic spilling in cyanobacteria is also observed in mutants having defects in their ability to synthesize glycogen are observed to excrete pyruvate and α-ketoglutarate into the growth medium, apparently to dissipate excess carbon fixed via the CBB cycle (Carrieri et al., 2012; Gründel et al., 2012). Presumably, this occurs because the assimilatory flux capacity of anabolic metabolism is exceeded by the production of these intermediates and the glycogen synthesis pathway normally absorbs this excess flux of reduced carbon, but in its absence, excretion of pyruvate and α-ketoglutarate occurs.

"PROTEOMIC LIMITATION" OF GROWTH RATES IS DUE TO CROWDING LIMITS WITHIN THE CELL

A common thread that emerges in the different modeling approaches discussed above is the necessity to include a constraint on the size or expression cost of the cellular proteome in order to derive critical features of microbial growth. Only when protein costs are explicitly included in the optimization models do we find that comprehensive predictions of metabolism possible. The case in point is the prediction of overflow metabolism in heterotrophs. Expression of metabolically inefficient, but less costly (protein expression cost) pathways leads to maximal growth rates under conditions of nutrient excess and accounts for the efficacy of overflow metabolism in maximizing cell growth rates. On the other hand, expression of metabolically more efficient (non-overflow) pathways catalyzed by enzymes with a higher proteomic cost, lead to maximal growth rates under conditions of nutrient limitation (Molenaar et al., 2009). However, the expression of more efficient, but more costly enzymes also comes at the expense of dedicating less proteomic resources toward the machinery of protein synthesis (ribosomes and affiliated proteins) so the efficiency of utilizing the limiting nutrient is maximized but only at the simultaneous cost of allocating less of the proteome to ribosomes and cell duplication, which accounts for the lowered rate of growth. This is consistent with the independent line of reasoning and experiment that led to the "bacterial growth laws" showing the allocation of proteomic resources enabled maximal bacterial growth and accounts for diverse regulatory features controlling bacterial metabolism and gene expression (Scott et al., 2010; Scott and Hwa, 2011; You et al., 2013). Similarly, when metabolism and gene expression are simultaneously integrated into the FBA model for *E. coli* growth, overflow metabolism is explained in terms of differing proteomic costs. Furthermore, growth regimes that are distinguished by nutrient limitation at lower growth rates and "proteome-limited" at maximal growth rates, fall out of the analysis (O'Brien et al., 2013). As discussed throughout, this apparent "proteome-limitation" and the associated "proteomic costs," appear to be a consequence of the physical constraints of intracellular crowding in bacteria. The underlying, yet still unproven assumption, is that expression of metabolically efficient pathways has a higher proteomic cost. Maximal rates of growth occur where all nutrients are in surplus and, under these surplus conditions, intracellular factors place an upper bound on the fastest achievable growth rates. At lower nutrient levels, growth rates are constrained by the limiting nutrient(s) and growth rate follows a dependence on nutrient concentration according to the quasi-Michaelis–Menten formulation of microbial growth (Monod, 1949). Thus, at saturating levels of nutrient (and for autotrophs and saturating light intensities), maximal growth rates are limited by intrinsic factors within the cell, whereas nutrient and light availability limit growth rates below this threshold.

What sets the upper bound on the maximal growth rate rates under saturating nutrient and light conditions for cyanobacteria? There is compelling evidence that the packing density of molecules and the solvent capacity of the cytoplasm along with the packing density of membrane complexes in microbial cell membranes all place severe limits on the size of the proteome in a microbial cell (Beg et al., 2007; Tadmor and Tlusty, 2008; Vazquez et al., 2008; Klumpp et al., 2013). The packing of photosynthetic membranes is very dense in chloroplast (Kirchhoff et al., 2011) and in cyanobacteria (Folea et al., 2008). In other words, the cost of a protein can be evaluated in terms of the space it occupies because expression of that protein comes at the expense of limiting the available cytoplasmic or membrane space allocated for the expression of other proteins. Atkinson argued that one of the fundamental selective constraints in the evolution of cells was the conservation of "solvent capacity" with the view that enzymes are optimized to be efficient, in part, to avoid "wasting solvent capacity" that would otherwise occur by the accumulation of high concentrations of metabolic intermediates (Atkinson, 1977). He argued that natural selection has favored enzymes in a pathway should have lower K_m values to have the fast substrate-product conversion fluxes even when metabolite concentrations are low. With such efficient enzymes, high flux rates through metabolic pathways can be maintained even at low metabolite concentrations. Of course this also assumes that the utilization of products of those pathways is equally efficient to avoid product accumulation. But this gets back to the larger point of proteome allocation: if the product of a biosynthetic pathways composed of efficient enzymes is an amino acid, then it would important that the expression of protein synthetic machinery is maintained at a high enough level (fraction of the proteome) to utilize the amino acids being produced. Conversely, if there is not a large enough allocation of amino acid biosynthetic enzymes (or the enzymes feeding nutrient precursors to those biosynthetic enzymes), then the proteomic allocation to

protein synthetic machinery would be excessive, and in a cellular environment where to total proteome is finite, the allocation would be suboptimal. The concentration of soluble protein in the cyanobacterial cytoplasm is estimated to be ~500 mg/mL (Moal and Lagoutte, 2012), similar to the high concentrations estimated for other bacteria (Zimmerman and Trach, 1991; Cayley et al., 2000; Ellis, 2001; Cayley and Record, 2003), although accurate estimates are technically challenging. The dense crowding of the bacterial cytoplasm is mainly due the volume occupied by protein and RNA, with the latter mostly associated with ribosomes (Neidhardt et al., 1990). The proportions of protein, RNA and metabolites represent about 55, 20, and 3% of the dry mass of the cell (Neidhardt et al., 1990). Nevertheless, the impact of macromolecular crowding on the metabolome may be critical because of the limitation it imposes upon the availability of water for solvation of metabolites (Cayley et al., 2000; Cayley and Record, 2003). That said, macromolecular diffusion rates are disproportionately decreased by crowding relative to small smaller solutes (Mika et al., 2010).

Macromolecular processes may be rate-determined by crowding limitations and this may affect the rate that the components of the cell can be duplicated. These limitations can be expressed in terms of characteristic transit times for the occurrence of productive collisions of metabolic and macromolecular reactants, which is controlled by the intercellular diffusional parameters involved. Theoretical analysis using this approach and using the simplifying assumption of spherical cell shapes, calculates the optimal size for a generic bacterial cell to be slightly larger than one micron, close to actual sizes in nature (Soh et al., 2013). For example, the highly crowded nature of the bacterial cytoplasm requires the expression of high concentrations of translation factors such as EF-Tu (Klumpp et al., 2013). This is because molecular crowding restricts the macromolecular diffusion of translational complexes that cooperate with the ribosome in protein synthesis and thus high concentrations are required to overcome this diffusional restriction and to achieve maximal cell growth rates (Klumpp et al., 2013). The attachment and localization of the enzyme ferredoxin NADP reductase (FNR) to the phycobilisome rods (Schluchter and Bryant, 1992) places it in proximity to PSI, which is calculated to be critical for the fast reduction of $NADP^+$ by linear electron transport (Moal and Lagoutte, 2012). Again, a mechanistic strategy has evolved to overcome diffusional limitations, this time realized by localizing the reactants in close proximity to one another. Recently, chemical analysis of the plastoquinone pool in cyanobacteria has indicated that the overall balance of the reduced and oxidized forms of plastoquinone are remarkably stable, which is in stark contrast to the dynamic changes evident in the fluorescence transients attributed to the redox state of the plastoquinone pool's ability to re-oxidize the electron acceptor side of photosystem II following its reduction by photochemical charge separation (Schuurmans et al., 2014). Conceivably, this disparity may reflect the spatial inhomogeneity in the redox state of the plastoquinone population with the reduced form accumulating near photosystem II at a rate faster than the rate that it can diffuse away to the cytochrome b_6f complex for oxidation. Overall, crowding of the cytoplasm and membrane systems with macromolecules suggests that the necessary modeling constraint of "proteome-limitation" discussed above may have a physical basis in terms of available space in the cytoplasm and membrane. This space limitation hypothesis is in contrast to the other alternative constraint hypothesis expressed in terms of the costs of ATP expenditure required for protein synthesis or limitations in growth due to the energetic costs for macromolecular precursor synthesis (Akashi and Gojobori, 2002). While metabolic efficiency may be at a premium under certain circumstances such as low nutrient environments (Molenaar et al., 2009; O'Brien et al., 2013), it is likely that the highly crowded cellular milieu places a premium on the space and solvent capacity occupied by the myriad molecular constituents of the cell.

While there is a good argument that proteomics costs in the form of limited space constrain maximal growth rates, one can ask whether the same constraints apply to growth in the nutrient-limited region of the growth versus nutrient availability curve? It is possible that under certain conditions, the expression of additional nutrient uptake and assimilation proteins could partially or entirely alleviate the nutrient limitation, but this up-regulated expression may begin to compete for cellular occupancy space with other important functional classes of protein. Nevertheless, there are also conditions where no amount of increased expression of uptake proteins can alleviate a deficiency of an essential nutrient if the nutrient is present only in vanishingly small amounts. Thus, it is conceptually useful to consider the two domains of growth limitation as metabolically limited and proteomically limited (**Figure 1**), referring to the nutrient-limited submaximal growth rate and the nutrient saturated maximal growth rate, respectively (O'Brien et al., 2013).

HYPOTHESIS: PROTEOMIC LIMITATIONS WITHIN THE CYANOBACTERIAL CELL CONSTRAIN MAXIMAL GROWTH RATES AND PHOTOSYNTHETIC ADAPTATION

The overall hypothesis is that growth rates are constrained by the limits on the total amount of proteins and other macromolecules that can be fit inside a cyanobacterial cell. Space and crowding constraints combined with the restrictions on surface-to-volume ratios are hypothesized to be the fundamental physical restrictions on the composition and function of the microbial cell, including cyanobacteria. As suggested below, this results in a novel and testable explanation for reductions in growth under conditions of osmotic stress. Surface-to-volume ratios, combined with cell shape, determine the capacity for nutrient and waste exchange across the cell boundaries and give an upper limit on the size of microbial cells (Soh et al., 2013). Packing, solubility, and solute concentrations limit how much material can be confined within the cell volume and membrane domains, which thereby limits the sum total of expressed proteomic resources, as discussed above. In effect, optimizing the allocation of protein resources for maximal cell growth rate is a zero sum game in the sense that production of more proteins in one functional class occurs at the expense of the ability to produce proteins in another functional class. This restriction is explicitly accounted for in the phenomenological models that minimally partition the proteome into three different sectors, the Q, P, and R sectors with the relative proportions determined by the growth rate (Scott et al., 2010; Scott and Hwa, 2011; You et al., 2013). This proteomic restriction is also explicitly incorporated into the course-grained models of heterotrophic microbial growth

(Molenaar et al., 2009) and the extended detailed FBA models of *E. coli* growth (O'Brien et al., 2013). Moreover, this leads to a prediction regarding the effects of osmotic stress on growth rates in cyanobacteria and other microbes as suggested recently (Klumpp et al., 2013).

HYPOTHESIS 1

Lowered rates of cell growth in salt-adapted cultures are due to global reductions in the concentrations of macromolecules and metabolites in the cytoplasm. There is strong evidence that growth rates in *E. coli* are inversely proportional to osmotic stress and that this is due to molecular crowding that is exacerbated by the necessity for the accumulation of compatible osmolytes in the cytoplasm (Cayley et al., 2000; Cayley and Record, 2003). During osmotic adaptation, *E. coli* cells enlarge in volume, keeping the protein content per cell relatively constant. The cells increase potassium and osmolyte and also increase the total amount of water per cell (Zimmerman and Trach, 1991). Combined with the proposal that diffusion of critical macromolecules (e.g., EF-Tu) limits growth rates (Cayley et al., 2000; Cayley and Record, 2003; Klumpp et al., 2013), the dilution of these critical molecules due to greater cell volume may account for the lower growth rates. There is a wide diversity of osmotic and salt tolerance characteristics and adaptive mechanisms among different cyanobacteria (Hagemann, 2011). Although information on changes in cyanobacterial cell size following adaptation to osmotic stress seems to be scant, at least one instance of larger cell size has been reported (Erdmann et al., 1992). Salt-tolerant and halophilic cyanobacteria generally synthesize compatible osmolytes (e.g., glucosylglycerol in *Synechocystis* sp. PCC6803) and accumulate potassium to maintain osmotic balance when challenged by high salt conditions. Immediately following an upshift in environmental salt concentrations, there is a decline in metabolic activities, such as in PSET, and there is a mobilization of many stress response genes. After this initial period, compatible osmolytes accumulate, damage is repaired and cyanobacterial cells regain nearly normal levels of metabolic activities and resume growth, albeit at a lower rate (Hagemann, 2011). The crowding of the cytoplasmic volume due to the accumulations of high concentrations of potassium and compatible osmolyte, while essential for osmotic adaptation, is hypothesized to reduce the growth rate of the cells due to occupancy of solvent space. Analysis of molecular crowding with respect to the concentration of translation factors necessary to sustain high growth rates in bacteria led to a similar hypothesis regarding the effect of increasing external osmotic stress (Klumpp et al., 2013). One of several predictions is that osmotic-stress adapted cyanobacterial cells ought to have either lower amounts of protein per cell, or similar amounts of protein per cell, but cells with larger volumes, as with *E. coli* (Zimmerman and Trach, 1991) and the resultant dilution of cellular protein should correlate with reductions in growth rate.

HYPOTHETICAL MODELING OF THE CONSEQUENCES OF PROTEOMIC LIMITATIONS IN THE GROWTH AND ALLOCATION OF PROTEOMIC RESOURCES IN CYANOBACTERIA

To evaluate the imposition of the constraint of a finite proteome in relation to photosynthetic growth of a microbe, an optimization model was constructed based upon the "autocatalytic replicator" models of Molenaar et al. (2009). The original publication provided the source code of the models and this code was restructured to emulate, in a highly simplified manner, autotrophic metabolism. In contrast to the highly detailed models used in FBA, these models are "course-grained" models. The models are implemented in the GAMS language (Andrei, 2013) and were set up to compute the optimal allocations of proteins necessary to achieve maximal growth rates (Molenaar et al., 2009). The original heterotrophic microbial growth model consists of a simple set of relations between enzymes and metabolites and is arranged in a way that satisfies basic assumptions of balanced cell growth, cell volume, and composition, and are defined by a set of equations and corresponding stoichiometry matrix (Molenaar et al., 2009). The present model uses the same strategy, but involves a rudimentary model for autotrophic metabolism (**Figure 3**). In essence, the previous heterotrophic model and present autotrophic model each represent a system of enzymes and transporters that feed precursors to ribosomes and membrane lipids. The GAMS language allows definition of mathematical sets and subsets, such as proteins and enzymes, allowing for versatile configurations and operations in the model, which in this case involved optimization

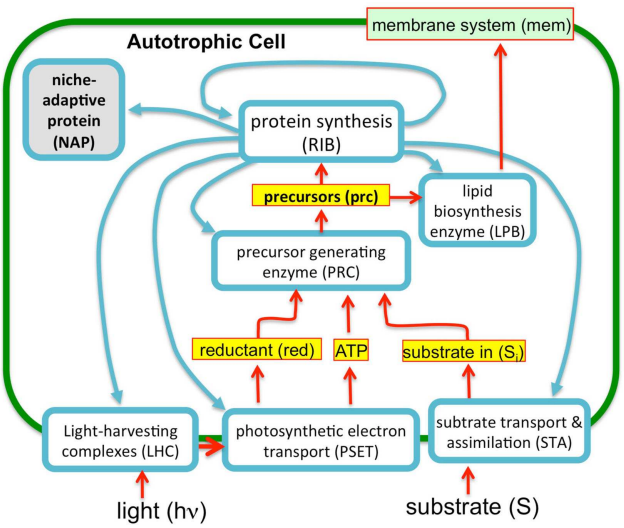

FIGURE 3 | A simplified autocatalytic replicator model of cyanobacterial growth. The model consists of a simple set of enzymes (blue outlined boxes), metabolites (yellow boxes), and membrane structural components (green objects) representing functional classes of molecules (e.g., enzyme "ribosome" represents are ribosomal proteins and those affiliated with protein synthesis). The proteins interrelated by a stoichiometric matrix and kinetic equations as described in the text. The model optimizes the allocation of finite proteomic resources among the different proteins (as protein synthesis, blue arrows) to achieve maximal growth rates. Consistent with the observation that microbial growth rates scale in direct proportion to the number of ribosomes, this "ribosome centric" model defines a set of "enzymes" (light blue boxes) that feed precursors to ribosomes and are, in turn, subject to synthesis by the ribosomes. Growth rates correspondingly correlate with flux rates for the generation of precursors for protein synthesis. The model was implemented in GAMS software environment (Andrei, 2013) using a previous model of heterotrophic growth (Molenaar et al., 2009).

of the size of the subsets. A ribosome subset of the proteins generates all proteins and the system is autocatalytic in the sense that ribosomes beget more ribosomes. This is possible due to the cooperation of ribosomes with the other proteins that contribute to the generation of precursor (e.g., amino acids) feeding the ribosomes and membrane structures. In these highly simplified models, the "ribosome enzyme" actually represents all proteins involved in protein synthesis, or in the terminology of the Scott models, the R sector (**Figure 2**), which is comprised of all "ribosome affiliated" proteins. The system of equations includes expressions that describe the different allocations of active ribosomes toward different sets of enzymes (substrate assimilation, activating enzymes, and the ribosomes themselves). In other words, the total pool of ribosomes sums to one with different fractions of the total ribosome pool working on different subsets of protein. The fractional distribution of ribosomes working on the synthesis of different subsets of proteins is optimized in these models with the objective function being the maximization of growth rate, μ. The actual proportions of the enzymes produced during the optimization of the model are not likely quantitatively accurate since a fitting of real kinetic parameters was not attempted either in the original or present models. However, in the case of the original heterotrophic models, the overall trends were informative since, for example, the switch between non-overflow and overflow metabolism could be emulated and the notion of a proteomic constraint was crucial, as discussed above. In the current form, substrate uptake involves the aggregate of all potentially limiting inorganic substrate transport and assimilation proteins (STA) and a PSET chain that generates ATP and reductant. The goal of the model is, essentially, to feed precursors to the ribosomes. The products of the STA and PSET proteins are combined by the action of precursor biosynthesis enzymes (PRB). The main difference with the original models of Molenaar et al. is the mechanism to generate the pool of precursors (prc) necessary for the synthesis of protein and lipid, which is now satisfied by the parallel action of the PSET and STA proteins. Collectively, these constitute the "core" proteins of the autotrophic replicator. In addition to these core proteins, a subset of proteins called "niche-adaptive proteins (NAP)" were modeled here. These are supposed to represent species-specific proteins that enable the organism to thrive in a particular niche and may not be present in other species. An additional difference with the heterotrophic models is the assumption that, apart from light, the external substrate is not a source of energy. As with the original model, there are constraints in the model to provide limits on the size of the cell, the fraction of proteins occupying the lipid membrane and cytoplasm. The model is schematically shown in **Figure 3**.

EFFECTS OF LIGHT INTENSITY ON GROWTH RATES AT DIFFERENT CONCENTRATIONS OF AVAILABLE INORGANIC SUBSTRATE

The behavior of the model under different light and substrate conditions was explored by iteratively varying these two environmental conditions. Each iteration computed the optimal distribution of proteomic resources among each of the different enzyme groups with the overall mathematical objective of maximizing the growth rate. **Figure 4** shows the increase in growth rate as a function light intensity at three different substrate levels. The simulated growth rate as a function of light intensity was observed, as expected, to exhibit a saturation behavior that is modulated by substrate availability. Although the model is too simple to specify it, the substrate could represent inorganic carbon and each of the three curves would represent the light saturation behavior of autotrophic cell as a function of inorganic carbon availability. Besides this lack of specificity, the present model has the additional limitation of not considering important physiological characteristics, such as the photosystem II to photosystem I ratio, which are known to be regulated as a function of irradiance and inorganic nutrient availability. Nevertheless, the current model does provide a first approximation of predicted optimal physiological responses to alterations in environmental conditions. When the allocation of the proteome is examined for one of the curves (high

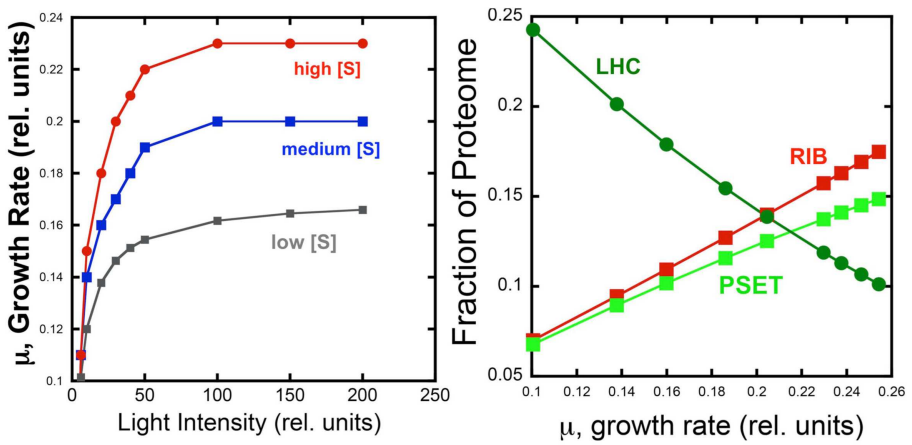

FIGURE 4 | Simulated effect of light intensity and inorganic substrate concentration on photoautotrophic growth rate and proteome allocation. Growth rates increase with light intensity, but rates saturate at different levels set by different levels of substrate concentration (Left). Allocation of the proteome to ribosomes (RIB) and photosynthetic electron transport (PSET) and light-harvesting complexes (LHC) as a function of growth rate (Right Panel) using data from the high light simulation shown in the left panel.

light) in this computational experiment, it is found that the fraction of light-harvesting complexes (LHC) decreases when plotted as a function of growth rate. This is expected since light is limiting growth early in the light saturation curve and at low growth rates with these conditions is reflected by the near linear decrease in the fraction of the proteome composed of LHC due to the fact that a smaller antenna provides the sufficient excitation of the PSET. It is worth noting that PSET is also predicted to be changing in relative abundance as a function of growth rate during light-limited growth, so that the amount of LHC providing excitation energy is adjusted to a "moving target" of different PSET levels. The model predicts that PSET increases with increasing growth rates, which fits with the expectation that it should parallel the rate of carbon fixation and thus biomass accumulation *in vivo*. A similar predicted increase in the allocation of proteome toward PSET is also observed for substrate limitation simulation (not shown). Since most chlorophyll in cyanobacteria is associated with reaction centers, the prediction of higher PSET with increasing growth rates leads to the prediction that faster growing cyanobacteria should have higher chlorophyll contents on a per cell basis. Evidence for higher levels of chlorophyll per cell in cyanobacteria have indeed been observed for light-limited (Deblois et al., 2013) and nitrogen-limited growth of marine cyanobacteria, at least in the range of moderate to high growth rates (Dittrich, 2013). It is also worth noting that while the predicted proteomic allocations of model may eventually be more directly tested experimentally, the model is mute on the regulatory features that lead to optimal distribution of the proteome amongst the functional sectors. Consistent with the growth hypothesis regarding ribosome content in direct proportion to μ (Schaechter et al., 1958; Worden and Binder, 2003), the allocation toward the RIB fraction of the cell increases linearly with growth rate.

HYPOTHESIS 2

Species-specific differences in the maximal growth rates of cyanobacteria are due to different proteomic allocations into niche-specific proteins.

The reported doubling times of cyanobacteria ranges from ~3 h to one or more days. We can use the above considerations to formulate the following hypothesis for why different species of cyanobacteria have different maximal growth rates: depending upon the niche they are evolutionarily adapted to, different species may have more or less total allocation of protein resources to NAPs. Hypothetically, cyanobacteria that are adapted to complex environments will need to express additional proteins beyond the core set of proteins needed for autotrophy. Cyanobacteria with smaller "fixed" fractions of NAPs will have the capacity for faster growth because they will be able to devote a greater fraction of the proteome to this core set of proteins, although they would correspondingly have less capacity to adapt to non-ideal environments. The fastest growing cyanobacteria would thus have the most proteomic resources devoted to core functions of autotrophic metabolism and minimal allocation of proteomic resources to specialized nutrient uptake and assimilation, defense mechanisms, and other NAPs. To simulate this, the model (**Figure 3**) includes a fraction of the proteome that is fixed and representing the NAP. To explore this idea, the simulation was performed at two different levels of NAP with the outcome showing that a large investment in NAP indeed predicts a large decrease in growth rate as might be expected (**Figure 5**, top panels). This observation is formally identical to the production of heterologous protein in the original heterotrophic models (Molenaar et al., 2009). A further observation is that the relative proportions of the other proteomic sectors is fairly similar under in the high NAP and low NAP cell types if the allocation to NAP protein is excluded (i.e., if the RIB, LHC, PSET, STA, PRB, and LPB are summed to 100%) (**Figure 5**, bottom panels). This suggests that the metabolic balance between these functional sectors is balanced in each cell type and that all sectors "expand" when the NAP proteins are removed from the optimization. Although not modeled, deviations from this trend would be anticipated to the extent that the NAP proteins utilize additional cellular resources (e.g., reductant) beyond the synthesis of the precursors that go into forming the NAP class of proteins.

The last set of simulations that were performed to investigate the consequence of engineering cells to divert metabolic precursors toward an excreted "energy" product (**Figure 6**). This scenario might apply to cells that are engineered for biofuel production, for example. The simulation is highly simplified in the sense that it only considers the diversion of ATP and reductant toward a hypothetical excreted product and ignores the more realistic inclusion of diversion of a fraction of the material substrates (S) toward this end. A more realistic model will result in proteome allocations that depend upon the chemical characteristics of the excreted product, most notably, the C/H ratio of its chemical formula. Nevertheless, the findings are interesting and show that under these simplified circumstances, the model predicts a resultant re-distribution of the proteome in accord with what is observed in experiment. It has been shown that the cyanobacteria engineered to excrete sugar have adjusted their metabolism to have increased total photosynthetic capacity presumable to compensate for the genetically imposed drain on their metabolism (Ducat et al., 2012).

CONCLUSION

The statistician, George Box stated that "essentially, all models are wrong, but some are useful" (Box and Draper, 1987). The autotrophic replicator model (ARM) is probably best considered a preliminary construction should be useful for bringing current ideas on heterotrophic microbial growth to the topic of autotrophic growth. It should also be useful since testable hypotheses can be derived from the model and packing constraint assumption presumed by the model. The present model was developed on the basis of previous models where it was concluded that inclusion of "macromolecular costs" is critical for accurate representation of optimal cellular metabolism and this is evident when trying to account for heterotrophic overflow metabolism (Molenaar et al., 2009). This constraint was also included in the ARM. It was also concluded that the best hypothesis for macromolecular costs was probably not the energy costs, but rather packing constraints limiting the size of the proteome. Accordingly, allocation of proteomic resources is essentially a zero sum game with the consequence that increased investment in proteins for niche adaptation, for example, result in slower growth rates due to correspondingly smaller investments

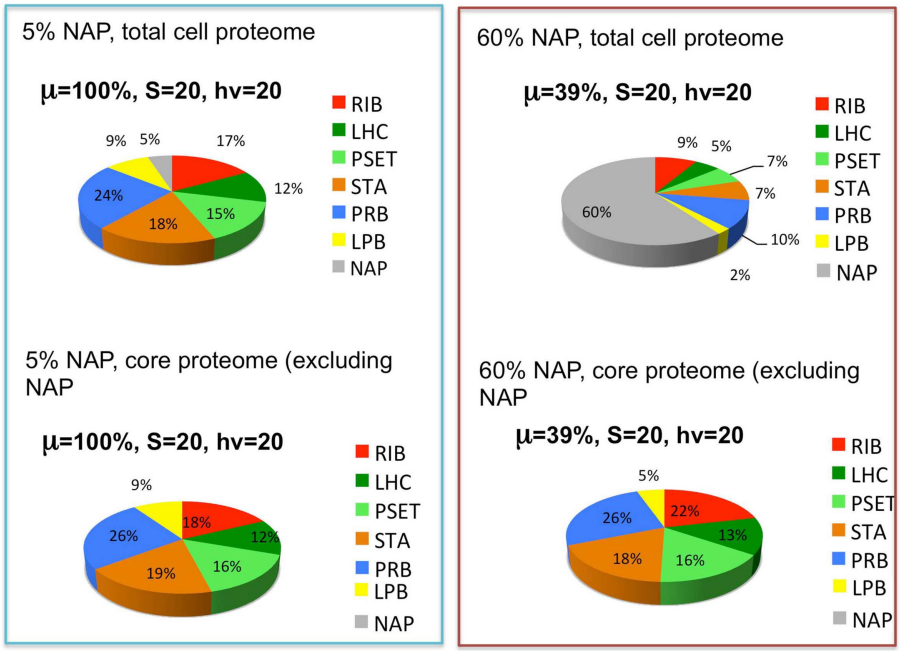

FIGURE 5 | The effects of the expression of non-core, "niche-adaptive protein (NAP)" on growth and expression of core autotrophic functions. Upper graphs depict the simulated distribution of the proteome holding the NAPs at 5% (upper left) or 60% (upper right) of the total proteome and the corresponding growth rates (μ) at saturating levels of light (hν) and substrate (S). Lower graphs show that the relative proportions of the other sectors are similar despite the reduction of their net amount due to displacement by the NAPs. Sectors correspond to functional protein groups: inorganic substrate transport and assimilation proteins (STA), photosynthetic electron transport chain (PSET) that generates ATP and reductant, precursor biosynthesis enzymes (PRB). The main difference with the original models of Molenaar et al. (2009) is the energy source (light) and mechanism to generate the pool of precursors (prc) necessary for the synthesis of protein and lipid, which is now satisfied by the parallel action of the PSET and STA proteins. Collectively, these constitute the "core" proteins of the autotrophic replicator (see **Figure 3**).

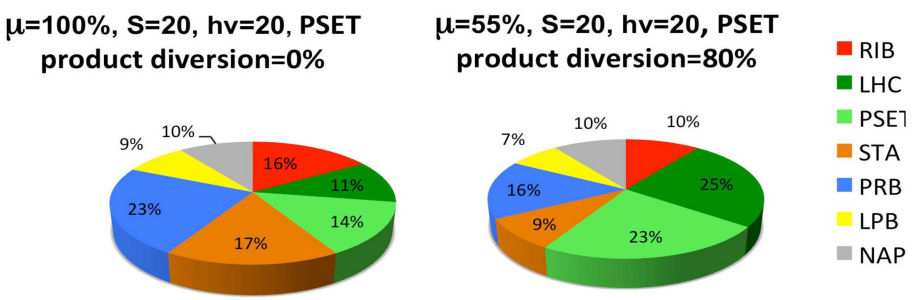

FIGURE 6 | Simulated diversion of energy precursors to excreted products alters the allocation of the proteome. Product diversion is defined as the excretion of 80% of the energy precursor for engineered product synthesis. Only ATP and reductant are considered in this highly simplified model, whereas a more realistic model will depend upon the chemical characteristics of the excreted product, most notably, the C/H ratio of its chemical formula. See **Figure 5** and text for definitions of the functional protein groups of the model.

in proteomic resources dedicated to cell duplication (e.g., ribosomes and energy generation). Proteomic limitation of maximal growth hypothesis lends itself to experimental tests including growth slowdowns due to loss of solvent space during osmotic stress as surmised, but not tested earlier (Klumpp et al., 2013). Such thinking also provides a heuristic explanation to better explain why some strains of cyanobacteria and algae exhibit much faster maximal growth rates than others. The autotrophic lifestyle requires an enormous investment in the synthesis of photosynthetic membranes and the enzymes of inorganic carbon uptake and carbon fixation. The ARM formulated is useful for glimpsing of trends in adaptation and engineering in cyanobacteria that can later be more accurately obtained from more sophisticated FBA models or from refinement of the current models to more accurately represent the kinetic and stoichiometric relationships anticipated in a real cyanobacterial cell. Besides the growth

hypothesis regarding the linear relationship between ribosome number and growth rate, additional testable predictions can be made including the abundance of photosynthetic machinery as a function of growth rate, species type, and environmental condition. Improvements would also include modeling different ATP and NADPH demands under different conditions and the corresponding ability to adjust the (photosystem I/photosystem II) ratios. It would also need to include alternative electron transport (e.g., cyclic electron flow) and specific nutrient uptake mechanisms such as the CO_2 concentrating mechanism. Such considerations are already made in FBA approaches (Knoop et al., 2010; Nogales et al., 2012), which would then need to be extended to include macromolecular expression (FBA-ME) (O'Brien et al., 2013). Furthermore, improvements can be made by trying to utilize the rich modeling literature regarding cyanobacterial and algal growth that have been effectively applied especially for understanding growth dynamics in marine environments (Ross and Geider, 2009).

Optimal growth ultimately requires that allocation of the proteins constituting different functional modules of the proteome result in a set of flux balances between the production and utilization of cellular metabolites. It has been argued here that the main reason for protein allocation being a zero sum game is that molecular crowding places a ceiling on the amount of protein in a bacterial cell. In the ARM, the amount and activity of the PSET proteins is assumed to be sufficient to supply ATP and reductant at rates that match the demand of enzymes involved in the generation precursors (PRC) for macromolecular synthesis. Natural selection has tuned the regulation to ensure optimal protein levels to achieve this balance. As noted, the ARM does not specify mechanisms, but it seems likely that metabolic intermediates that accumulate or are depleted under conditions of imbalance are good candidates to serve as allosteric modulators of gene expression. This type of regulation is observed, as one example, for control of the inorganic carbon uptake mechanism proteins (Nishimura et al., 2008; Daley et al., 2012). It is also almost certain that other selective forces modify expression levels of proteins to not only satisfy flux balance, but also achieve system robustness. For example, the PSET appears to be expressed at levels that exceed the amounts needed to supply its main products, ATP and NADPH, to the CBB cycle based upon measurement of photochemical quenching capacity at steady state ambient growth light conditions. One possibility is that expression levels of the PSET proteins have evolved to cope with fluctuations in light intensity and have resulted higher expression levels to be able to the PSET to absorb peaks in energy input without the generation of damaging reactive intermediates.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fbioe.2015.00001/abstract

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Cyanobacteria as cell factories to produce plant secondary metabolites

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Cyanobacteria represent a promising platform for the production of plant secondary metabolites. Their capacity to express plant P450 proteins, which have essential functions in the biosynthesis of many plant secondary metabolites, makes cyanobacteria ideal for this purpose, and their photosynthetic capability allows cyanobacteria to grow with simple nutrient inputs. This review summarizes the advantages of using cyanobacteria to transgenically produce plant secondary metabolites. Some techniques to improve heterologous gene expression in cyanobacteria are discussed.

Keywords: plant secondary metabolites, phenylpropanoid, cyanobacteria, P450 proteins, photosynthetic growth

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Benefits of Plant Secondary Metabolites to Human Health

Secondary metabolites produced by plants confer protection against stresses such as infections, wounding, UV irradiation, and ozone (Douglas, 1996), and allow plants to adapt to continuously changing environmental conditions (Korkina, 2007). Secondary metabolites are largely derived from primary metabolites, such as amino acids and carbohydrates, which are modified by methylation, hydroxylation, or glycosylation (Crozier et al., 2006).

Increasing evidence suggests that plant secondary metabolites, especially the largest group, phenylpropanoids, and their derivatives, are powerful antioxidants that directly scavenge reactive oxygen and nitrogen species (ROS/RNS) (Perron and Brumaghim, 2009). A balance between oxidant and antioxidant systems is critical for maintaining cellular functions. The excessive production of ROS/RNS inside cells results in oxidative stress, loss of cell function, and apoptosis or necrosis. Detoxification of ROS/RNS by enzymatic and non-enzymatic antioxidants minimizes cell damage (Ratnam et al., 2006; Reuter et al., 2010). The antioxidant activity of five phenylpropanoids (i.e., verbascoside, forsythoside, arenareoside, ballotetraside, and caffeoyl malic acid) extracted from the perennial herb *Ballota nigra* were investigated against superoxide, hydrogen peroxide, hypochlorite, and hydroxyl radicals generated in cell-free systems (Fraga et al., 2010). The ability of these phenylpropanoids to scavenge free radicals was comparable to that of *N*-acetyl cysteine, an established antioxidant drug (Nordberg and Arner, 2001).

Plant secondary metabolites have become the focus of intensive research, due to their beneficial effects on human health as anticancer, antioxidant, anti-virus, and anti-inflammatory agents. However, these compounds are mainly isolated from plant extracts or from cultivated plant cells at relatively high cost and low yield. It is expensive and technically challenging to chemically synthesize these molecules. Therefore, there is a strong need to develop novel, efficient, and economical methods to produce beneficial plant secondary metabolites.

Cyanobacteria are Suitable for Producing Plant Secondary Metabolites

Several inherent properties of cyanobacteria make them attractive candidates for the biosynthesis of plant secondary metabolites, such as their photosynthetic activity, their amenability to genetic engineering, and their ability to live in tough environments.

Large-scale cyanobacterial cultivation is frequently performed in phototrophic conditions because this approach is cheaper, results in less contamination, and consumes CO_2 (Chen et al., 2011). Commercially used open ponds or closed photobioreactors have been developed for large-scale biomass production (Olaizola, 2000; López et al., 2006; Eriksen, 2008; Ugwu et al., 2008; Rodolfi et al., 2009; Singh and Gu, 2010). The nutrient inputs for cyanobacteria are simple: sunlight, CO_2, H_2O, N, P, and a few mineral nutrients, without carbohydrate feedstocks (Yu et al., 2013).

A wide variety of enzymes and pathways are involved in plant secondary metabolite production. Of these enzymes, cytochrome P450 monooxygenases participate in the pathways to produce compounds such as phenylpropanoids, alkaloids, terpenoids, cyanogenic glycosides, and glucosinolates (Mizutani and Ohta, 2010). They contribute various oxidative modifications of the carbon skeleton using NADPH or NADH as reducing equivalents (Sligar, 1999). P450 sequences have been found in the genome of most known cyanobacterial species (Ke et al., 2005); *Anabaena* sp. PCC 7120 has six P450 genes (Robert et al., 2010) and *Synechocystis* sp. PCC 6803 has one (*cyp120A1*/*slr0574*) (Ke et al., 2005). Because most eukaryotic P450 proteins are membrane-bound proteins, it is challenging to heterologously express these proteins in other prokaryotes, such as *E. coli*, which lack developed internal membrane systems. By contrast, cyanobacteria have an intracellular membrane system, i.e., the thylakoids, which function in electron transport. This makes cyanobacteria highly suitable hosts in which to express P450 enzymes (Melis, 1999).

Using Cyanobacteria to Produce Plant Secondary Metabolites

Several cyanobacteria have been engineered as cell factories for the production of plant secondary metabolites (**Table 1**). Metabolic distributions of produced plant secondary metabolites are summarized in **Figure 1** based on their biosynthetic pathways.

Tricarboxylic Acid Cycle

To produce ethylene, an ethylene-forming enzyme gene (efe) from *Pseudomonas syringae* was inserted into the *Synechococcus elongatus* PCC 7942 chromosome at the psbAI locus, and the recombinant strain produced ~512 μg ethylene L^{-1} h^{-1} $OD730^{-1}$ (Takahama et al., 2003). An artificial chimeric enzyme complex containing two ethylene-generating enzymes from *Solanum lycopersicum* (tomato) was introduced into *S. elongatus* PCC 7942, and the strain produced ethylene with a titer of ~3.9 μg ethylene L^{-1} h^{-1} $OD730^{-1}$ (Jindou et al., 2014).

TABLE 1 | Plant secondary metabolites produced by genetically engineered cyanobacteria.

Products	Yield	Host	Reference
Ethylene	~512 μg L^{-1} h^{-1} $OD730^{-1}$[b]	*S. elongatus* PCC 7942	Takahama et al. (2003)
	~3.9 μg L^{-1} h^{-1} $OD730^{-1}$	*S. elongatus* PCC 7942	Jindou et al. (2014)
Isoprene	50 μg (g dry cell)$^{-1}$ day^{-1}	*Synechocystis* sp. PCC 6803	Lindberg et al. (2010)
	~125 μg (g dry cell)$^{-1}$ day^{-1}	*Synechocystis* sp. PCC 6803	Bentley et al. (2014)
Caffeic acid[a]	7.2 mg L^{-1}	*Synechocystis* sp. PCC 6803	Xue et al. (2014b)
ρ-coumaric acid[a]	82.6 mg L^{-1}	*Synechocystis* sp. PCC 6803	Xue et al. (2014a)
Mannitol	0.15 g L^{-1} day^{-1}	*Synechococcus* sp. PCC 7002	Jacobsen and Frigaard (2014)
Limonene	56 μg L^{-1} day^{-1}	*Synechocystis* sp. PCC 6803	Kiyota et al. (2014)
	50 μg L^{-1} h^{-1}	*Synechococcus* sp. PCC 7002	Davies et al. (2014)
Carotenoid[a]	8.4 mg (g dry cell)$^{-1}$	*Synechocystis* sp. PCC 6803	Kudoh et al. (2014)

[a] *Time over which the yield was achieved is not provided in the publication.*
[b] *The yield is calculated from ethylene gas production of 451 nL mL^{-1} h^{-1} $OD370^{-1}$ reported by Takahama et al. (2003). The authors believe that the rate of 37 mg L^{-1} h^{-1} previously attributed to this study (Lan and Liao, 2011; Shen and Liao, 2012; Wang et al., 2012) is incorrect.*

2-C-Methyl-D-Erythritol 4-Phosphate Pathway

Isoprene, which protects plants from abiotic stresses (Sharkey et al., 2008) and serves as a renewable biofuel, was produced in *Synechocystis* sp. PCC 6803 with a titer of 50 μg (g dry cell)$^{-1}$ day^{-1} by expressing an isoprene synthase gene (*ispS*) from *Pueraria Montana* (Lindberg et al., 2010). By co-expressing seven genes of a heterologous mevalonic acid biosynthetic pathway from *Enterococcus faecalis* and *Streptococcus pneumoniae* in that *ispS* transformant, the yield of isoprene production increased 2.5-fold (Bentley et al., 2014). To produce limonene, a limonene synthase gene (*LMS*) from *Schizonepeta tenuifolia* was expressed in *Synechocystis* sp. PCC 6803, and the titer was 41 μg L^{-1} day^{-1}. By overexpressing three genes (*dxs*, *crtE*, and *ipi*) in the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway to increase the supply of limonene substrate, geranyl pyrophosphate (GPP), limonene production was improved by 1.4-fold (Kiyota et al., 2014). In another study, 50 μg L^{-1} h^{-1} limonene was produced by a *Synechococcus* sp. PCC 7002 strain expressing codon optimized *Mentha spicata* limonene synthase gene (*mslS*) (Davies et al., 2014). Carotenoids are naturally produced terpenoid-type molecules by cyanobacteria through MEP pathway. Recently, Kai et al. overexpressed a key enzyme, 1-deoxy-D-xylulose-5-phosphate synthase (DXS), in *Synechocystis* sp. PCC 6803, and the carotenoid level in the strain was 1.5 times higher than that in the wild-type strain (Kudoh et al., 2014).

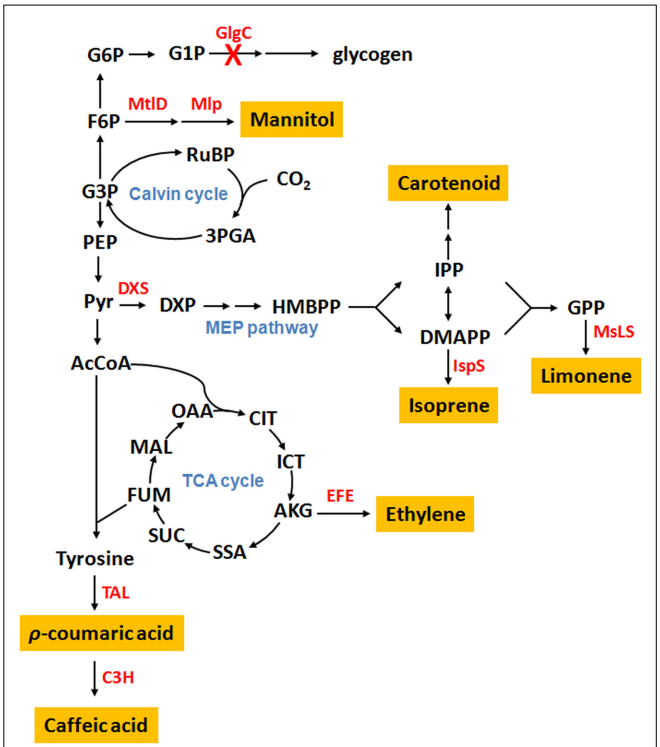

FIGURE 1 | Cyanobacterial metabolic pathways for the production of plant secondary metabolites. Abbreviation: G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; RuBP, ribulose-1,5-diphosphate; 3PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; DXP, 1-deoxy-D-xylulose 5-phosphate; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; AcCoA, acetyl-CoA; OAA, oxoacetate; CIT, citrate; ICT, isocitrate; AKG, α-ketoglutarate; SSA, succinic semialdehyde; SUC, succinate; FUM, fumarate; MAL, malate; GlgC, ADP-glucose pyrophosphorylase; MtlD, mannitol-1-phosphate dehydrogenase; Mlp, mannitol-1-phosphatase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; IspS, isoprene synthase; MsLS, *Mentha spicata* limonene synthase; EFE, ethylene-forming enzyme; TAL, tyrosine ammonia-lyase; C3H, ρ-coumarate 3-hydroxylase; H.

Other Metabolites

Our group recently constructed a *Synechocystis* sp. PCC 6803 strain genetically engineered to produce caffeic acid, in which an *Arabidopsis* ρ-coumarate 3-hydroxylase (encoded by *ref8*) was expressed. With the addition of substrate, ρ-coumaric acid, to the medium, the titer reached 7.2 mg L^{-1} (Xue et al., 2014b). In another report, the direct precursor of caffeic acid, ρ-coumaric acid, was produced at a concentration of 82.6 mg L^{-1} by a *Synechocystis* sp. PCC 6803 mutant, which harbored a tyrosine ammonia-lyase (TAL) gene (*sam8*) from *Saccharothrix espanaensis* and lacked a hypothetical laccase gene (Xue et al., 2014a). Mannitol was produced in *Synechococcus* sp. PCC 7002 with a titer of 0.15 g L^{-1} day^{-1} by heterologously expressing mannitol-1-phosphate dehydrogenase (*mtlD*) from *E. coli* and mannitol-1-phosphatase (*mlp*) from *Eimeria tenella*. By genetically inactivating glycogen synthesis, the yield increased 3.2-fold (Jacobsen and Frigaard, 2014).

Some Considerations for the Genetic Manipulation of Cyanobacteria

Although many species of cyanobacteria have been successfully engineered to express heterologous genes and produce valuable compounds, as summarized above, this approach remains challenging due to epigenetic suppression, poor transcription, and protein post-translational modifications. Here, we highlight some factors that need to be considered when genetically engineering cyanobacteria to produce plant secondary metabolites.

Inactivation of Glycogen Synthesis Pathway

Glycogen is one of the dominant carbon sinks for cyanobacteria. Up to 60% of dry cell weight is converted to glycogen when cyanobacteria are growing in nitrogen-limited media (Allen and Smith, 1969). Therefore, inactivation of glycogen synthesis pathway should allow a greater proportion of carbon partitioning to non-native products. In a recent study, the *glgC* gene (coding for glucose-1-phosphate adenylyltransferase) was knocked out from *S. elongatus* PCC 7942 strain producing isobutanol. The amount of total fixed carbon flux toward isobutanol production was increased by 2.5-fold after deletion of *glgC* under constant high light condition (150 μE s^{-1} m^{-2}) (Li et al., 2014). Another example is a *Synechoccocuus* sp. PCC 7002 strain expressing a mannitol biosynthetic pathway, which produces mannitol equivalent to 10% of cell dry weight under 250 μE s^{-1} m^{-2}. By blocking glycogen biosynthesis, the yield increased to 32% (Jacobsen and Frigaard, 2014).

Laccase Knockout

Laccases are enzymes that oxidize many phenolic compounds and are present in a large variety of species. Oxidized substrates become free radicals, which are unstable and can be modified by other non-enzymatic reactions, such as hydration, polymerization, and disproportionation (Thurston, 1994). In a recent study, a putative laccase gene, *slr1573*, was found in *Synechocystis* sp. PCC 6803. After knocking out *slr1573* from its genome, the production of *p*-coumaric acid increased by more than 25-fold in the strain expressing TAL enzyme (Xue et al., 2014a). Although reports of laccase functions in cyanobacteria are limited, this gene might be a barrier for phenylpropanoids production using cyanobacteria.

Codon Optimization

A codon usage bias exists for most protein-coding genes expressed in heterologous hosts. It is critical to optimize the codon usage in order to obtain high levels of overexpression of heterologous genes (Steen et al., 2010; Bond-Watts et al., 2011; Paddon et al., 2013). Several factors need to be considered when designing new gene sequences, including host codon usage frequency (Angov et al., 2011), AT/GC ratio (Gustafsson, 2009),

mRNA secondary structure (Tang et al., 2011), repeat sequences (Li et al., 2011), and restriction sites for cloning (Raab et al., 2010). Our group recently constructed a transgenic *Synechocystis* sp. PCC 6803 that heterologously expressed *ref8* from *Arabidopsis thaliana*, which encodes a P450 enzyme ρ-coumarate 3-hydroxylase, and was capable of producing caffeic acid (Xue et al., 2014b).

Transgene Stability

Several reports describe the instability of transgenes in genetically engineered cyanobacteria. During subculturing of an ethylene-producing *S. elongatus* PCC 7942 transformant, a duplicated sequence in the *efe* gene was found that resulted in a truncated and non-functional gene (Takahama et al., 2003). In another study, a *Synechocystis* sp. PCC 6803 strain was genetically modified to produce lactic acid by integrating a lactate dehydrogenase gene in the genome. Wild-type phenotypic colonies appeared during segregation, and further analysis identified a nonsense mutation in the transgene (Angermayr et al., 2012). Similarly, in an effort to produce isopropanol, four enzymes in the isopropanol biosynthetic pathway from *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *E. coli* were expressed in *S. elongatus* PCC 7942. The authors repeatedly found a missense mutation in one gene (*atoD*), which reduced the enzymatic activity (Kusakabe et al., 2013). The mechanism underlying the instability has not been determined. A common approach is to select transformants carrying single unrearranged transgenes and to keep these as backups.

Markerless System

The standard method of genetically engineering cyanobacteria involves transformation of a plasmid carrying genes of interest and integration of these foreign genes into the cyanobacterial genome at specific sites through double-crossover homologous recombination (Vermaas, 1996). Antibiotic resistances are used as selectable markers for positive transformants. In some cases, when multiple gene integrations are required in one strain, the number of available antibiotic markers restricts the number of insertions, and thus markerless genomic mutations are desirable. Currently, the most widely used markerless technique for cyanobacteria was developed using a plasmid containing *sacB* (a levansucrase gene) and an antibiotic resistance cassette (Lagarde et al., 2000). In the first transformation, a target region in the genome is replaced by the *sacB*-antibiotic resistance cassette and antibiotic resistance is used for positive selection. The second transformation is performed by replacing the *sacB*-antibiotic resistance cassette with the gene of interest. Sucrose is added to the medium for negative selection. The levansucrase encoded by *sacB* converts sucrose to levans, a toxic polymer that kills the bacteria. Consequently, only markerless mutants can survive in the presence of sucrose. For example, a *Synechocystis* sp. PCC 6803 mutant was constructed for ethanol production by integrating pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*) genes into the genome. An *aphX/sacB* selection cassette was used to generate a markerless transformant, which is able to produce ethanol at a titer of 5.2 mmol OD730 unit^{-1} L^{-1} day^{-1} (Dexter and Fu, 2009). Recently, an alternative strategy was developed using a one-step gene replacement approach (Viola et al., 2014). The plasmid designed for this strategy harbors an *nptI* (kanamycin resistance gene)-*sacB* selection cassette flanked by 5′ and 3′ fragments of the gene of interest, which have overlapping segments. After transformation, the *nptI-sacB* cassette with the exogenous gene is integrated into the genome through double-crossover recombination, and complete segregations are selected based on kanamycin resistance. Then, a second single crossover event occurred between the overlapping fragments, leading to the excision of the *nptI-sacB* cassette. Mutants that had undergone the second recombination were screened on sucrose in the absence of kanamycin.

Another successful counter selection method based on acrylate toxicity was developed for *Synechococcus* sp. PCC 7002, in which *sacB* counter selection system did not work (Begemann et al., 2013). After one transformation step, a native *acsA* gene (encoding an acetyl-CoA ligase) is replaced by the gene of interest, and the loss of acetyl-CoA ligase function overcomes growth inhibition by acrylate. Thus, positive transformants can be screened on growth medium with addition of acrylate. After reinsertion of the *acsA* gene into a neutral site on genome, multiple gene integrations can be achieved.

Limitations of Cyanobacteria for Producing Plant Secondary Metabolites

In addition to the potential advantages of using cyanobacteria to produce plant secondary metabolites mentioned above, this technology is still in its infancy and numerous challenges need to be addressed. For instance, production titers from engineered cyanobacteria are much lower than that from heterotrophic fermentation, and efficient, large volume bioreactors need to be designed. Another consideration is the protein post-translational modifications. Because some plant enzymes in the secondary metabolite biosynthesis pathways require post-translational modifications (e.g., glycosylation), they could be non-functional when expressed in cyanobacteria that are not equipped with these machineries.

Conclusion

Cyanobacteria can be used as cell factories to convert solar energy into high value products, such as plant secondary metabolites, which are beneficial to human health. Their high photosynthetic efficiency and ease of genetic manipulation make cyanobacteria a better choice for this purpose than other organisms. Recently, researchers have put efforts into engineering cyanobacteria to produce plant secondary metabolites from sunlight and CO_2. However, there are still challenges for engineering applications of cyanobacteria, such as improvement of product titers, bioprocess scale-up, and product recovery.

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Engineered transcriptional systems for cyanobacterial biotechnology

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Cyanobacteria can function as solar-driven biofactories thanks to their ability to perform photosynthesis and the ease with which they are genetically modified. In this review, we discuss transcriptional parts and promoters available for engineering cyanobacteria. First, we go through special cyanobacterial characteristics that may impact engineering, including the unusual cyanobacterial RNA polymerase, sigma factors and promoter types, mRNA stability, circadian rhythm, and gene dosage effects. Then, we continue with discussing component characteristics that are desirable for synthetic biology approaches, including decoupling, modularity, and orthogonality. We then summarize and discuss the latest promoters for use in cyanobacteria regarding characteristics such as regulation, strength, and dynamic range and suggest potential uses. Finally, we provide an outlook and suggest future developments that would advance the field and accelerate the use of cyanobacteria for renewable biotechnology.

Keywords: constitutive promoters, cyanobacteria, cyanobacterial promoters, inducible promoters, promoter engineering, regulated promoters, synthetic biology, transcriptional engineering

Cyanobacteria are interesting chassis for renewable, solar-powered production of fuels and high-value products, primarily due to their photosynthetic capabilities and the relative ease of genetically transforming and engineering them (Heidorn et al., 2011; Wang et al., 2012; Berla et al., 2013). Their ability to fix carbon is useful not only because of their capacity to produce carbon-based fuels (Angermayr et al., 2009) but also as it could be used to capture CO_2 released from fossil fuels, which is with all certainty a major cause of global warming (IPCC, 2013). Further, their complex metabolism could be harnessed to generate natural products (Kehr et al., 2011) or engineered to produce high-value bioactive products (Lassen et al., 2014).

The emerging field of synthetic biology offers tools and methodology to enable and accelerate the development of cyanobacteria as biotechnological host chassis. However, to do this, well-characterized biological parts such as promoters (herein defined as an entire transcriptional regulatory region with a transcriptional start site), terminators, translational elements, and coding sequences must be made available for cyanobacteria (Heidorn et al., 2011; Wang et al., 2012; Berla et al., 2013). Transcriptional components like promoters, transcription factors (TFs), and RNA polymerases (RNAPs) are of particular importance as they govern the first control point in gene expression. Further, to maximize their usefulness in synthetic biology applications, these components should retain proper functioning in cyanobacteria and fulfill certain requirements. Therefore, in addition to discussing the latest promoters and other transcriptional parts, this review also covers special considerations unique to cyanobacteria and general part requirements. In addition, several factors other than transcription are also important for the regulation of gene expression e.g. the initiation of translation and the engineering thereof (Salis et al., 2009), the regulation of translation initiation by small regulatory RNAs (sRNAs) (Desnoyers et al., 2013; Lalaouna et al., 2013) or by riboswitches (Nakahira et al., 2013; Berens and Suess, 2014), and the modulation of translation and protein production efficiency through codon bias (Quax et al., 2013). This text focuses on factors affecting the production or decay of mRNA; nonetheless, factors that affect translation in cyanobacteria are of great importance and hence merit their own review. For additional aspects of cyanobacterial synthetic biology and other biological components, the reader is referred to previously published reviews (Heidorn et al., 2011; Wang et al., 2012; Berla et al., 2013) and a recent paper presenting a modular vector system for engineering cyanobacteria (Taton et al., 2014).

SPECIAL CHARACTERISTICS THAT MAY AFFECT TRANSCRIPTIONAL ENGINEERING IN CYANOBACTERIA

DIFFERENCES IN RNA POLYMERASES

The bacterial RNAP consists of an apoenzyme made up of five subunits, $\beta\beta'\alpha_2\omega$. When it binds a sigma factor and forms the complete holoenzyme, $\beta\beta'\alpha_2\omega\sigma$, it gains the ability to bind a promoter specifically and initiate transcription (Saecker et al., 2011). Cyanobacterial RNAP consists of the same subunits as the generic, enterobacterial RNAP, except that the β' subunit is split into two parts: the γ and the β' subunits. The cyanobacterial γ subunit corresponds to the N-terminal part of the enterobacterial β' subunit, whereas the cyanobacterial β' subunit corresponds to the C-terminal part of the enterobacterial β' (Schneider and Haselkorn, 1988; Xie et al., 1989). It is unknown what the effect of the split β' is, if any, but differences in how *Escherichia coli* (*E. coli*) and *Calothrix* sp. PCC 7601 RNAP transcribe the P*lac* and the P*lacUV5* promoters *in vitro* have been observed (Schyns et al., 1998), and the β' split or an insertion in the cyanobacterial β' subunit were suggested causes. Later, it was suggested

that the insertion is a jaw-like DNA-binding domain that interacts with the promoter (Imashimizu et al., 2003). Further, a recent study examined the differences in Mn^{2+} tolerance between *E. coli* and cyanobacterial RNAP. While Mn^{2+} is toxic for most bacteria as it can replace the RNAP active-site Mg^{2+} ion, cyanobacteria need Mn^{2+} at higher intracellular concentrations for maintaining the photosystems. By comparing the activities of *E. coli* and *Synechococcus elongatus* sp. PCC 7942 (*Synechococcus* 7942) RNAP systems *in vitro*, it was concluded that the cyanobacterial RNAP transcribes its DNA slower but with higher fidelity (Imashimizu et al., 2011). The same study also suggested that the β′ insertion of cyanobacterial RNAP could be responsible for the slower but more precise transcriptional elongation. Finally, a recent study investigated the function of the omega subunit in cyanobacteria. It was found to be non-essential in *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), as is generally the case in bacteria. Nonetheless, its absence negatively affected the association of RNAP to the primary sigma factor, leading to the downregulation of highly expressed genes (Gunnelius et al., 2014).

SIGMA FACTORS AND PROMOTER TYPES

Sigma switching is an adaptive mechanism that allows bacteria to adapt to new environmental conditions or different types of stress, as different sigma factors have different promoter preferences. Most alternative σ-factors belong to the σ^{70}-family, of which σ^{70} itself is the primary sigma factor. However, there are examples of σ-factors belonging to the σ^{54}-family, which generally require ATP-driven activators to unwind the promoter DNA (Seshasayee et al., 2011). Cyanobacteria only have sigma factors belonging to the σ^{70}-family (Khudyakov and Golden, 2001; Fujisawa et al., 2010) but those on the other hand can be divided into three groups. Group 1 consists of the primary sigma factor SigA, which corresponds to σ^{70} in *E. coli*, and handles transcription under normal growth conditions. Group 2 consists of non-essential sigma factors that provide a mechanism for environmental adaptation (Imamura and Asayama, 2009). For instance, the SigB factor is expressed in *Synechocystis* 6803 after heat shock or salt stress to transcribe a set of initial stress genes, in connection with the downregulation of SigA expression (Tuominen et al., 2003). Group 3 sigma factors are involved in specific stress-survival regulons such as sporulation (Imamura and Asayama, 2009).

Cyanobacterial promoters can be divided in three different types that differ in the DNA-sequence elements that they contain. Type I promoters are typical σ^{70}-promoters with the transcriptional start site (TSS) at +1 (by definition), a −10 element (consensus sequence 5′-TATAAT-3′), and a −35 element (consensus sequence 5′-TTGACA-3′). Under normal growth conditions, type I promoters are chiefly transcribed by SigA, although group 2 sigma factors may also recognize type I promoters. Type II promoters are usually connected to stress or adaptation responses and thus are normally transcribed by group 2 sigma factors, although depending on the specific promoter they may also be recognized by SigA. Type II promoters have a −10 element but typically lack the −35 element, instead, these promoters rely on the binding of upstream transcriptional activators (Imamura and Asayama, 2009). As an example, the type II *glnB* P2 promoter in *Synechocystis* 6803 has an upstream motif for NtcA binding and subsequent upregulation of transcription during nitrogen deprivation. It is mainly recognized by the group 2 sigma factor SigC (Imamura et al., 2006). Type III promoters do not have regular −10 and −35 elements and are probably mostly involved in stringent responses involving type III sigma factors, but may, depending on the promoter, be recognized by any of the sigma factor groups (Imamura and Asayama, 2009). For engineering purposes, it makes sense to choose promoter types depending on under what growth conditions expression is desired. Finally, overexpression of sigma factors is a strategy to affect transcription globally, for example, to activate certain stress responses. This was recently done in *Synechocystis* 6803, where SigB overexpression was observed to enhance temperature and butanol tolerance (Kaczmarzyk et al., 2014).

STABILITY AND DEGRADATION OF mRNA

Cellular activities are constantly regulated through the maturation or degradation of mRNAs and regulatory RNAs by a number of different ribonucleases (RNases), leading to average mRNA half-lives around a few minutes in most prokaryotes (Evguenieva-Hackenberg and Klug, 2011). These RNases differ in their target specificities and have different roles in the turnover of mRNA. Endoribonucleases typically initiate degradation, which is completed by the action of exoribonucleases. Generally, bare mRNAs not occupied by ribosomes, mRNAs with accessible 5′ monophosphate ends, or with AU-rich sequences are targets of initial endonucleolytic attacks (Deutscher, 2006). In *E. coli*, the essential single-strand endonuclease RNase E is thought to initiate most attacks on mRNA, mainly as a part of an RNA degradation complex known as the degradosome. In addition to RNase E, the *E. coli* degradosome consists of polynucleotide phosphorylase (PNPase), the RNA helicase RhlB (for unwinding secondary structures), and the glycolytic enzyme enolase (Mackie, 2013). In bacteria, 3′-end polyadenylated RNAs are targeted for degradation, which in *E. coli* is carried out by the 3′ to 5′ exonucleases PNPase, RNase R, or RNase II. PNPase, however, has a dual role in that it can also synthesize heteromeric but adenine-rich poly(A) tails. Normally though, RNA polyadenylation is handled by poly(A)-polymerase (PAP), which produces homomeric poly(A) tails (Slomovic and Schuster, 2011). Further, it has been observed that sRNAs, normally complexed with the RNA chaperone Hfq in *E. coli*, can regulate the stability of mRNAs. sRNA-mediated mRNA degradation can occur passively, when pairing of sRNA-Hfq to the mRNAs 5′ untranslated region (5′-UTR) blocks translation and leaves the mRNA vulnerable to RNase attacks. Active degradation takes place when a sRNA-Hfq-RNase E complex binds an mRNA, or when the sRNA-Hfq complex binds an mRNA and thereby creates a target site for the double-stranded endonuclease RNase III, which causes cleavage of both the mRNA and the sRNA (Saramago et al., 2014).

Cyanobacteria possess an RNase E with the conserved N-terminal endoribonucleolytic domain intact and a C-terminal domain that is highly divergent from that of the *E. coli* enzyme. Further, it was shown that the catalytic N-terminal domain of the *Synechocystis* 6803 RNase E functions in the same way as its *E. coli* counterpart, and that it even cleaves *E. coli* RNase E target RNAs in the same position as the *E. coli* enzyme. However, the C-terminal half of the *Synechocystis* 6803 RNase E cannot function as a scaffold for assembling the *E. coli* degradosome complex

(Kaberdin et al., 1998). Indeed, cyanobacterial RNase E does not form an *E. coli*-like degradosome complex. Instead, it was recently found that the *Anabaena* (*Nostoc*) sp. PCC 7120 (*Anabaena* 7120) and the *Synechocystis* 6803 RNase E enzymes form a complex with PNPase through a nonapeptide located at the C-terminus of RNase E. Alignments of the RNase E genes from 60 different cyanobacterial strains revealed that this nonapeptide subregion is highly conserved, implying that this RNase E-PNPase complex is a general feature of cyanobacteria (Zhang et al., 2014). Further, the authors suggested that the cyanobacterial RNase E-PNPase complex indicates close functional integration of RNA cleavage, polyadenylation and phosphorolysis, and that it may be an efficient RNA decay machine.

High-throughput sequencing-based studies have found massive transcription of different types of non-coding RNAs (ncRNAs) in cyanobacteria (Mitschke et al., 2011a,b; Xu et al., 2014), which suggests that ncRNAs are important for the regulation of cyanobacterial gene expression. Further, there are several examples of the modulation of mRNA stability by interactions with ncRNAs in cyanobacteria. For instance, long anti-sense RNAs (asRNAs) of 3.5 and 7 kb were found to block *Prochlorococcus* sp. RNase E from cleaving mRNA *in vitro* as it formed a protective asRNA-mRNA duplex (Stazic et al., 2011). In addition, a *Synechocystis* 6803 asRNA binding to the 5′-UTR of the *psbA2* transcript was found to block RNase E-mediated mRNA degradation *in vitro* by masking an AU-rich box and the ribosome binding site (RBS). Also, the transcription of the *psbA2* asRNA was correlated with the expression of the *psbA2* mRNA, both being upregulated by light (Sakurai et al., 2012). Interestingly, the *psbA2* 5′-UTR's AU-box and RBS were previously identified to be targets of dark-induced RNase E-mediated mRNA degradation (Horie et al., 2007). This illustrates how the interplay between RNases and regulatory RNAs functions as an important regulation mechanism of gene expression on several different levels. It is not yet clear what role, if any, the cyanobacterial Hfq plays in asRNA or sRNA-mediated regulation of mRNA stability or gene expression. Cyanobacterial Hfq differs from the *E. coli* Hfq in its RNA binding sites and it cannot mediate sRNA-dependent regulation in *E. coli* (Boggild et al., 2009). However, *Anabaena* 7120 Hfq has been implicated in the regulation of the *nir* operon (Puerta-Fernandez and Vioque, 2011), and *Synechocystis* 6803 Hfq was recently found to form a complex with type IV pili on the cytoplasmic membrane (Schuergers et al., 2014). The authors of the latter study speculated that cyanobacterial Hfq may be involved in membrane-associated post-transcriptional regulation. Clearly, more research is required to shed light on the role of Hfq in cyanobacteria. Finally, we conclude that the stability of mRNAs is an important factor to consider for transcriptional engineering, and may even be used as a design parameter. For instance, different elements affecting mRNA stability could be excluded or deliberately included to increase or decrease the amount of mRNA for different genes, even if they are transcribed from the same promoter.

CIRCADIAN RHYTHM EFFECTS ON GENE EXPRESSION

The circadian rhythm provides a means for cells to co-ordinate metabolic activities with the dark and light cycles of night and day, and therefore, it is of special importance for photosynthetic organisms. It is a global actor on gene expression that is driven by its core oscillator, which consists of the three proteins KaiA, KaiB, and KaiC that drive a KaiC phosphorylation cycle (Ishiura et al., 1998; Johnson et al., 2008). It has been found that about half, or 30–64%, of all genes are rhythmically expressed in *Synechococcus* 7942, and DNA topology has been suggested to be one of the regulation mechanisms (Dong et al., 2010). A recent study, also in the circadian rhythm model organism *Synechococcus* 7942, identified the response regulator RpaA as the master regulator through which the core oscillator exerts its influence on global gene expression patterns and cell division (Markson et al., 2013). It was found that RpaA binds and regulates genes involved in a large range of activities, including its own gene *rpaA* and the *kaiBC* clock genes, TFs, σ-factors, the DNA-binding nucleoid protein HU, regulators of cell division, and genes involved in the general metabolism. These wide-ranging effects make circadian rhythm an important and potentially useful factor to take into consideration for cyanobacterial biotechnology. However, it was recently observed that gene expression patterns in *Synechocystis* 6803 that varies temporally with light/dark cycles may not be connected to a circadian rhythm, as the periodical expression behavior stopped under constant dark or light conditions (Beck et al., 2014). On the other hand, it is a possibility that the prolonged growth of this strain under continuous light conditions has affected its circadian rhythm. Finally, for engineering purposes, there may be advantages in connecting the expression of certain genes of interest to the circadian rhythm. Expression only during the day could be advantageous for enzymes requiring an electron flow from the photosystems, or only during the night for oxygen-sensitive enzymes.

GENE DOSAGE AND CYANOBACTERIAL GENOME COPY NUMBERS

Gene dosage is a design criterion that merits consideration for any transcriptional system. The number of promoters per cell is not only important from a strength of expression perspective, where a higher gene dosage usually leads to higher expression levels (Lutz and Bujard, 1997), but also important for regulation. For example, the cellular concentration of repressors may be sufficient to repress a promoter under low copy number, but may be insufficient and cause a higher basal promoter activity level when the target promoter exists in too many copies. The location of the expression construct is a factor that is connected to the copy number, as the copy numbers of plasmids and genomes between different strains normally differ. Cyanobacterial strains have multiple genome copy numbers, as exemplified by *Synechocystis* 6803, that was found to have a chromosome copy number of 12 (Labarre et al., 1989) or even up to between 40 and 200, depending on the growth phase, as newer data suggest (Griese et al., 2011). Hence, genetic circuits inserted into the genomes of different cyanobacterial strains might behave differently solely because of gene dosage-related effects. Another less obvious factor is that the gene copy number of a gene inserted into the bacterial chromosome will depend on the distance to the origin of replication. The closer it is to the origin, the higher the gene copy number will be because of more frequent replication, and vice versa, the closer it is to the replicative terminus, the lower the copy number will be (Klumpp et al., 2009).

DESIRED PROPERTIES OF TRANSCRIPTIONAL PARTS

DECOUPLING AND MODULARITY

An ideal promoter would drive the same level of transcription independently of the biological components it expresses, making

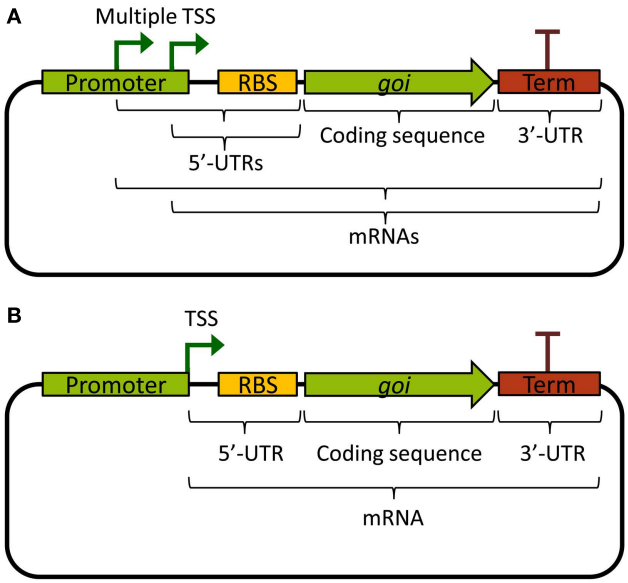

FIGURE 1 | Basic expression constructs differing in the promoters. **(A)** Example of a promoter with multiple TSS, producing multiple different mRNAs, and which contributes excess sequence to the 5′-UTRs. **(B)** Example of a well-defined standardized promoter that ends with its TSS and hence does not contribute excess sequence to the 5′-UTR. TSS: transcriptional start site; UTR: untranslated region; RBS: ribosome binding site; *goi:* gene of interest; Term: transcriptional terminator.

rational design of new genetic circuits possible based on its previous characterization. Unfortunately, from a biological engineer's perspective, promoters are not always truly modular or well defined. Often, there are multiple transcriptional start sites, producing mRNAs with different 5′ ends, or the promoter sequence continues downstream of the TSS, contributing excess sequence to the 5′-UTR (**Figure 1A**). This leads to unpredictable effects on mRNA stability, as the mRNA sequence itself will affect its stability through differential association with RNases (see Stability and Degradation of mRNA). Further, the 5′-UTR is important for ribosome binding and initiation of translation, and interactions between the part of the promoter sequence that is included in the 5′-UTR and the RBS, or the first part of the coding sequence, could lead to the formation of ribosome-blocking secondary structures (De Smit and Vanduin, 1990). Indeed, a recent combinatorial study where many different promoters and 5′-UTRs were combined with two different fluorescent protein reporters found that the largest part of the variation in translation efficiency could be explained by the choice of promoter, and that mRNA abundance was mostly explained by the 5′-UTR sequence (Mutalik et al., 2013b). This, of course, is a problem for the reliable reuse of characterized promoters in metabolic engineering; the mRNA levels of an expressed gene will depend on the combination of a gene's specific 5′-UTR, which may depend on both the promoter and the RBS, and the coding DNA sequence itself.

To solve these problems, standardized promoters that always end with their TSS have been suggested (**Figure 1B**). Going further, the same study also developed a bi-cistronic system for translation that prevents 5′-UTR secondary structures from blocking translation of the gene of interest, which works also for different coding sequences (Mutalik et al., 2013a). Other ways of solving the problem of cross-talk between promoters and 5′-UTRs includes adding self-cleaving ribozymes to the RBS, which will truncate the mRNA and remove any contribution to the 5′-UTR from the promoter (Lou et al., 2012). These two solutions can be viewed as functional insulation or decoupling, two engineering concepts vital to the success of rational design of genetic circuits. Alternatively, to circumvent cross-talk problems in rational design for metabolic engineering, it would be possible to use combinatorial gene expression optimization approaches (Du et al., 2012; Kim et al., 2013). However, these methods often require high-throughput screening of circuit functionality in the final production host, which is challenging for most cyanobacterial strains due to special growth requirements and longer generation times as compared with common biotechnological chassis such as *E. coli* or yeast. Nonetheless, smaller combinatorial optimization strategies are feasible and may even be preferable, for instance when well-characterized, functionally insulated parts are lacking. This was recently illustrated when 2,3-butanediol production was optimized in *Synechococcus* 7942 by varying 5′-UTRs (Oliver et al., 2014). Finally, it is important to note that decoupling and modularity are important concepts also for other transcriptional components than promoters. In general, it is not desirable that expressed TFs or RNAPs bind or non-specifically transcribe promoters outside the engineered circuit. One way to remove or minimize such unintended cross-talk is the use of orthogonal parts.

NATIVE AND ORTHOGONAL PARTS

Natural biological systems and their components are generally not decoupled, but have evolved to perform their function inside the cellular environment, in the myriad of interactions that occur with other biomolecules and on different levels of regulation (Young and Alper, 2010). Because of this, the implementation of natural biological systems is often difficult to understand and consequently difficult to use or engineer. Unknown interactions or cross-talk between natural components and other parts of the cell may cause a system to fail or perform less than optimally (Cardinale and Arkin, 2012). To reduce the risk for interactions with native transcriptional systems, orthogonal components or whole systems could be introduced. Orthogonal parts can be defined as components that are functionally decoupled from other parts and/or systems, enabling them to operate without unintended cross-talk. As an example, a recently developed group of T7 RNAPs is orthogonal to the host's own transcriptional machinery, since they do not recognize the host's promoters, and vice versa, since the host's RNAP does not recognize the T7 promoters. Further, each T7 RNAP was engineered to recognize and transcribe a specific T7 promoter sequence, while displaying only limited cross-talk with other, non-cognate, T7 promoters (Temme et al., 2012). Therefore, these engineered T7 RNAPs are not only orthogonal to the host's own transcriptional system but are also highly orthogonal to each other. Orthogonal transcriptional parts could be mined from strains of bacteria that are sufficiently divergent from the new host to minimize the risk of cross-talk, or from other domains of life. Ultimately, synthetic transcriptional

components could be designed *de novo* to be both well defined and functionally decoupled, thereby displaying a maximum degree of orthogonality.

PROMOTERS USED FOR REGULATED OR CONSTITUTIVE GENE EXPRESSION

Regulated promoters, especially repressed promoters that can be induced to higher activities when desired, are valuable tools as they can be used both for basic research and for the development of metabolically engineered strains. Further, it is also possible that repressed promoters could increase the genetic stability of engineered cyanobacterial strains (Jones, 2014). Interestingly, in support of this notion, an *E. coli* study found that the genetic stability of genetic circuits decreased exponentially with increased expression levels (Sleight et al., 2010).

In many cases, promoters that are used for constitutive expression are not truly constitutive in the sense that their activity is not always constant. This could include, for example, native promoters that appear constitutive under certain growth conditions, or orthogonal promoters that are constitutive due to the absence of their specific regulators. Further, regulated or constitutive promoters endowed with core promoter elements close to the conserved σ^{70} consensus sequences can be expected to be broad-host-range, as they are expected to be similar in other bacteria (Wösten, 1998). In this section, we discuss selected promoters of interest for transcriptional engineering in cyanobacteria, and present summaries of relevant characteristics in **Table 1**. Finally, for the later discussion of regulated promoters, we here define two different ratios of use when analyzing promoter performance. First, we define the repression ratio as the activity of the promoter in the absence of its repressor divided by its repressed activity. Second, we define the induction ratio as the activity of the promoter when induced or activated divided by its repressed or non-activated activity. We make this distinction as all repressors are not inducible but still potentially useful in, for example, genetic inverters (NOT gates) or toggle switches (Gardner et al., 2000).

Recently, P*cpc560*, a "super-strong" transcriptional regulatory region consisting of the 560 bp upstream of the start codon of the c-phycocyanin beta subunit gene *cpcB* in *Synechocystis* 6803 [also previously used due to its high level of expression (Xu et al., 2011)], was characterized (Zhou et al., 2014). P*cpc560* contains two predicted promoters and was found to be dependent on an upstream sequence containing 14 predicted transcription factor binding sites (TFBS) for its high activity. Further, it was used to express two heterologous genes to up to 15% of the total soluble protein content. However, it is not clear to what extent the transcriptional activity of the promoter, as compared with the translational efficiency of the native 5′-UTR of *cpcB*, contributes to the high expression levels. Also, it would be interesting to investigate whether the strong enhancement of gene expression from the promoter fragment containing the predicted 14 TFBS is isolated to transcriptional efficiency. P*cpc560* may prove very useful, both for the design of new, "super-strong" cyanobacterial promoters, and for direct applications, as the lack of strong expression has previously been identified as a bottleneck in cyanobacterial biotechnology (Angermayr and Hellingwerf, 2013; Formighieri and Melis, 2014).

Different versions of the strong promoters P*trc* or P*tac*, synthetic chimeras of the *E. coli trp* and *lacZYA* operon promoters that differ in the core spacer length (Brosius et al., 1985), have frequently been used for constitutive cyanobacterial expression (in the absence of the *lac* repressor, see e.g., Huang et al., 2010; Angermayr et al., 2014; Formighieri and Melis, 2014) or LacI-regulated expression. These promoters have the advantage of being orthogonal to cyanobacteria, both in the promoter sequence and regarding the LacI TF. While Ptrc works well as a broad-host-range promoter (Huang et al., 2010), since the core promoter is close to a consensus σ^{70}/SigA promoter, repression of P*trc* by LacI differs a lot among different studies and strains of cyanobacteria. Generally, different variants of *Ptrc* have worked better for LacI-regulated expression in *Synechococcus* 7942 (Geerts et al., 1995; Niederholtmeyer et al., 2010), while LacI repression of P*trc* has been found to be very leaky or non-existent in *Synechocystis* 6803 (Huang et al., 2010; Guerrero et al., 2012). Other LacI-regulated promoters have been found to be more well functioning in *Synechocystis* 6803, such as P*A1lacO-1* with an IPTG-induction ratio of eight (Guerrero et al., 2012). P*trc2O*-2 was well repressed with a repression ratio of 408 but could, on the other hand, not be induced (Camsund et al., 2014). Solutions to repression or induction issues in LacI-regulated promoters could be found in promoter engineering to improve repression (Camsund et al., 2014) or by using different mutants or versions of LacI (Markiewicz et al., 1994; Satya Lakshmi and Rao, 2009; Gatti-Lafranconi et al., 2013) to improve either repression or induction.

Several wide dynamic-range TetR-regulated promoters for use in *Synechocystis* 6803 were recently designed, exemplified by L03 with a 290-fold induction ratio under red light (Huang and Lindblad, 2013). This was done by systematically varying a few base-pairs in the $P_L$tetO-1 (Lutz and Bujard, 1997) derived BBa_R0040 promoter (The iGEM Registry of Standard Biological Parts, http://parts.igem.org/) that was previously shown to be very weak in *Synechocystis* 6803 (Huang et al., 2010). This orthogonal transcriptional system could also be expected to be broad-host-range, as the core promoter of L03 is close to a consensus σ^{70}/SigA promoter. The inducer anhydrotetracycline is light sensitive, which could be seen as limiting the system. On the other hand, this enables selective expression during the night or in darkness, rendering the system light regulated.

Metal-ion inducible promoters are a type of well-regulated native promoters. These have evolved to maintain the cellular homeostasis of important metal co-factors that become toxic at higher concentrations. For an exhaustive review of metal-ion inducible promoters, we refer the reader to Berla et al. (2013), while here we mention one wide-dynamic-range example. P*nrsB*, the promoter of the *nrsBACD* operon, is involved in maintaining Ni^{2+} homeostasis in *Synechocystis* 6803 through the NrsRS two-component system (Lopez-Maury et al., 2002). It was induced about 350-fold when comparing gene expression from cultures grown in medium without supplemented metals to cultures supplemented with 15 μM Ni^{2+} (Peca et al., 2007).

As promising as the abovementioned regulated promoters may seem, in many cases, the use of small molecule or metal inducers in large-scale cyanobacterial biotechnology can be problematic. The use of heavy metals can be both detrimental to culture growth

Table 1 | Selected promoters used for regulated and constitutive cyanobacterial expression.

Promoter	Origin	TF	Characteristics and references
P*cpc560*	*Synechocystis* 6803	14 TFBS predicted	"Super strong"; heterologous production up to 15% of total soluble proteins (Zhou et al., 2014).
P*trc*	Synthetic chimera of *E. coli* P*trp* and P*lacZYA*	LacI	Originally by Brosius et al. (1985). Used in *Synechococcus* 7942 with an induction ratio of 36 (Geerts et al., 1995), or in *Synechocystis* 6803 with an induction ratio of 1.6. Broad-host-range constitutive in the absence of LacI (Huang et al., 2010).
P*trc2O-2*	Version of P*trc* with dual *lac* operators	LacI	Strong, tightly repressible but not inducible system (repression ratio of 408) (Camsund et al., 2014).
P*A1lacO-1*	Derived from P*A1* of phage T7	LacI	Originally by Lutz and Bujard (1997). Induction ratio of eight in *Synechocystis* 6803 (Guerrero et al., 2012).
L03	Modified from phage λ P_L-derived P_L *tetO-1*	TetR	Induction ratio of 290 under red light conditions in *Synechocystis* 6803 (Huang and Lindblad, 2013).
P*nrsB*	*Synechocystis* 6803	NrsRS	Induction ratio of about 350 using 15 μM Ni^{2+} in *Synechocystis* 6803 (Peca et al., 2007).
P*psbA2*	*Synechocystis* 6803	Unknown	Clearly activated after shift from low to high light (10–500 μmol photons m^{-2} s^{-1}) in *Synechocystis* 6803 (Lindberg et al., 2010).
P*cpcG2*	*Synechocystis* 6803	CcaSR	Clearly activated by green light when *Synechocystis* 6803 cultures are grown in red light (Abe et al., 2014).
P*psbA*	*Amaranthus hybridus*	–	Used for constitutive expression in *Synechococcus* 7002 (Jacobsen and Frigaard, 2014). Broad-host-range close to consensus σ^{70} promoter.
Plastocyanin promoter	*Spirulina platensis* strain C1	–	Used for constitutive expression in *Synechococcus* 7942 (Jeamton et al., 2011). Broad-host-range close to consensus σ^{70} promoter.
J23 library	Synthetic	–	A synthetic library of minimal and constitutive σ^{70} promoters, exemplified by BBa_J23101 (iGEM Registry). Spans a wide range of expression levels in *Synechocystis* 6803 (Camsund et al., 2014). Broad-host-range close to consensus σ^{70} promoters.

TF, transcription factor; TFBS, transcription factor binding sites.

and an environmental hazard. Further, the addition of small molecule inducers in large scale can be expensive, present practical problems of mixing or leakage into surrounding water bodies. In cases when regulation of gene expression is still necessary, but small molecule inducers must be avoided, quorum sensing (Li and Satish, 2012), circadian rhythm (see section above), or light-regulated gene expression (Camsund et al., 2011) might be preferable. Unfortunately, efforts at introducing orthogonal quorum sensing-based regulation in cyanobacteria have not yet been successful (Guerrero et al., 2012). Light-regulated gene expression has already been used to some extent through, e.g., the high light inducible *psbA2* promoter in *Synechocystis* 6803 (Lindberg et al., 2010). In another more recent *Synechocystis* 6803 study, the CcaSR green-light sensitive two-component system that regulates the *cpcG2* promoter was used to optimize a light-sensitive expression induction system (Abe et al., 2014).

Finally, constitutive promoters may be used as an alternative when regulated promoters are not necessary, or used in expression libraries to fine-tune metabolic circuits. A strong promoter from the plant *Amaranthus hybridus* chloroplast, *PpsbA*, has been used for constitutive expression in *Synechococcus* PCC 7002 (*Synechococcus* 7002) and a range of different bacteria, thanks to its similarity to the σ^{70} consensus promoter sequence (Jacobsen and Frigaard, 2014). Another plant promoter used for constitutive expression in cyanobacteria is the phycocyanin promoter (PC promoter) from *Spirulina platensis* strain C1 that is a close to consensus σ^{70} promoter. The PC promoter was found to drive transcription in both *E. coli* and *Synechococcus* 7942 and is likely to be broad-host-range (Jeamton et al., 2011). Finally, the BioBrick J23 promoter library offers a range of synthetic, minimal, and hence orthogonal, constitutive promoters that may be used for fine-tuning expression levels. It can be exemplified by the BBa_J23101 promoter (iGEM Registry) that has been suggested as an expression standard for bacteria (Kelly et al., 2009). In a recent study, several members from the J23 library previously characterized in *E. coli* (iGEM Registry) were selected for characterization in *Synechocystis* 6803, where they were found to span a wide range of expression levels (Camsund et al., 2014). Further, as they are σ^{70} promoters, they can be expected to function in a wide range of cyanobacteria.

OUTLOOK AND SUGGESTIONS FOR FUTURE DEVELOPMENT

There is clearly a need for more robust and well-regulated orthogonal promoters to help accelerate cyanobacterial biotechnology.

The availability of more orthogonal promoters and TFs will decrease the dependence on strain-specific promoters and enable the sharing of parts, which is of great importance as many different cyanobacterial strains are commonly used, and because different strains grow in different environments. As done recently for *E. coli* (Stanton et al., 2014), exogenous repressors could be mined from large sequence databases, codon optimized for several common strains of cyanobacteria and synthesized, and used to regulate novel synthetic promoters engineered from near-consensus σ^{70} promoters to ensure activity in most cyanobacteria. Examples of potentially useful repressors could be the yeast activator Gal4, which has been shown to function as a repressor in bacteria (Paulmier et al., 1987), or the LuxR quorum sensing activator, which was used as an acyl-homoserine lactone-activated repressor in bacteria (Egland and Greenberg, 2000). Further, the development of more sophisticated genetic circuits for fine-tuned metabolic engineering will require regulated promoters that can respond to internal metabolites. This can be exemplified by the dynamic sensor-regulator system (DSRS) developed recently for production of fatty acid-based products in *E. coli* (Zhang et al., 2012). The DSRS made use of a TF that sensed the levels of a key intermediate molecule and regulated other pathway promoters accordingly to minimize the accumulation of potentially toxic enzymes or intermediates. This not only increased the yield of the final product but also resulted in increased genetic stability of the constructs. For cyanobacteria, whose metabolisms are highly dependent on light as an energy source, photons could be seen as an internal metabolite and orthogonal light-regulated TFs (Camsund et al., 2011) could be used as sensors for cyanobacterial DSRS. Furthermore, partially or fully synthetic TFs can now be engineered to bind different synthetic operators, exemplified by engineered zinc-finger DNA-binding proteins (Dhanasekaran et al., 2006) or the recently implemented CRISPR-Cas9 system for CRISPR interference (CRISPRi) (Qi et al., 2013). These customizable TFs could greatly expand the potential toolbox of transcriptional parts for engineering cyanobacteria.

Finally, the most orthogonal gene expression system is one that does not rely on the host's own RNAP at all or otherwise minimally. By using an orthogonal RNAP that does not recognize the host's own promoters, and for which the host's RNAP does not recognize the orthogonal promoters, the risk for cross-talk is strongly reduced, and combined with likewise orthogonal TFs, the system is almost completely decoupled from the host's own transcriptional systems. One such orthogonal RNAP is the phage T7 RNAP and its promoters. T7 RNAP does not recognize the host's promoters, and vice versa, the host's RNAP does not recognize the T7 promoters (Temme et al., 2012). Further, it is conceivable that marine cyanophages like Syn5 (Zhu et al., 2013), which differ from T7 RNAP in among other things a greater salt tolerance, could fill the same role as an orthogonal RNAP. To conclude, it is our expectation that the development of a broad range of widely applicable cyanobacterial genetic parts will help to enable the use of cyanobacteria as large-scale green producers of the renewable products of the future.

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Protein network signatures associated with exogenous biofuels treatments in cyanobacterium *Synechocystis* sp. PCC 6803

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Although recognized as a promising microbial cell factory for producing biofuels, current productivity in cyanobacterial systems is low. To make the processes economically feasible, one of the hurdles, which need to be overcome is the low tolerance of hosts to toxic biofuels. Meanwhile, little information is available regarding the cellular responses to biofuels stress in cyanobacteria, which makes it challenging for tolerance engineering. Using large proteomic datasets of *Synechocystis* under various biofuels stress and environmental perturbation, a protein co-expression network was first constructed and then combined with the experimentally determined protein–protein interaction network. Proteins with statistically higher topological overlap in the integrated network were identified as common responsive proteins to both biofuels stress and environmental perturbations. In addition, a weighted gene co-expression network analysis was performed to distinguish unique responses to biofuels from those to environmental perturbations and to uncover metabolic modules and proteins uniquely associated with biofuels stress. The results showed that biofuel-specific proteins and modules were enriched in several functional categories, including photosynthesis, carbon fixation, and amino acid metabolism, which may represent potential key signatures for biofuels stress responses in *Synechocystis*. Network-based analysis allowed determination of the responses specifically related to biofuels stress, and the results constituted an important knowledge foundation for tolerance engineering against biofuels in *Synechocystis*.

Keywords: biofuels, network, WGCNA, tolerance, *Synechocystis*

INTRODUCTION

Human society has been dependent on fossil fuels for centuries. However, fossil fuels are not an infinite resource, and the possibility of their running out in the future and the increasing concerns over energy security and global climate change pose an urgent call for developing renewable ways to produce fuels. Among all alternatives, photosynthetic cyanobacteria have recently attracted significant attention as a promising "microbial cell factory" to produce renewable biofuels due to their capability to utilize solar energy and CO_2 as the sole energy and carbon sources, respectively (Ducat et al., 2011; Quintana et al., 2011; Robertson et al., 2011). Cyanobacteria contain considerable amounts of lipids in the thylakoid membranes and possess higher photosynthetic efficiency and faster growth rate compared to eukaryotic green algae and higher plants (Quintana et al., 2011). In addition, cyanobacteria have a relatively simple genetic background and are amenable to modification by metabolic engineering and synthetic biology (Wang et al., 2012a). Recent efforts have led to successful production of various biofuels in engineered cyanobacterial cells, such as ethanol (Deng and Coleman, 1999), butanol and isobutanol (Atsumi et al., 2009), alkanes (Choi and Lee, 2013), and biodiesel (Da Ros et al., 2013). However, the current biofuel productivity in the cyanobacterial systems is several orders of magnitude lower than their native producing microbes (Jin et al., 2014). In addition to ongoing efforts to optimize the existing pathways and to discover and construct novel pathways, one option to achieve high productivity is to improve cellular tolerance to toxic biofuel products synthesized by the cyanobacterial hosts (Dunlop, 2011; Zingaro and Papoutsakis, 2012).

Although response mechanisms against biofuels have been extensively studied in many native biofuel-producing microbes (Couto et al., 1997; Dunlop, 2011), it remains unclear for cyanobacteria. As part of our long-term goal to construct more robust and product-tolerant photosynthetic "*chassis*" for synthesizing various renewable biofuels, our laboratory has applied integrated transcriptomic, proteomic, and metabolomic approaches

Abbreviations: COG, cluster of orthologous groups of proteins; CTR, core transcriptional response; HPLC, high performance liquid chromatography; iTRAQ, isobaric tag for relative and absolute quantitation; KEGG, Kyoto encyclopedia of genes and genomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PCA, principal component analysis; PLS-DA, partial least square-discriminant analysis; PPI, protein–protein interaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TO, topological overlap; TOM, topological overlap matrix; WGCNA, weighted gene co-expression network analysis.

to determine the metabolic profiles of a model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) stressed under various biofuels (Liu et al., 2012; Qiao et al., 2012; Wang et al., 2012b; Tian et al., 2013; Zhu et al., 2013). Consistent with early genome-level studies in other microbes (Nicolaou et al., 2010; Dunlop, 2011), our previous results showed that *Synechocystis* cells employed a combination of multiple resistance mechanisms in dealing with biofuels stress (Wang et al., 2012b). In addition, the comparative proteomic analysis provided strong evidence that proteins involved in multiple aspects of photosynthesis (i.e., photosystems I and II, cytochrome, and ferredoxin) were up-regulated in ethanol-treated *Synechocystis* (Qiao et al., 2012), suggesting there could be unique response mechanisms employed by cyanobacteria to combat biofuel toxicity.

Although initial efforts using a conventional approach of analyzing individual genes/proteins according to fold change and statistical significance has led to determination of the responses associated with each of the biofuels in *Synechocystis* (Liu et al., 2012; Qiao et al., 2012; Wang et al., 2012b; Tian et al., 2013; Zhu et al., 2013), it becomes clear that network-focused rather than individual gene/protein-focused methodologies would be more appropriate to obtain a complete picture of cellular response (Lehtinen et al., 2013). In addition, the network analysis defines modules and their possible biological roles based on connectivity of proteins or genes rather than using any artificial cutoff, which may avoid information loss related to genes/proteins of low abundance or small fold changes, such as signal transduction genes. In recent years, network analysis has been applied to cyanobacterial studies. For example, Singh et al. (2010) constructed a *Bayesian* network of *Synechocystis* using transcriptomic data and defined a set of genes as the core transcriptional response (CTR) that are commonly regulated under most of environmental perturbations (Singh et al., 2010). McDermott et al. (2011) developed a predictive *in silico* model of diurnal and circadian behavior of *Cyanothece* 51142 using transcriptomic data, and the results showed that incorporation of network topology into the model could improve the ability to explain the behavior (McDermott et al., 2011). Recently, Wang et al. (2013b) utilized a weighted gene co-expression network analysis (WGCNA) approach to establish transcriptional networks for four cyanobacterial species under metal stresses, and a further cross-species network comparison led to the discovery of several core response modules and genes that may be essential to all metal stresses, as well as species-specific hub genes for metal stresses (Wang et al., 2013b). The studies demonstrated that network-based analysis could be a powerful tool in deciphering cellular responses.

In this study, to further identify responses specifically related to biofuels stress that could be used as potential targets for rational tolerance engineering, a topological analysis of global proteins co-expression network combined with protein–protein interaction (PPI) network was first performed to uncover a core set of proteins commonly responsive to both biofuels stress and environmental perturbations. Then, a WGCNA was applied to identify responses specifically related to biofuels stress. The combination of both analyses allowed the identification of the protein network signatures associated with exogenous biofuels treatments, and provided new insights into the molecular mechanisms against biofuels stress in *Synechocystis*.

MATERIALS AND METHODS

PROTEOMIC DATA SOURCES

A total of five iTRAQ LC-MS/MS datasets of *Synechocystis* sp. PCC 6803 from our previous study were re-analyzed at a peptide level. Growth of *Synechocystis* under ethanol, butanol, hexane, salt stress conditions with dosages of 1.5% (*v*/*v*), 0.2% (*v*/*v*), 0.8% (*v*/*v*), 4% (*w*/*v*), and nitrogen starvation, which led to ~50% growth reduction were then determined. For each condition, cells were harvested at two time points (24 and 48 h) that were corresponding to middle-exponential and exponential-stationary transition phases in the growth time courses for proteomics analysis. Each biological replicates sample has two technical replicates. Due to the page limitation, for details about the environmental perturbation and biofuel stress experiments and original proteomic datasets please find from several previous publications (Liu et al., 2012; Qiao et al., 2012, 2013; Huang et al., 2013; Tian et al., 2013).

PROTEOMIC DATA ANALYSIS

The mass spectroscopy analysis was performed using a AB SCIEX TripleTOF™ 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA), coupled with online micro flow HPLC system (Shimadzu Co, Kyoto, Japan) as described previously. Genome sequence and annotation information of *Synechocystis* sp. PCC 6803 were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes). The details for the experimental design, execution, and proteomic data analysis can be found in the original publications (Liu et al., 2012; Qiao et al., 2012, 2013; Huang et al., 2013; Tian et al., 2013).

PROTEIN CO-EXPRESSION NETWORK CONSTRUCTION

To construct the association network from proteomic data, we used a multi-step procedure for network construction: first, we performed a procedure for data normalization identical with Principal component analysis (PCA) (See below); second, correlation values were calculated between present values for all pairs of peptides. In this study, we used peptides rather than proteins to construct the protein co-expression network. One reason is lots of related peptides from the same protein are always observed in discordance, which may be due to different post-translational modifications or isoforms. Correlation is calculated as the *Pearson* correlation coefficient for all pairwise peptides. Third, in order to generate a reliable protein co-expression network, high correlation coefficients ($r > 0.9$) was used, where only gene pairs with a correlation coefficient higher than 0.9 were considered connected. Finally, we combined protein co-expression network with experimentally determined PPI network (Sato et al., 2007). In this process, known PPI between observed proteins already in the co-expression network were added as new edges to the network.

TOPOLOGICAL ANALYSIS

Topological analysis of networks was performed using Cytoscape software. Bottleneck and hub proteins were defined as the top 20% of proteins ranked by the values of betweenness and degree

centrality, respectively (McDermott et al., 2011, 2012). The degree and betweenness centrality metrics were defined according to the methods described by McDermott et al. (2012). Briefly, degree centrality is a metric of the connectedness of a node, and betweenness centrality is a metric that measures how often paths between nodes must traverse a given node. Generally, degree centrality is the fraction of edges for a particular protein out of all possible interactions for that protein in the network, and betweenness is the number of shortest paths between all pairs of proteins in the network that pass through a specific node.

PRINCIPAL COMPONENTS ANALYSIS

The proteomics data were converted to a ratio versus control conditions. The data were then log2 scaled, and each unit reflects a twofold change in abundance. In order to avoid influence caused by missing data, peptides with any missing data in any condition were removed. Remaining core peptides identified in all conditions were subjected to PCA and partial least square-discriminant analysis (PLS-DA) by SIMCA-P 11.5 software. Averaging was taken for all technical replicates of samples, as in general good reproducibility was observed between replicates (Liu et al., 2012; Qiao et al., 2012; Tian et al., 2013).

WEIGHTED GENE CO-EXPRESSION NETWORK ANALYSIS

Weighted gene co-expression network analysis approach was used to establish a co-expression network from the LC-MS/MS proteomic data (Langfelder and Horvath, 2008). The co-expression network was created first by calculating weighted *Pearson* correlation matrices corresponding to peptide abundance expression, and then by following the standard procedure of WGCNA to create the networks. Briefly, weighted correlation matrices were transformed into matrices of connection strengths using a power function. These connection strengths were then used to calculate topological overlap (TO) (Langfelder and Horvath, 2008). The topological overlap matrix (TOM) is computed as $\mathrm{TOM}_{ij} = (l_{ij} + a_{ij})/[\min(k_i,k_j) + 1 - a_{ij}]$ where l_{ij} is defined as the dot product on row i and column j in adjacency matrix $[a]$ and k_i (the connectivity) is the summation of row i in adjacency matrix $[a]$ (Gibbs et al., 2013). Hierarchical clustering based on TO was used to group proteins with highly similar co-expression relationships into modules. Protein dendrograms were obtained by average linkage hierarchical clustering, while the color row underneath the dendrogram showed the module assignment determined by the Dynamic Tree Cut method. The network for each module was generated with the minimum spanning tree with a dissimilarity matrix from WGCNA. The modules with $r > 0.55$ and a p-value <0.1 were extracted (Wang et al., 2013a).

FUNCTIONAL ENRICHMENT ANALYSIS

Metabolic pathway enrichment analysis was conducted according to Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cluster of Orthologous Groups of proteins (COG) database using the following formula:

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

N is the number of all proteins with KEGG pathway annotation information, M is the number of proteins with a given KEGG pathway annotation, n is the number of the associated proteins with KEGG pathway annotation information, and m is the number of the associated proteins with a given KEGG pathway annotation. All pathway mapping was manually checked for each of the proteins. We also calculated functional enrichment by considering each group or module of interest versus all proteins in the network as a background, as the ratio of m/n versus M/N.

RESULTS AND DISCUSSION

OVERVIEW OF PROTEOMICS ANALYSIS

The proteomic datasets used in this study are listed in **Table 1**. Briefly, the datasets contain four sets of quantitative iTRAQ–LC-MS analyses of *Synechocystis* grown under five stress conditions, i.e., biofuel stresses of ethanol, butanol and hexane, and environmental perturbations of high salt and nitrogen starvation. For each condition, treated and corresponding wild-type control cells were harvested at two time points (i.e., 24 and 48 h). Each biofuel-stressed dataset has two technical replicates. For overall data quality, reproducibility and full description of the proteomic datasets, please refer to several previous publications (Liu et al., 2012; Qiao et al., 2012, 2013; Huang et al., 2013; Tian et al., 2013).

In previous studies, all identified peptides were matched to proteins in the *Synechocystis* genome, and then further analysis was conducted using protein-based quantitative data (Liu et al., 2012; Qiao et al., 2012, 2013; Huang et al., 2013; Tian et al., 2013). However, recent studies showed that the peptide-based proteomic data can be a better choice in constructing protein network since peptides derived from the same protein were shown to have a statistically higher TO and concordance in abundance, which is potentially important for inferring protein abundance (Gibbs et al., 2013). In addition, using peptide-based data also avoids issues related to multiple mapping of the same peptide (Cox and Mann, 2011; Gibbs et al., 2013). In this study, we thus used the peptide-based raw proteomic data and subjected them directly to Mascot analysis. After data filtering to eliminate low-scoring spectra, only the peptides that were identified in both control and the stress-treated samples (so that the ratio calculation is possible) were included for further analysis, resulting a final dataset consisting of 11,179 unique peptides, which are corresponding to 1,971 proteins.

Table 1 | The proteomic datasets used in this study.

Condition[a]	Unique spectra	Peptides	Unique peptides	Proteins
Ethanol (Qiao et al., 2012)	21,066	7,337	7,192	1,523
Butanol (Tian et al., 2013)	18,745	6,355	6,252	1,300
Hexane (Liu et al., 2012)	19,217	6,995	6,875	1,389
Salt (Qiao et al., 2013)	23,822	8,379	8,257	1,702
N-starvation (Huang et al., 2013)	23,674	8,404	8,282	1,703

[a] *References for each dataset are provided.*

Comparison between various stress conditions showed that a total of 3,840 peptides that correspond to 900 (22.7%) proteins were identified in all conditions, possible core stress responses in *Synechocystis*. Functional classification of these commonly identified proteins showed that they were found in almost all aspects of *Synechocystis* metabolism (Additional File S1 in Supplementary Material). Comparison of these possible core stress response proteins with the CTR identified previously (Singh et al., 2010) showed that 230 of the 399 CTR proteins were also responsive in all stress conditions of this study. In addition, the comparison allowed identification of the proteins associated with each individual or multiple stress conditions (**Figure 1**). For example, ethanol-, butanol-, and hexane-stressed datasets shared a common set of 4,166 peptides, corresponding to 1,091 proteins, while each contained 1,474, 752, and 987 unique peptides, respectively; the environmental perturbations of high salt and nitrogen starvation shared a common set of 8,221 peptides, while each contained only 36 and 61 unique peptides, respectively. The great difference in terms of the number of unique peptides between biofuels stress and environmental perturbation suggested that different response strategies could be employed in *Synechocystis* (Singh et al., 2010).

CONSTRUCTION OF PROTEIN CO-EXPRESSION NETWORK TO IDENTIFY COMMON RESPONSES

The network approach combined with topological analysis of global "omics" datasets has been proven to be a significant tool to identify responses under multiple different conditions (McDermott et al., 2012), in many organisms such as cyanobacteria, pathogenic bacteria, yeast, worm, fly, and human cell culture (McDermott et al., 2012). Previous analyses of stress responses to exogenous biofuels in *E. coli* (Dunlop, 2011; Wang et al., 2013a; Jin et al., 2014) and cyanobacteria (Liu et al., 2012; Qiao et al., 2012; Tian et al., 2013) using conventional methodologies showed that both general stress responses such as up-regulation of heat shock proteins and membrane modification, and possible biofuel-specific responses can be induced by individual biofuel stress. To further decipher metabolic responses using a network-based approach, we first constructed a protein co-expression network using proteomic datasets to determine the general stress responses that were commonly responsive to both biofuels stress and environmental perturbations. Briefly, the protein co-expression network was constructed based directly on pairwise or low-order conditional pairwise association measures, such as the correlation or mutual information, to infer the connectivity between proteins (Nicolaou et al., 2010). This method has the advantage of low computational complexity, which is a more suitable approach for network analysis of relatively large number of quantitative peptide data in this study (Nicolaou et al., 2010). The workflow of the network construction was illustrated in **Figure 2**. First, we inferred the network using the similarities between expression profiles of all qualified peptides through *Pearson* correlation and then filtered the correlations to remove those with low correlation values. Here, we used a relatively high threshold of 0.9 to ensure a highly credible connection between peptides, while avoiding losing too many nodes. Second, we transformed the threshold correlation matrix into a peptide co-expression network using a *perl* script (available upon request). The nodes in the networks are peptides while the links between them (edges) represent co-expression properties. The result showed that most of the peptides from the same protein tended to cluster together, which can be viewed as a validation of the network quality. In several cases, we observed that peptides from the same proteins were located in discordance in the network, which is probably due to the different post-translational modifications to the same proteins (Gibbs et al., 2013). Third, as a previous study showed that incorporating a PPI network into a proteins co-abundance network could significantly improve target discrimination using topological measures than the networks without PPI (McDermott et al., 2012), we further integrated a PPI network of *Synechocystis* constructed by Sato et al. (2007) to the protein co-expression network we constructed by adding them directly as new edges in the protein co-expression network. The integrated network achieved has a total of 866 nodes and 20,226 edges, representing a majority of the proteins (866/900) we identified from all stress conditions.

Topological analysis was then conducted by calculating topological attributes of all the nodes in the network. It is well accepted that nodes with top degrees and betweenness are highly central in networks, and so-called hubs and bottlenecks are more likely to be important to the system than others with low topological attributes (Sato et al., 2007; Yao and Rzhetsky, 2008). Based on the same criteria used in several previous studies (McDermott et al., 2011, 2012), we determined bottlenecks as the proteins in the network with top 20% of the betweenness values and hubs as the proteins with top 20% of the degrees values. Interestingly, the result showed that most proteins (109/180) were with both top 20% betweenness and top 20% degrees, thus considered to be bottleneck-hubs (Sato et al., 2007), consistent with a previous study that showed high

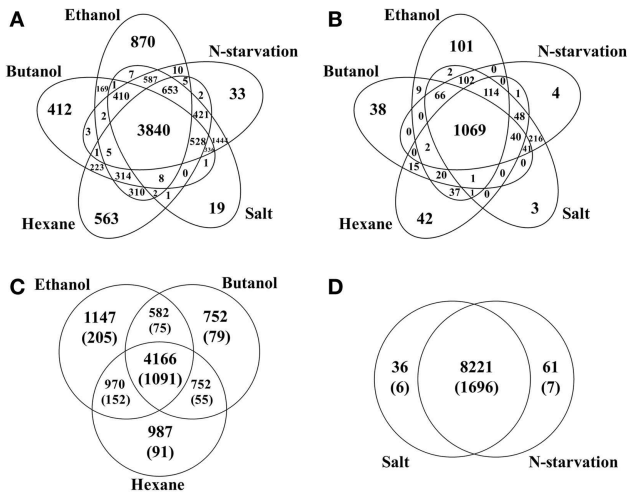

FIGURE 1 | Comparisons of peptides identified under different stress conditions. **(A)** Peptides distribution among conditions, including ethanol-, butanol-, hexane-, high salt, and nitrogen starvation treated samples. **(B)** Proteins distribution among conditions. **(C)** Peptides/proteins distribution among biofuels stress conditions. Protein-based information was provided inside parenthesis. **(D)** Peptides/proteins distribution between two environmental perturbations. Protein-based information was provided inside parenthesis.

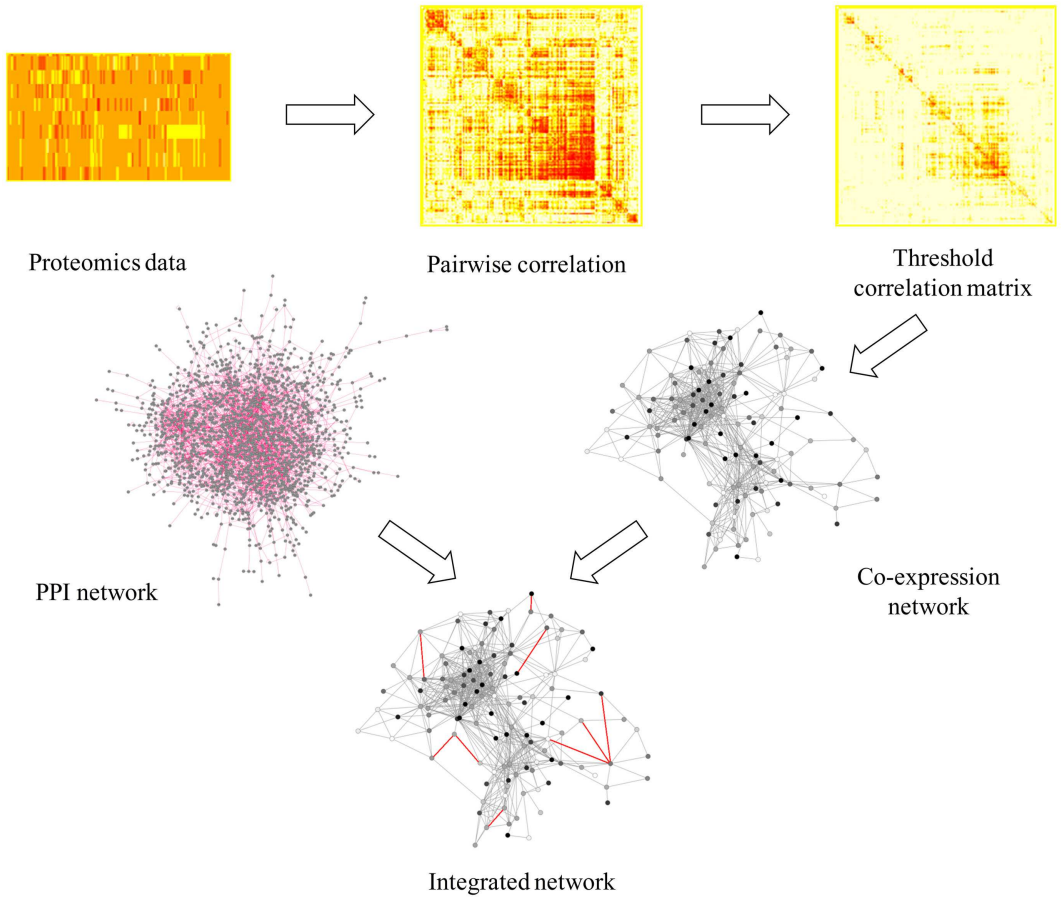

FIGURE 2 | A scheme of workflow to generate protein co-expression network. In integrated network, gray line represents co-expression information and red line represents PPI information.

correlation between betweenness of a node with its corresponding degrees (Goh et al., 2003).

FUNCTIONAL CHARACTERIZATION OF BOTTLENECK AND HUB PROTEINS

Early studies have found that common stress responses typically involve wide aspects of cell metabolism, including induction of oxidative stress response, heat shock proteins, efflux pumps, and accumulation of osmoprotective compounds (Nicolaou et al., 2010; Rutherford et al., 2010; Dunlop, 2011). As the hubs and bottlenecks identified from the integrated protein network are highly relevant to the stress responses, we conducted an enrichment analysis of bottlenecks and hubs among functional categories (**Figure 3A**). The results showed that both bottlenecks and hubs had a very similar pattern of being highly associated with several key functional categories, such as "[K] Transcription," "[L] Replication, recombination and repair," and "[O] Post-translational modification, protein turnover, chaperones." Meanwhile, the results also showed that the bottlenecks were highly associated with "[G] Carbohydrate transport and metabolism" functional category, while the hubs were highly associated with "[D] Cell cycle control, cell division, chromosome partitioning," "[N] Cell motility, " and "[U] Intracellular trafficking, secretion, and vesicular transport" functional categories.

Enrichments of the bottlenecks and hubs among several metabolic pathways were also observed (**Figure 3B**). Briefly, the enrichments are described below: (i) *ROS response pathway*: early studies showed organic solvent or environment stress induced production of reactive oxygen species (ROS) in many microbes (Houot et al., 2007; Stanley et al., 2010; Yang et al., 2010; Wang et al., 2013b). ROS accumulation could lead to DNA mutation, mRNA and protein denaturation, and membrane lipid peroxidation and ultimately cell death (Bhattacharya et al., 2004). Generation of antioxidants, such as superoxide dismutase (SOD) (Bhattacharya et al., 2004), glutaredoxin (Marteyn et al., 2013), carotenoids (Wilson et al., 2006), or tocopherols (Yang et al., 2008) that were capable of rapidly detoxifying ROS, has been considered as one of the key strategies to deal with stress in *Synechocystis* (Bhattacharya et al., 2004; Wilson et al., 2006; Yang et al., 2008; Marteyn et al., 2013). The results showed that the pathways related to antioxidants response, such as "Peroxisome" (ko04146) and "Glutathione metabolism" (ko00480), were enriched in higher TO proteins, consistent with the recent discovery that peroxiredoxins and glutathione-dependent peroxidase play major roles in combating

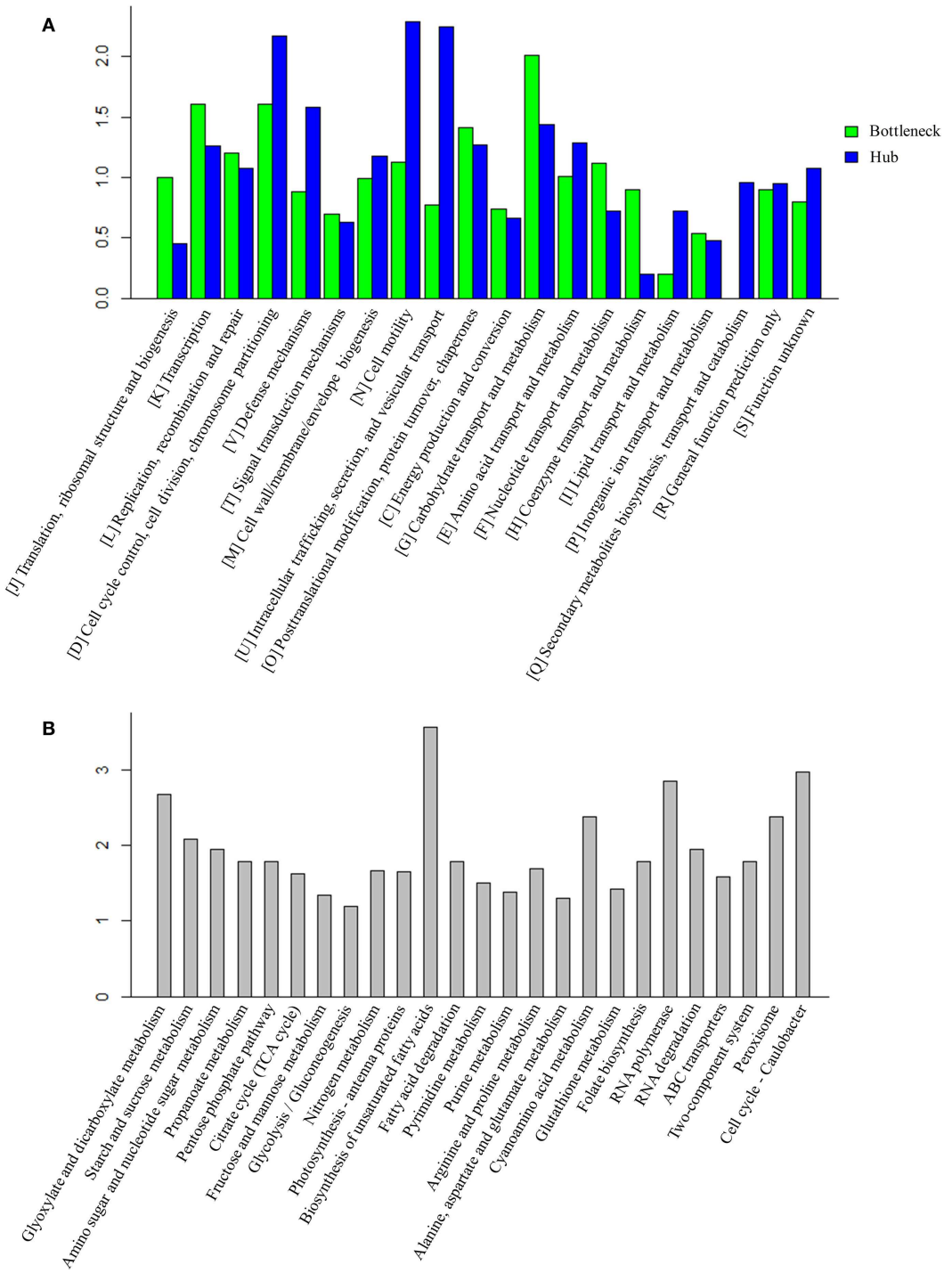

FIGURE 3 | Function analysis of bottlenecks and hubs proteins. **(A)** COG enrichment analysis of bottlenecks and hubs proteins. *X*-axis indicates the COG category and *Y*-axis indicates the ratio compared to background. **(B)** KEGG metabolic pathways enrichment analysis of bottlenecks and hubs proteins. Top 25 metabolic pathways are listed and ordered by function category. *X*-axis indicates the names of pathway and *Y*-axis indicates the ratio compared to background.

oxidative stress in cyanobacterium *Anabaena* (Banerjee et al., 2012). Peroxisomes could convert hydrogen peroxide to water and thus protect the microorganism from oxidative damage, while glutaredoxin could catalyze the reduction of protein disulfides and glutathione-protein mixed disulfides in a coupled system with glutathione, NADPH, and glutathione reductase (Li et al., 2005); (ii)

Transporters: transporters have been suggested as one important mechanism against solvent/biofuel toxicity in *Synechocystis* (Tian et al., 2013). For example, *slr1295* encoding an iron transport system substrate-binding protein was involved in butanol resistance (Zhu et al., 2013). In addition, a broad range of transporters with different substrate-specificities were also found involved in organic solvent tolerance in *E. coli* (Okochi et al., 2007). Moreover, transporters were also involved in tolerance to many environmental perturbations. For example, *ggtA* gene (*slr0747*) encodes a subunit of the transport system for the osmoprotective compound glucosylglycerol that is necessary for *Synechocystis* grown under salt stress (Hagemann et al., 1997). Our analysis found that "ABC transporters" (ko02010) was enriched in hubs and bottlenecks, suggesting that transporters play an essential role for cell survival when grown under a wide range of stresses; (iii) *Cell membrane permeability*: as a common resistance barrier against environmental stresses, changes of cell wall, or cell membrane composition can improve solvent tolerance to biofuels in many microbes (Ramos et al., 1997; Kajiwara et al., 2000; Zhao et al., 2003). In addition, an early study showed that unsaturation of fatty acids was associated with the ability of the photosynthetic machinery to tolerate salt stress in *Synechocystis* (Allakhverdiev et al., 1999). Consistent with this result, the network analysis also found that pathways "Biosynthesis of unsaturated fatty acids" (ko01040) and "Fatty acid degradation" (ko00071) were enriched in the bottlenecks and hubs.

WGCNA ANALYSIS TO DETERMINE THE BIOFUEL-SPECIFIC RESPONSES

To uncover biofuel-specific responses, we first converted the raw proteomic data into ratio data between the stress and the control conditions, and then used the log2 transformed ratio datasets for a PCA analysis. PCA score plot showed that almost all samples (i.e., different treatments, time points) were visibly separated, suggesting there are obvious differences in terms of the metabolic responses between various biofuels stress and environmental perturbations (**Figure 4A**). In addition, PCA score plot also revealed: (i) samples at 24 h of the middle-exponential phase tended to be clustered together, while samples at 48 h of the exponential-stationary transition phase were distinctly separated and became far away from the center when compared to those of 24 h, suggesting that more dramatic metabolic changes occurred after stress treatments of longer time; (ii) a greater separation along principal component 2nd between the biofuel-treated and environmental-treated samples was observed, and the biofuels-stressed profiles tended to be clustered together when compared to salt and nitrate starvation, suggesting there is a relatively high similarity between all biofuels-stressed samples than to environmental perturbations; (iii) a moving trend of the profiles along the principal component 1st seemed correlated with the carbon chain length of the biofuels tested in this study, although further proof is still needed; and (iv) finally, it was also observed that one of the salt treatment samples was clustered closely with biofuel-stressed samples, suggesting that just PCA analysis itself may not be enough to determine the biofuel-specific responses. Subsequently, we also performed a PLS-DA to further define differences between responses to various stresses. In the PLS-DA score plot, all biofuel samples are more tightly clustered together, completely separated with samples perturbed by environmental stresses (**Figure 4B**), indicating clear differences between the sample groups of biofuel and environmental stresses and suggestive of the different metabolic responses.

Weighted gene co-expression network analysis was employed as a method that can additionally define "modules" of co-expressed proteins explicitly and provide additional network statistics that describe the systems properties of metabolic networks (Langfelder and Horvath, 2008). The WGCNA analysis showed that a total of 17 metabolic modules were detected within the WGCNA co-expression networks of *Synechocystis*. Using a cut-off of correlation coefficients (r value > 0.55) and their confidence (p-values < 0.1), we found 5 out of 17 modules correlated with biofuels stress, among which 4 module's eigengenes were positively correlated while only 1 module eigengenes were negatively correlated with biofuels stress (**Figure 5**). A scatter plot of peptide significance versus module membership was plotted for these biofuel related modules (Additional Figure S1 in Supplementary Material), and the results also demonstrated high correlations between biofuel

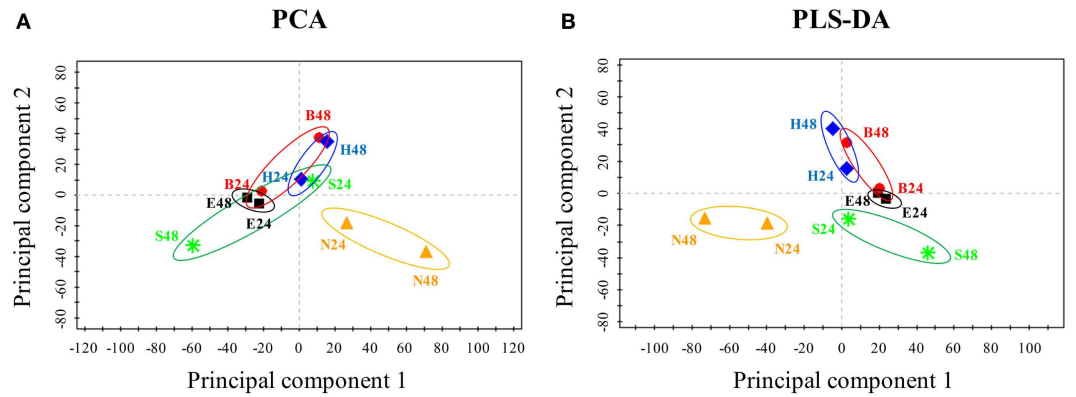

FIGURE 4 | Principal component analysis and PLS-DA score plot of the responses under various stress conditions in *Synechocystis*. (A) PCA score plot. Samples with different treatments were indicated by colors. *X*-axis, *Y*-axis were labeled with the first principal component and the second principal component, accounting for 33.4 and 16.3% total variation, respectively. **(B)** PLS-DA score plot.

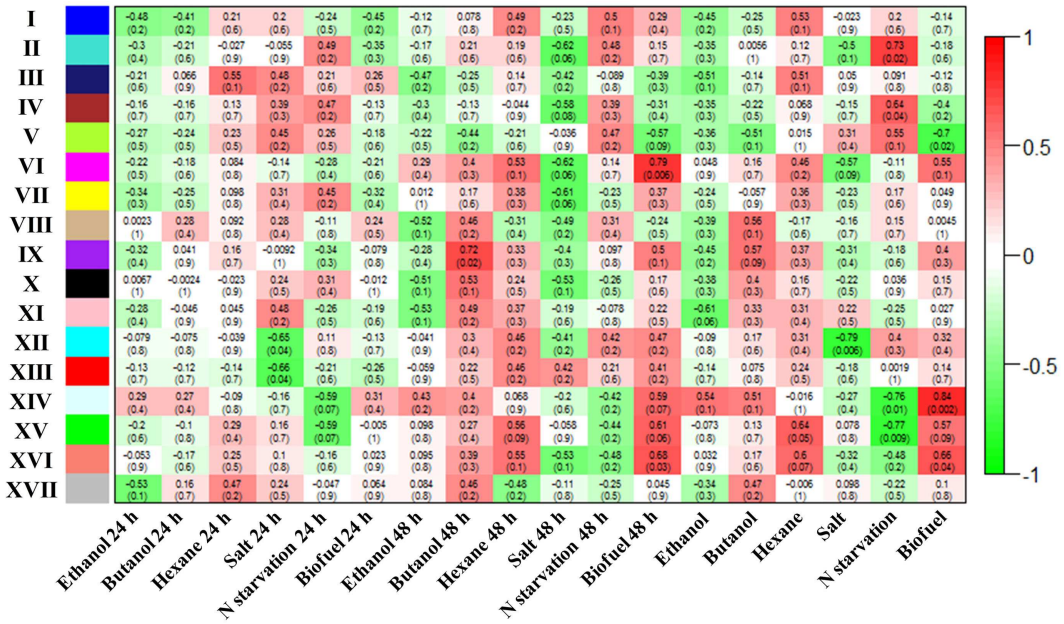

FIGURE 5 | Correlation between identified modules and stress conditions. Correlation of each module with conditions was indicated by colors. In addition, correlation efficient of modules with conditions are provided for each module, and the *p*-values are provided inside parenthesis.

and the respective module eigengenes. In contrast, the background peptides (module XVII) showed no correlation with any biofuel stress. Among all modules positively correlated to biofuels stress, module VI eigengenes were negatively correlated with salt stress; module XIV and XV eigengenes were negatively correlated with nitrate starvation; and module XVI eigengenes were negatively correlated with both salt and nitrate starvation stress.

Topological overlap is a similarity metric that incorporates information from neighboring nodes, making it robust to noisy correlations. TOM is a robust and biologically meaningful measurement that encapsulates the similarity of a co-expression relationship with all others in the network (Langfelder and Horvath, 2008). Within the network constructed by the WGCNA approach, we first trimmed the network with a moderate threshold, removing low-quality connections with strengths between nodes less than TO value of 0.1. The results showed that, even after the threshold removal, most of the connections were still kept in the network; for example, the XV module was only reduced to 250 nodes from the initial 264 nodes (data not shown). Topological analysis was then conducted to determine the top 10 hub and bottleneck peptides for each biofuel-correlated modules. The result showed most peptides with higher topological attributes were related to proteins of photosynthesis functions in the biofuel-correlated modules (data not shown).

We also determined the top one or two hub peptides with the greatest connectivity in each module, which were supposed to be biologically important under a more stringent threshold (**Figure 6**). In the XV module positively correlated with biofuels stress, a hub peptide "AITTAASR" from *sll1580* encoding "phycocyanin associated linker protein" was connected with peptides from proteins of the same operon, including *sll1577* "phycocyanin subunit B," *sll1578* "phycocyanin a subunit," and *sll1579* "phycocyanin associated linker protein." In the XIV module positively correlated with biofuels stress, two hub peptides, "TVVPANPLVQMK" and "VALVGDAAGTVTK" were identified. They were matched to the same Sll1091 protein of "a bacteriochlorophyll synthase subunit" and connected with a peptide from *sll1471* encoding "phycobilisome rod-core linker polypeptide." Together, the results suggested that light-harvesting proteins could be key components involved in biofuels stress response. Interestingly, one peptide "LTYYTPDYTPK" from Slr0009 of "ribulose bisphosphate carboxylase" that catalyzes the first reaction of CO_2 fixation was also identified as a hub peptide in the VI module positively correlated with biofuels stress. In addition, a hub peptide "VFNQYTELFSVGDLAQMVQK" of Slr1020 in the XVI module positively correlated with biofuels stress was connected to several peptides of proteins related to carbon fixation, such as Sll1342, Slr0394, and Sll1070. The finding of proteins related to CO_2 fixation function as important hub peptides under biofuels stress implicated that CO_2 metabolism could also be important in dealing with biofuels stress in cyanobacteria.

PATHWAY ENRICHMENT ANALYSIS OF BIOFUEL-SPECIFIC MODULES

For the peptides/proteins located in the biofuel-associated modules, we carried out an enrichment analysis for their distribution among functional categories and metabolic pathways. The detailed enrichment information for all COG and top 25 KEGG pathways is provided in Additional Files S3 and S4 in Supplementary Material. The results showed that several functional categories were significantly enriched, such as functional categories of "[I] Coenzyme transport and metabolism," "[G] Carbohydrate transport

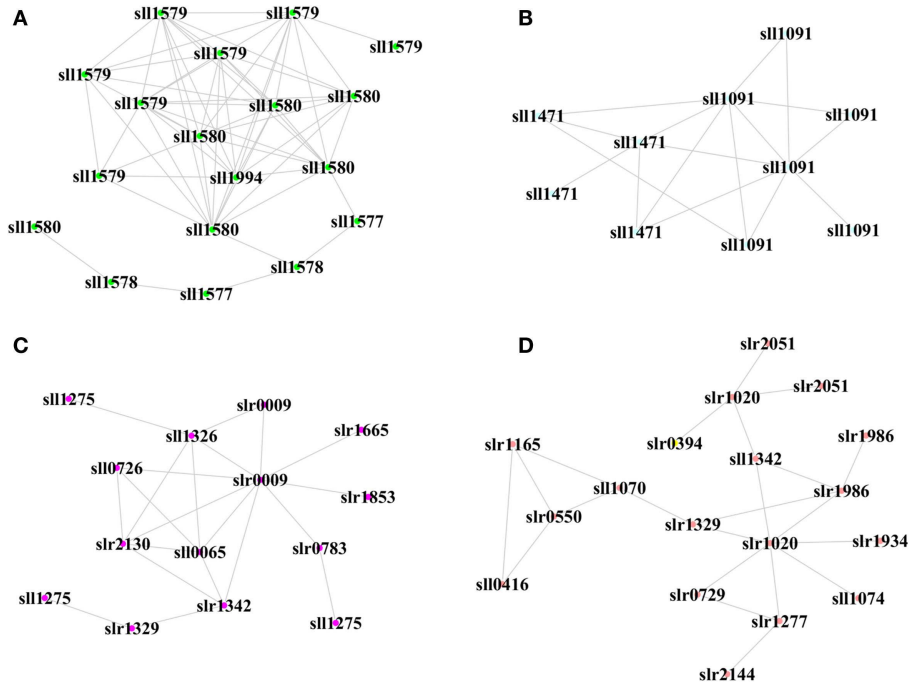

FIGURE 6 | Hub proteins and their neighbor proteins in biofuel-responsive modules. **(A)** Hub peptide from Sll1580 "phycocyanin associated linker protein" in the XV module. **(B)** Hub peptide from Sll1091 "43 kD bacteriochlorophyll synthase subunit" in the XIV module. **(C)** Hub peptide from Slr0009 "ribulose-bisphosphate carboxylase" in the VI module. **(D)** Hub peptide from Slr1020 "sulfolipid biosynthesis protein SqdB" in the XVI module. Each node represents a peptide from a protein.

and metabolism," "[C] Energy production and conversion," and "[M] Cell wall/membrane/envelope biogenesis" (**Figure 7A**).

The KEGG pathway enrichment analysis showed that "Lipopolysaccharide biosynthesis" (ko00540), "Photosynthesis – antenna proteins" (ko00196), "Porphyrin and chlorophyll metabolism" (ko00860), and "Carbon fixation in photosynthetic organisms" (ko00710) were significantly enriched (**Figure 7B**). Structures and components of the cell wall are known to be affected by solvents/biofuels (Nicolaou et al., 2010; Dunlop, 2011; Jin et al., 2014). Early analysis of cell morphology found visible cell aggregation under biofuel treatments (Liu et al., 2012; Qiao et al., 2012; Tian et al., 2013), while the aggregation was not obvious under environmental stress (Huang et al., 2013; Qiao et al., 2013). Consistently, an early study found that the mutants of *E. coli* with hyper tolerance under organic solvent stress tend to increase lipopolysaccharide content (Aono and Kobayashi, 1997). Phycobilisomes and phycocyanins are the most abundant proteins and the major light-harvesting antennae for photosynthesis in cyanobacteria (Nakamoto and Honma, 2006) and are directly attached to the thylakoid membranes, where they absorb photons and efficiently transfer excitation energy to the photosynthetic reaction centers (Nakamoto and Honma, 2006). Twelve of 13 identified proteins in pathway "Photosynthesis – antenna proteins" (ko00196), and 17 of 37 proteins in pathway "Photosynthesis" (ko00195) were up-regulated, respectively. In addition, two other photosynthesis related pathways, "Porphyrin and chlorophyll metabolism" (ko00860) and "Terpenoid backbone biosynthesis" (ko00900) that is a precursor biosynthetic pathway of accessory pigment-carotenoid, were also up-regulated specifically in response to biofuels. In early studies, carotenoid biosynthesis has been found up-regulated by strong light stress in *Synechococcus elongatus* PCC 7942 (Schafer et al., 2006) and in stress-tolerant mutants of *Haematococcus pluvialis* (Sandesh Kamath et al., 2008). Moreover, Slr1225 protein of phytoene synthase involved in carotenoid biosynthesis was also identified in the V module positively correlated to biofuels stress. Similarly, 11 of 16 proteins in "Carbon fixation in photosynthetic organisms" (ko00710) pathway were also up-regulated by the biofuel. These results were consistent with early studies that showed dissociation of phycobilins from the thylakoids in *Anabaena* P-9 under external free fatty acids biotic stress (Wu et al., 2006) and *S. elongatus* PCC 7942 (Ruffing and Jones, 2012), and the proteins associated with photosynthesis, especial PSII, up-regulated during free fatty acids production in *S. elongatus* PCC 7942 (Ruffing, 2013).

The KEGG pathway enrichment showed amino acid metabolism related pathways, including "Glycine, serine, and threonine metabolism" (ko00260), "Cysteine and methionine metabolism" (ko00270), "Lysine biosynthesis" (ko00300), and "Valine, leucine, and isoleucine biosynthesis" (ko00290) were significantly enriched (**Figure 7B**). The roles of amino acid in stress resistance was previously reported in *E. coli*, in which lysine, tryptophan, leucine, isoleucine, and valine were found related to resistance to acid and various biofuel products (Diez-Gonzalez and Karaibrahimoglu, 2004; Horinouchi et al., 2010; Wang et al., 2014), and the addition

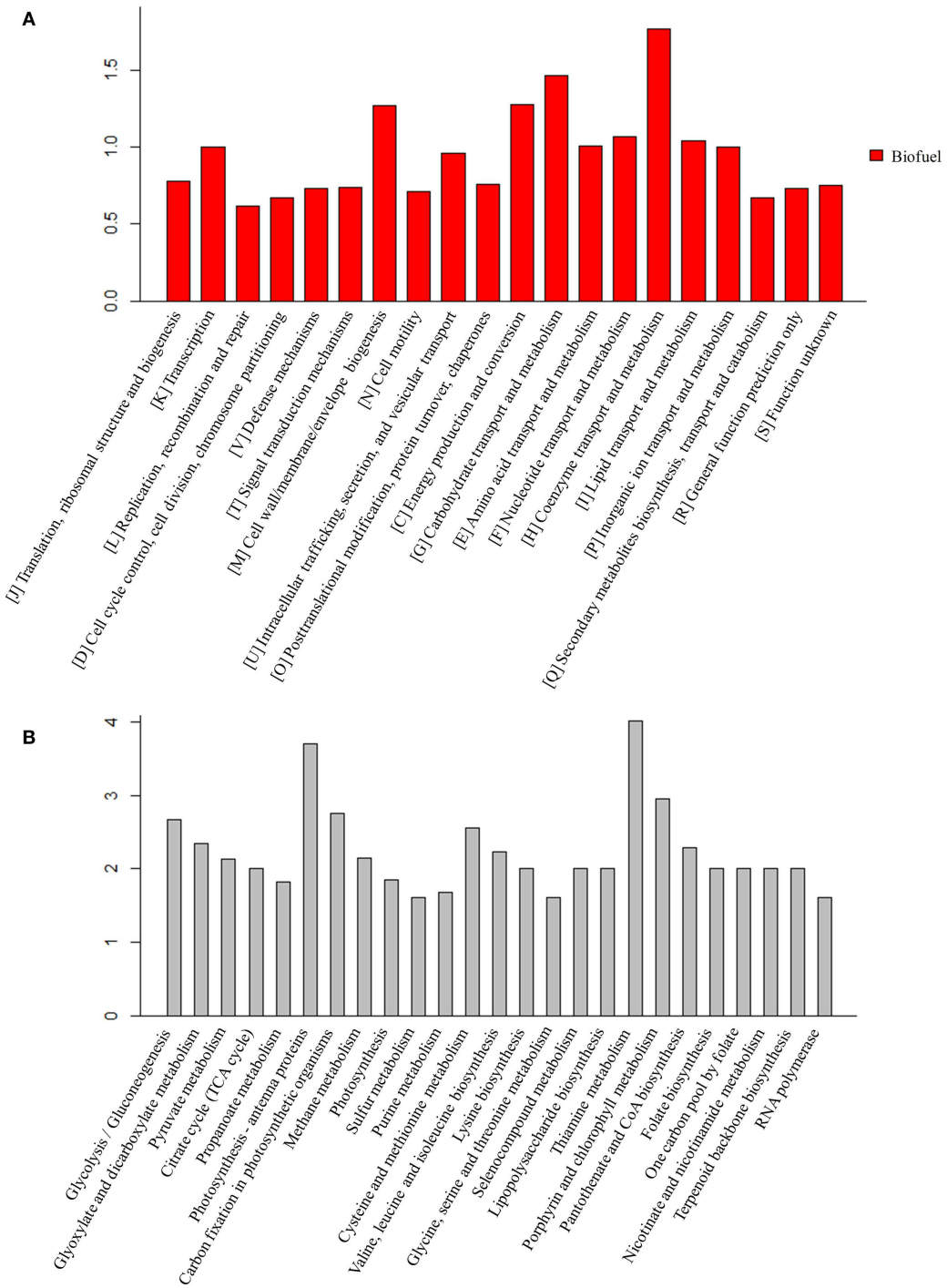

FIGURE 7 | Enrichment analysis of the proteins identified in biofuel-responsive modules. **(A)** COG enrichment analysis of proteins in biofuel-responsive modules. *X*-axis indicates the COG category and *Y*-axis indicates the ratio compared to background. **(B)** KEGG metabolic pathways enrichment analysis of proteins in biofuel modules. Top 25 metabolic pathways are listed and ordered by function category. *X*-axis indicates the names of pathway and *Y*-axis indicates the ratio compared to background.

of isoleucine can improve ethanol tolerance of *E. coli* (Horinouchi et al., 2010). It may be worth to determine whether similar roles are also played by amino acids in cyanobacteria.

The KEGG pathway enrichment showed carbohydrate metabolism related pathways, such as "Pyruvate metabolism" (ko00620), "Glycolysis" (ko00100), "Citrate cycle" (ko00020), and related

cofactors metabolism pathway "Thiamine metabolism" (ko00730) were also enriched (**Figure 7B**). Enrichment of "Glycolysis" (ko00100) was probably due to the fact that most of its proteins are the same as "Carbon fixation in photosynthetic organisms" (ko00710). In addition, although proteins functioning in "Pyruvate metabolism" were up-regulated under biofuels stress, no enzyme after acetyl-CoA in the citrate cycle was found in the biofuel-responsive modules, implying acetyl-CoA was probably more directed into fatty acid biosynthesis rather than the citrate cycle under biofuel stress conditions.

CONCLUSION

Although synthetic biology technologies have improved biofuel production significantly in photosynthetic cyanobacteria, current biofuels productivity in these renewable systems is still very low (Oliver and Atsumi, 2014). Meanwhile, it becomes clear that toxicity of the end-product biofuels to cyanobacterial cells may represent a major hurdle for further improving the efficiency and productivity of the processes. For rational construction of high-tolerant *chassis* (Alper et al., 2006), the knowledge on molecular mechanisms responsive to biofuels stress is necessary (Baer et al., 1987; Atsumi et al., 2010). To seek a better understanding of the biofuel-tolerance mechanisms, in this study, using the proteomic datasets collected from several previous studies (Liu et al., 2012; Qiao et al., 2012, 2013; Huang et al., 2013; Tian et al., 2013), we applied network-based methodologies to compare the stress responses induced by three biofuels stress and two environmental perturbations in *Synechocystis*, as the network-based strategy has the advantages of identifying low abundance or small changes and stress-specific response proteins (Singh et al., 2010; McDermott et al., 2011; Wang et al., 2013b). The comparison allowed identification of a set of common responsive proteins to all perturbations, many of which were identical to the core transcriptional genes determined previously in *Synechocystis* (Singh et al., 2010). In addition, the analysis revealed proteins related to cell surface lipopolysaccharide modification, photosynthesis (i.e.,

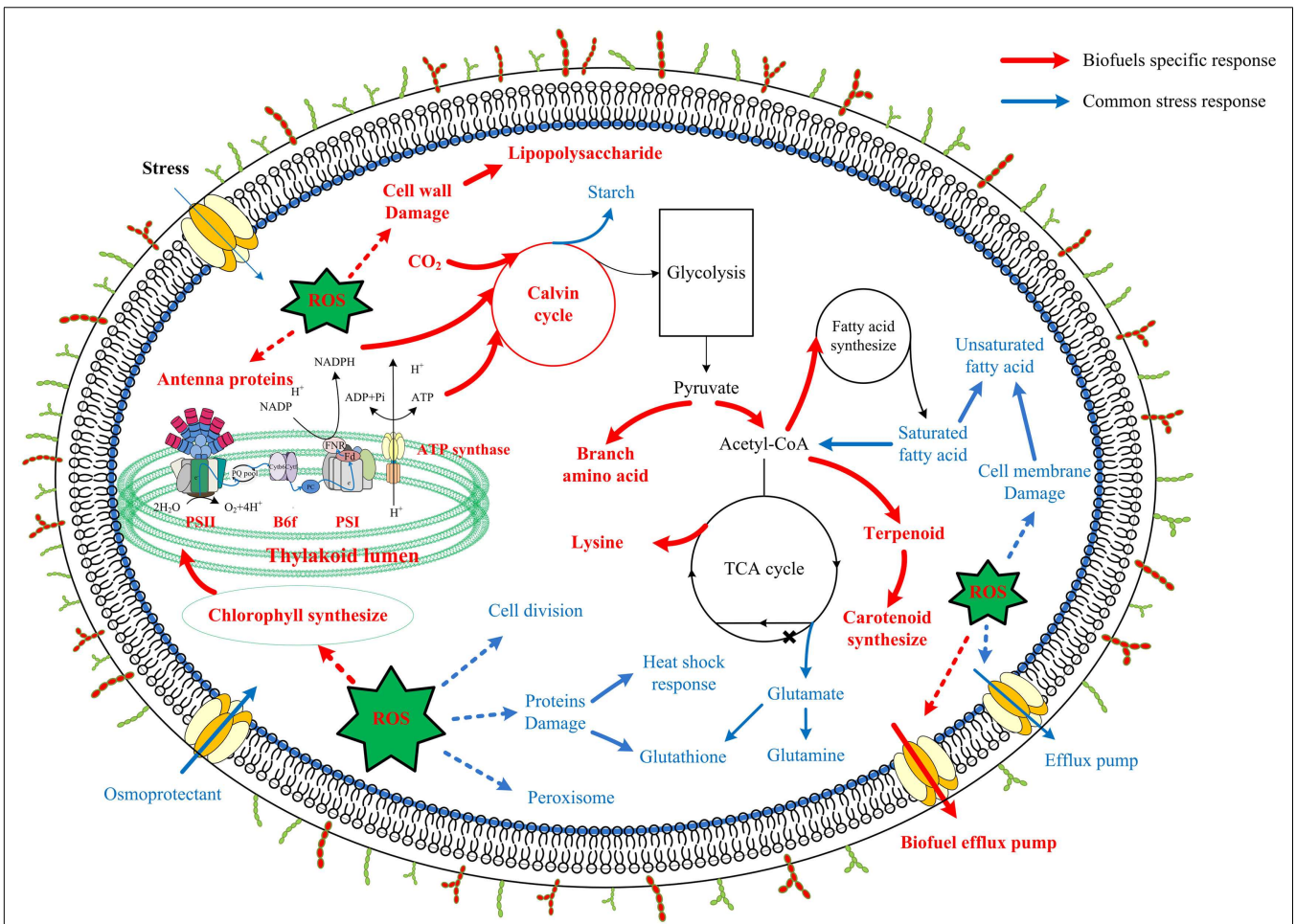

FIGURE 8 | Scheme of biofuel-responsive signatures in *Synechocystis*. The metabolic features presented were deduced either from proteins integrated network as common stress responses (blue) or from WGCNA analysis as biofuel-specific responses (red). Processes associated with photosynthesis, central carbohydrate metabolism, lipolysis and fatty acid metabolism, amino acid metabolism, ROS stress responses, as well as cell surface structure modification are indicated and labeled. Relevant transport systems are shown within the cell membrane as well. *According to a recent study in *Synechococcus*, function of 2-oxoglutarate dehydroase in citrate cycle was finished by Sll1981 protein of 2-oxoglutarate decarboxylase and Slr0370 protein of a succinic semialdehyde dehydrogenase (Zhang and Bryant, 2011; Xiong et al., 2014).

photosynthetic pigments, light-harvesting, and carbon fixation), and branch amino acid biosynthesis could be specific responses to biofuels. A scheme of metabolic signatures associated with exogenous biofuels treatments in *Synechocystis* was presented in **Figure 8**. Briefly, the biofuel-responsive signatures of *Synechocystis* may include enhanced activities associated with transporters, photosynthesis, CO_2 fixation, ROS detoxification proteins, and some amino acid and acetyl-CoA biosynthesis for fatty acid. The study provided a better view of metabolic responses caused by the biofuels stress, and also demonstrated that the network-based approach is a powerful tool to identify important target proteins responsive to biofuels stress.

AUTHOR CONTRIBUTIONS

Guangsheng Pei, Lei Chen, Jiangxin Wang, Jianjun Qiao, and Weiwen Zhang conceived of the study. Guangsheng Pei carried out the data analysis. Guangsheng Pei and Weiwen Zhang drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fbioe.2014.00048/abstract

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Engineering limonene and bisabolene production in wild type and a glycogen-deficient mutant of *Synechococcus* sp. PCC 7002

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The plant terpenoids limonene ($C_{10}H_{16}$) and α-bisabolene ($C_{15}H_{24}$) are hydrocarbon precursors to a range of industrially relevant chemicals. High-titer microbial synthesis of limonene and α-bisabolene could pave the way for advances in *in vivo* engineering of tailor-made hydrocarbons, and production at commercial scale. We have engineered the fast-growing unicellular euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 to produce yields of 4 mg L^{-1} limonene and 0.6 mg L^{-1} α-bisabolene through heterologous expression of the *Mentha spicata* L-limonene synthase or the *Abies grandis* (E)-α-bisabolene synthase genes, respectively. Titers were significantly higher when a dodecane overlay was applied during culturing, suggesting either that dodecane traps large quantities of volatile limonene or α-bisabolene that would otherwise be lost to evaporation, and/or that continuous product removal in dodecane alleviates product feedback inhibition to promote higher rates of synthesis. We also investigate limonene and bisabolene production in the Δ*glgC* genetic background, where carbon partitioning is redirected at the expense of glycogen biosynthesis. The *Synechococcus* sp. PCC 7002 Δ*glgC* mutant excreted a suite of overflow metabolites (α-ketoisocaproate, pyruvate, α-ketoglutarate, succinate, and acetate) during nitrogen-deprivation, and also at the onset of stationary growth in nutrient-replete media. None of the excreted metabolites, however, appeared to be effectively utilized for terpenoid metabolism. Interestingly, we observed a 1.6- to 2.5-fold increase in the extracellular concentration of most excreted organic acids when the Δ*glgC* mutant was conferred with the ability to produce limonene. Overall, *Synechococcus* sp. PCC 7002 provides a highly promising platform for terpenoid biosynthetic and metabolic engineering efforts.

Keywords: terpenoids, limonene, bisabolene, cyanobacteria, glycogen, *glgC*, metabolic sink

INTRODUCTION

In recent years, cyanobacteria have been successfully used as a platform to generate a range of commercially significant products, including isoprene (Lindberg et al., 2010; Bentley and Melis, 2012; Bentley et al., 2014), isobutanol (Atsumi et al., 2009), 2,3-butanediol (Oliver et al., 2013, 2014), and ethylene (Ungerer et al., 2012). In all cases, photoassimilated carbon was drawn from native metabolic pathways through the heterologous expression of one or more enzymes to create new carbon sinks. While demonstrating the proof-of-principle, these strategies are unlikely to attain high yields of novel end-products as carbon partitioning and regulation are hardwired into the organism's central metabolic networks. For cyanobacteria, one of the main metabolic engineering bottlenecks is the ability to redirect carbon from the major glycogen and protein sinks to pathways of interest. A careful rewiring of central metabolism is required to increase product yield at the expense of biomass accumulation, without negatively influencing photosynthesis and carbon fixation rates.

In the context of photoautotrophic biotechnology, the terpenoid biosynthetic pathway is of particular interest, as it produces the largest and most diverse array of naturally occurring organic compounds (typically of plant origin) (Davies et al., 2014). The plant terpenoids limonene ($C_{10}H_{16}$) and bisabolene ($C_{15}H_{24}$) are recognized as precursors to a range of commercially valuable products, with applications in biofuels, bioplastics, pharmaceutical, nutraceutical, and cosmetic industries (Duetz et al., 2003; Peralta-Yahya et al., 2011). In this study, we engineer the fast-growing euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 for the production of limonene and α-bisabolene through heterologous expression of the *Mentha spicata* L-limonene synthase and *Abies grandis* (E)-α-bisabolene synthase genes. In cyanobacteria, terpenoids are synthesized via the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, utilizing photosynthetically generated pyruvate and glyceraldehyde-3-phosphate (GAP) as the primary substrates (**Figure 1**). Limonene and bisabolene represent new carbon sinks that draw from the native terpenoid pathway at the levels of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP).

The two major competing pathways for cyanobacterial terpenoid production are protein and glycogen (the cyanobacterial

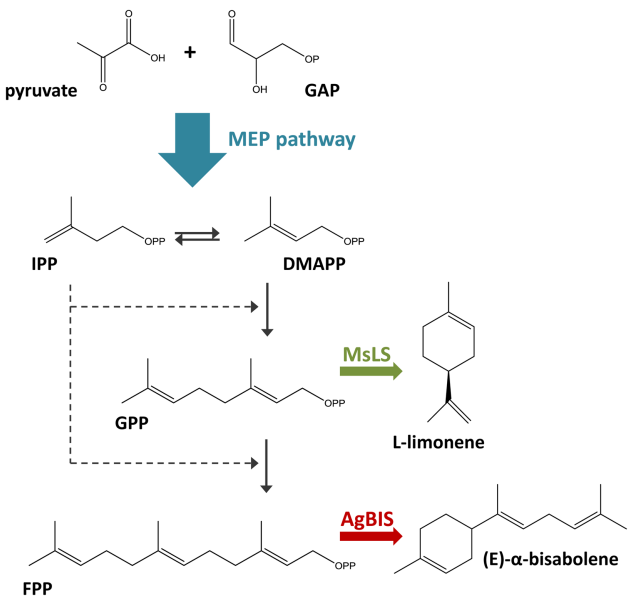

FIGURE 1 | Terpenoid biosynthesis via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MEP terpenoid biosynthetic pathway converts pyruvate and glyceraldehyde-3-phosphate (GAP) feedstock to the C5 isomers, isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP). The stepwise addition of IPP to DMAPP generates C10 geranyl pyrophosphate (GPP) and C15 farnesyl pyrophosphate (FPP), the respective precursors to monoterpenes and sesquiterpenes. In this study, the heterologous expression of the *M. spicata* L-limonene synthase (MsLS) and *A. grandis* (E)-α-bisabolene synthase (AgBIS) in *Synechococcus* sp. PCC 7002 was designed to draw on GPP and FPP pools, respectively, generated by the native cyanobacterial MEP pathway for the production of the non-native terpenoids L-limonene and α-bisabolene.

storage carbohydrate) biosynthesis. Recent studies in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 have demonstrated that upon simultaneous inactivation of protein and glycogen sinks (*via* nitrogen-deprivation in the Δ*glgC* glycogen-deficient mutant), a suite of organic acids are excreted due to the resulting metabolic imbalance (Carrieri et al., 2012; Grundel et al., 2012; Hickman et al., 2013). In our study, we sought to elucidate how such major shifts in flux distribution will affect the overall metabolic partitioning toward the terpenoid pathway in *Synechococcus* sp. PCC 7002, using the newly engineered limonene and α-bisabolene terpenoid sinks as the reporting products.

MATERIALS AND METHODS

CLONING AND PLASMID CONSTRUCTION

The *E. coli* DH5α strain was used for routine subcloning according to standard protocols. The (−)-4*S*-limonene synthase gene from *M. spicata* (GenBank accession Q40322) (Colby et al., 1993) was optimized for codon-usage in *Synechococcus* sp. PCC 7002 (DNA 2.0, USA) without the predicated chloroplast transit peptide (cTP), and is referred to in this work as MsLS. The first 168 bases of Q40322 encoding the cTP were replaced with an ATG codon for translation initiation in *Synechococcus* sp. PCC 7002, retaining residues R58/R59 that are required for catalytic activity (Williams et al., 1998). The (*E*)-α-bisabolene synthase gene from *A. grandis* (GenBank accession O81086) (Bohlmann et al., 1998; Trapp and Croteau, 2001) was also optimized for codon-usage in *Synechococcus* sp. PCC 7002 (DNA 2.0, USA), and is referred to as AgBIS. Oligonucleotide primers used to amplify these codon-optimized genes are as follows (introduced restriction sites for cloning are indicated in bold, and lower case letters represent start or stop codons): MsLS, MsLS_**NcoI**_F, 5′-CTTCGCTGAA**CCatgG**AACGTCGCTC-3′, and MsLS_**BamHI**_R, 5′-TGACG**GGATCC**ttaAGCGAAGG-3′; AgBIS, AgBIS_**NcoI**_F, 5′-AGATTAATT**CCatgG**CCGGTGTGA-3′; and AgBIS_**BamHI**_R, 5′-GCC**GGATCC**ttaTAACGGCAAC-3′.

A neutral site (NSI) for the integration of transgenes was identified in the *Synechococcus* sp. PCC 7002 genome between two open-reading frames encoding hypothetical proteins (SYNPCC7002_A0935 and SYNPCC7002_A0936), which we have termed NSI. This was used as the site of integration for the limonene synthase (MsLS) and α-bisabolene synthase (AgBIS) genes. Oligonucleotide primers designed to amplify 750 bp up-stream (us) and down-stream (ds) regions flanking NSI, which were used for double homologous recombination in *Synechococcus* sp. PCC 7002 are as follows (introduced restriction sites are indicated in bold): NSIus, 935us_**NsiI**_F, 5′-AGTTCAC**ATGCAT**AAAGTCAA-3′, and 935us_**EcoRI**_R, 5′-CGTATAG**GAATTC**TTACTCAG-3′; NSIds, 936ds_**SalI**_F, 5′-GCATACT**GTCGAC**CTATTTTAT-3′; and 936ds_**SphI**_R, 5′-AGTTGAC**GCATGC**AGAGGTGG-3′. These regions were cloned into a pAQ1 plasmid containing the *Synechocystis* sp. PCC 6803 *cpcBA* (*cpc*) promoter (Xu et al., 2011). The MsLS and AgBIS transgenes, along with a spectinomycin-resistance cassette (SmR), were cloned between the NSI-flanking regions immediately downstream of the *cpc* promoter, generating plasmids pNSI-cpc-MsLS-SmR and pNSI-cpc-AgBIS-SmR. To demonstrate that any phenotypes arising from the expression of MsLS or AgBIS were not a direct result of interference with the NSI region of *Synechococcus* sp. PCC 7002 genomic DNA, we also constructed the control plasmid pNSI-cpc-YFP-SmR, containing a YFP gene in place of the MsLIM or AgBIS terpene synthase (TPS) genes.

CYANOBACTERIAL STRAINS, GROWTH CONDITIONS, AND TRANSFORMATION

Wild type and transformant *Synechococcus* sp. PCC 7002 strains were maintained on solid A^+ media (Stevens et al., 1973) supplemented with 8.25 mM Tris–HCl (pH 8.2) and 0.3% sodium thiosulfate. Where appropriate, spectinomycin was used at a concentration of 50 $\mu g\,mL^{-1}$ and kanamycin at 100 $\mu g\,mL^{-1}$. Liquid cultures were grown in A^+ media supplemented with 8.25 mM Tris–HCl (pH 8.2). For nitrogen-deprivation, $NaNO_3$ was omitted from the growth media and replaced mol:mol with NaCl. Nitrogen-deplete media is referred to as $A^+(-N)$. Cultures were grown in 250 mL Erlenmeyer flasks on an orbital shaker at 37°C, in an incubator with an atmosphere of 1% CO_2 in air, and constant illumination at 250 $\mu mol\ photons\ m^{-2}\ s^{-1}$ of photosynthetically active radiation (PAR). Liquid starter cultures were grown in the presence of antibiotics; however, during experimental procedures antibiotics were eliminated. Transformation of *Synechococcus* sp. PCC 7002 with the plasmid constructs described above was performed

according to established procedures for cyanobacteria (Eaton-Rye, 2011) to generate the strains LS (containing MsLS) and BIS (containing AgBIS). Complete chromosomal segregation for the introduced transgenes was achieved through propagation of multiple generations on selective agar and verified by colony-PCR using the primers: NSI_us_F, 5′-CTAGCACAAATGAAGCCCGAC-3′, and NSI_ds_R, 5′-GCAGATATAAGCAACGGTACAG-3′ (**Figure 2**). The Δ*glgC* strain was obtained from D. A. Bryant, which was generated via the insertional disruption of the *glgC* open reading frame (SYNPCC7002_A0095) with a kanamycin-resistance cassette (KmR) (Guerra et al., 2013). Oligonucleotide primers used to verify *glgC* disruption in transformant strains are: glgC_F, 5′-TCACGTAGTCGGGTTTGATGTC-3′, and glgC_R, 5′-CACTAAAGTCCACGACACGACC-3′. The Δ*glgC* strain was also transformed with the MsLS and AgBIS plasmid constructs to generate strains Δ*glgC*:LS and Δ*glgC*:BIS, respectively.

TERPENOID EXTRACTION AND QUANTIFICATION

Synechococcus cultures used for terpenoid production assays were grown photoautotrophically in 100 mL batches, with an inoculation density of $OD_{730\,nm} = 2.0$. Where indicated, a 5-mL dodecane (analytical grade, Sigma-Aldrich, USA) overlay was applied to the culture at $t = 0$ to trap terpenoids during the 96-h experimental time course. At 96 h, cells were harvested by centrifugation to separate the biomass from the media. Terpenoid extraction was performed on the cell pellet using the chloroform:methanol:water biphasic separation protocol described by Bligh and Dyer (1959). To determine if any accumulated terpenoids had partitioned to the media or adhered to the flask walls, the supernatant was returned to the original Erlenmeyer flask and a 5-mL dodecane overlay applied for 1 h for terpenoid extraction.

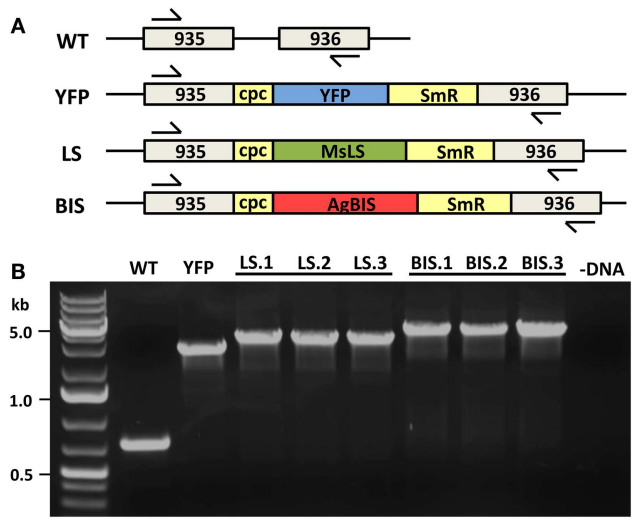

FIGURE 2 | MsLS and AgBIS transgene integration to the *Synechococcus* chromosome. **(A)** The YFP, MsLS, and AgBIS transgene constructs were introduced to a neutral site (NSI) of genomic DNA (between open-reading frames A0935 and A0936) via double homologous recombination. Constructs contained the *Synechocystis* sp. PCC 6803 *cpcBA* (*cpc*) promoter to drive transgene expression, and a spectinomycin-resistance selectable marker (SmR). Arrows indicate the position of genomic DNA primers used to check for integration and complete chromosomal segregation in transformant lines. **(B)** Verification of compete chromosomal segregation for the introduced transgenes in YFP, LS, and BIS transformants by colony-PCR using the above-mentioned primers. Three independent transformant lines of LS (LS.1, LS.2, and LS.3) and BIS (BIS.1, BIS.2, and BIS.3) were verified.

Aliquots of 200 μL were harvested from the dodecane overlay at each experimental time point. These samples, and the chloroform extracts from the cell pellet, were analyzed directly using 1 μL of sample injected into a GC-FID (Agilent 7890A) equipped with a DB-5-ms column (Agilent Technologies, Santa Clara, CA, USA; 30 m × 0.25 mm; 0.25 μm film thickness) and operated using the temperature program: 60–310°C (at 12°C min^{-1}), and H_2 carrier gas at 1.5 mL min^{-1}. Quantification of limonene or α-bisabolene from the dodecane or chloroform extracts was performed using standard curves constructed by GC peak integration of serial dilutions of limonene (Sigma-Aldrich, USA) and bisabolene (Alfa Aesar, USA) standards in the appropriate solvent. Due to the reverse solvent effect (Grob, 1983; Grob and Schilling, 1983), limonene was observed as a broad peak on the chromatogram when mixed with dodecane. Dilution of the sample with a solvent of lower molecular weight than the solute typically eliminates this problem. However, dilution pushed limonene below the limits of detection in some samples, and so limonene peaks were integrated using the area measured under the broad limonene peak. The bisabolene standard contained a number of discrete peaks, and ~40% of the total combined peak areas were attributed to other sesquiterpenes with different retention times relative to α-bisabolene. The weight contribution by α-bisabolene was adjusted accordingly for the standard curves. Mass spectral analysis was conducted using a Varian 3800 GC and Varian 1200 quadrupole MS/MS equipped with a Rxi-5ms column (30 m × 0.25 mm; 0.25 μm film thickness); temperature program: 60–310°C (at 12°C min^{-1}); He carrier gas at 1.2 mL min^{-1}; mass spectra, 70 eV, EI mode; ion source temperature 215°C; scan mass range, 18–500.

EXCRETED ORGANIC ACID MEASUREMENTS

Synechococcus cultures used for excreted metabolite assays were grown photoautotrophically in 50 mL batches with an inoculation density of $OD_{730\,nm} = 2.0$. Cells were washed twice with $A^+(-N)$ media to remove media nitrogen before inoculation in either nitrogen-replete or nitrogen-deplete media for experimentation. Aliquots of cell suspension (1 mL) were removed from the culture at appropriate time points and centrifuged to pellet cell biomass. The resulting supernatant was filtered through a 0.45-μm silicon membrane and the filtrate used directly for high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. A Surveryor Plus (Thermo Scientific, Waltham MA) HPLC equipped with an Aminex fermentation monitoring column [(150 mm × 7.8 mm); Bio-Rad, Hercules, CA, USA] was used for HPLC analyses. 25 μL of filtrate was injected for isocratic elution using 8 mM H_2SO_4 as the mobile phase with a flow rate of 0.5 mL min^{-1} and a column operating temperature of 45°C. A refractive index (RI) detector operating at 50°C was used for metabolite identification and quantification. Metabolite peaks were integrated and quantified using standard curves constructed from serial dilutions of authentic standards

(Sigma-Aldrich, USA). Proton NMR spectra were generated with a JEOL ECA 500 MHz spectrometer; 128 scans were used with water-suppression.

BIOMASS, CHLOROPHYLL *a*, CARBOHYDRATE, AND WHOLE CELL ABSORPTION SPECTRA MEASUREMENTS

Cyanobacterial biomass accumulation was measured as dry cell weight (DCW), where 2 mL samples of culture were pelleted and washed with 8.25 mM Tris–HCl (pH 8.2) to remove any residual salt from the growth medium. Pellets were then resuspended in 2 mL 8.25 mM Tris (pH 8.2), and dried overnight at 80°C in pre-weighed aluminum dishes prior to measuring the DCW. The dry weight of 2 mL 8.25 mM Tris (pH 8.2) solution (without cells) was subtracted from the cell dry weight to eliminate Tris buffer contributions. Chlorophyll *a* concentrations in cell suspensions were determined spectrophotometrically in 90% methanol extracts according to Meeks and Castenholz (1971). Total reducing sugars (in glucose equivalents) were quantified by the anthrone assay, as described by Meuser et al. (2011), as a measure of the carbohydrate content of cells. For whole cell absorption spectra, cell suspensions were normalized to an $OD_{730\,nm}$ of 0.3 and measured spectrophotometrically (400–800 nm).

RESULTS

CONSTRUCTION OF *SYNECHOCOCCUS* sp. PCC 7002 STRAINS CARRYING MsLS AND AgBIS TRANSGENES

A "NSI" region of the *Synechococcus* sp. PCC 7002 genome was identified between two open reading frames encoding hypothetical proteins and used as a site for transgene integration (described in Materials and Methods). The *cpcBA* promoter from *Synechocystis* sp. PCC 6803 (Xu et al., 2011) was used to drive expression of the TPS transgenes. Numerous lines were selected for each new strain generated to ensure that any phenotypes arising were a consistent feature of the specific transformation. **Figure 2** shows the successful integration of the MsLS and AgBIS constructs at the NSI locus, as evidenced by the gel shift of the amplified colony-PCR product in transformant strains relative to the wild type. Integration of the YFP construct was also confirmed in the YFP transformant, which was designed as a control strain to identify phenotypes in the LS and BIS strains that may be attributed to chromosomal disruption at the NSI locus, rather than expression of the TPS transgenes. Importantly, the lack of a wild type-sized band in all transformant lines indicated that homoplasmy for the introduced transgenes was achieved.

IDENTIFICATION OF LIMONENE AND α-BISABOLENE AS END-PRODUCTS OF TRANSFORMANT STRAINS

The use of an organic solvent overlay has proven to be a successful method for harvesting terpenoids from microbial cultures (Newman et al., 2006; Anthony et al., 2009; Alonso-Gutierrez et al., 2013; Bentley et al., 2013). GC–FID analyses of solvent overlays from LS (**Figure 3A**) and BIS (**Figure 3B**) transformants showed prominent peaks with similar retention times to commercial standards of L-limonene (4.40 min) and bisabolene (9.89 min), respectively, which were absent from the wild type. **Figure 4** shows the mass spectral analyses of putative L-limonene and α-bisabolene peaks from transformant solvent extracts in comparison to the corresponding reference spectra from the NIST Mass Spectral Library (upper panels). The solvent also extracted additional products common to all strains, including wild type (**Figure 3**, ~10.5 min retention time), which may be membrane lipids (accumulated levels were below detection limits for mass spectral analysis). A comparison of L-limonene and bisabolene commercially available standards with NIST reference spectra is also shown in **Figure 4** (lower panels). The major peak (9.89 min) in the bisabolene standard (**Figure 3B**) was identified as α-bisabolene, the peak at 9.94 min was bisabolol, while the small peaks with retention times <9.98 min were all sesquiterpenes based on a mass to charge (m/z) of 204.

TRANSFORMANT FITNESS AND EFFECT OF DODECANE ON THE RATES OF PHOTOAUTOTROPHIC GROWTH

Photoautotrophic growth rates, as measured by chlorophyll and biomass accumulation, were comparable between wild type and the YFP control strain (**Figure 5A**, left panels). This verified that NSI is a suitable "neutral" site for transgene integration in *Synechococcus* sp. PCC 7002 under the defined experimental conditions, because cell growth rate was not adversely affected by the genetic manipulation at this locus. Rates of chlorophyll and biomass accumulation in the LS and BIS transformants were also comparable to that of the wild type (**Figure 5**, left panels), confirming that expression of the MsLS and AgBIS transgenes, and subsequent intracellular accumulation of limonene and α-bisabolene, did not adversely affect cell fitness under our experimental conditions.

Dodecane was chosen as the solvent of choice for longer-term terpenoid extraction experiments due to its relatively low volatility, which enabled continuous extraction over multiple days (evaporative loss was determined as negligible over the five day cultivation period, data not shown). The C_{12} chain length also allowed chromatographic separation from C_{10} limonene and C_{15} bisabolene. The rate of limonene and α-bisabolene biosynthesis during the growth phase was measured over a 96-h time course using a 5% (v/v) dodecane overlay. Notably, the presence of the dodecane overlay did not greatly affect chlorophyll content or biomass yield in any of the strains (**Figure 5**, right panels), indicating that atmospheric CO_2 was able to efficiently diffuse though the dodecane layer and dissolve in the liquid media at concentrations that were not limiting for photoautotrophic growth under these conditions.

LIMONENE AND BISABOLENE BIOSYNTHETIC RATES AND CELLULAR LOCALIZATION

Three independent lines of the LS transformant consistently produced yields over 4 mg limonene L culture^{-1}, with the highest rate of 50 μg L culture^{-1} h^{-1} recorded over the exponential growth phase when cells were actively dividing (**Figure 6A**). As cultures reached higher cell densities, light limitation due to cell shading began to limit photosynthetic growth (**Figure 5A**), which likely translated into reduced rates of limonene biosynthesis. Assuming that carbon comprised ~50% of the total biomass, the carbon partitioning to limonene was estimated at 0.3%. Yields of α-bisabolene were considerably lower, where average yields in BIS transformant lines ranged between 0.5 and 0.7 mg α-bisabolene L culture^{-1} (**Figure 6B**). The highest α-bisabolene production rate

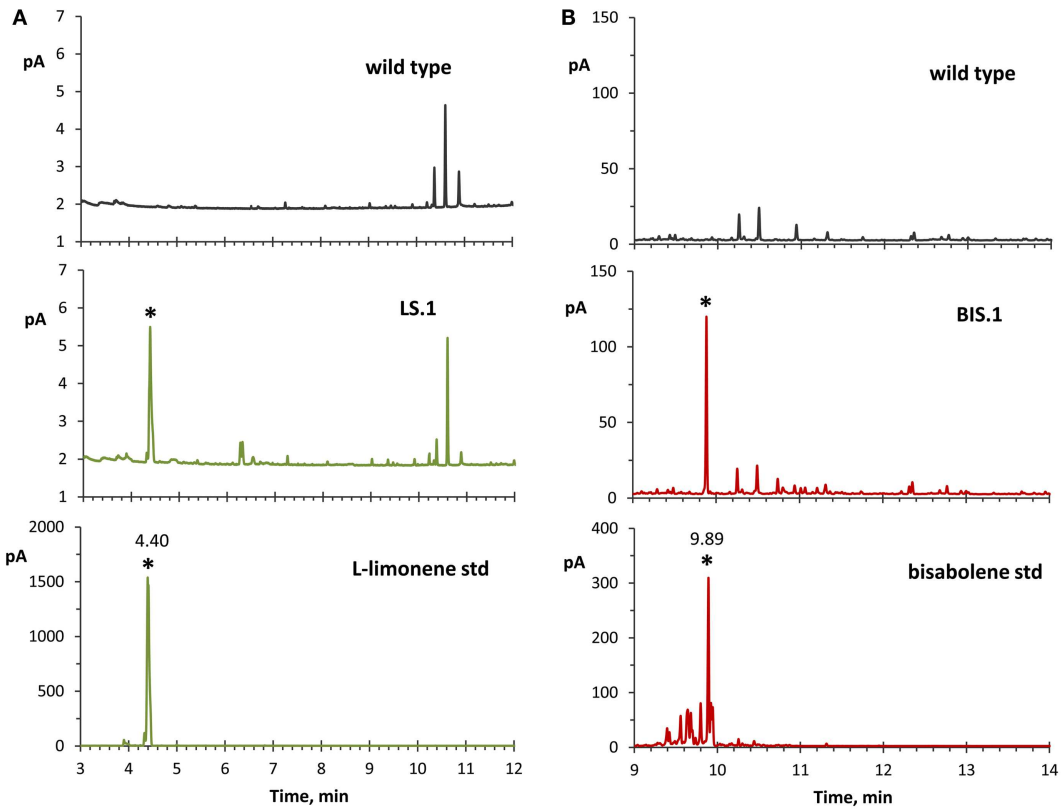

FIGURE 3 | GC–FID analyses of solvent overlay-extracted limonene and bisabolene from transformant strains. **(A)** Hexane-overlay extraction from the LS transformant (LS.1 line as a representative) showed accumulation of a product that was absent in the wild type, and with a similar retention time (4.40 min) to a commercial L-limonene standard. **(B)** Dodecane overlay extraction from the BIS transformant (BIS.1 line as a representative) revealed a peak with a similar retention time (9.89 min) to a bisabolene standard, which is absent in the wild type. For these experiments, dodecane was used for bisabolene extraction because of the extended period of time required to accumulate detectable levels of bisabolene, whereas hexane was applied for better limonene peak resolution due to the reverse solvent effect observed with dodecane.

was 6 μg L culture^{-1} h^{-1} over the active growth phase, equivalent to ~0.06% of assimilated carbon partitioning as α-bisabolene.

A methanol:chloroform extraction was performed on cells harvested at the termination of the 96-h time course to assess whether any limonene or α-bisabolene remained in the cells after the dodecane extraction procedure. On average, 11 μg limonene L culture^{-1} was extracted from LS cells (representing 0.25% of the total limonene yield), and 40 μg α-bisabolene L culture^{-1} from BIS cells (8% of the total bisabolene yield) (**Figure 6C**). Therefore, almost 2.5-fold (mol:mol) more α-bisabolene remained inside cells after dodecane extraction, compared to limonene. Relative to the overall terpenoid yields from LS and BIS transformants, a much greater fraction of α-bisabolene was retained intracellularly compared to limonene. This may be explained, in part, by the mass difference between these two compounds, with the smaller limonene molecule having a greater rate of diffusion across the cell membrane.

Surprisingly, similar amounts of limonene and α-bisabolene were extracted from harvested cells regardless of the presence or absence of the dodecane overlay (**Figure 6C**). Additional dodecane extractions of the spent media performed after cell harvesting did not reveal any terpenoids that partitioned to the media. The higher overall product yields displayed by cultures overlayed with dodecane may be due to: (i) the trapping and solubilization of limonene and α-bisabolene, which prevented evaporative loss, and/or (ii) the sequestration of limonene and α-bisabolene from the cells, which alleviated a negative feedback inhibition mechanism.

PHYSIOLOGICAL CHARACTERIZATION OF *SYNECHOCOCCUS* sp. PCC 7002 Δ*glgC*

At a light intensity of 250 μmol photons m^{-2} s^{-1} PAR, the Δ*glgC* strain displayed slightly impaired photoautotrophic growth in nutrient-replete media, as measured by $OD_{730\,nm}$ and chlorophyll content (**Figure 7A**, left panels). In contrast to the wild type, Δ*glgC* did not grow photoautotrophically in nitrogen-deplete medium (**Figure 7A**, right panels) due to its inability to degrade the light-harvesting phycobilisomes as a source of nitrogen (Carrieri et al., 2012; Grundel et al., 2012; Guerra et al., 2013; Hickman et al., 2013). Accordingly, the absorbance spectrum of Δ*glgC* cells in nitrogen-deplete media shows the presence of phycobilin, which is absent in the wild type after nitrogen-deprivation (**Figure 7B**). As a result, the Δ*glgC* culture retained the blue–green hue under nitrogen-deprivation, while the wild type appeared more yellow–green due to the unmasking of chlorophyll *a* as the blue-pigmented

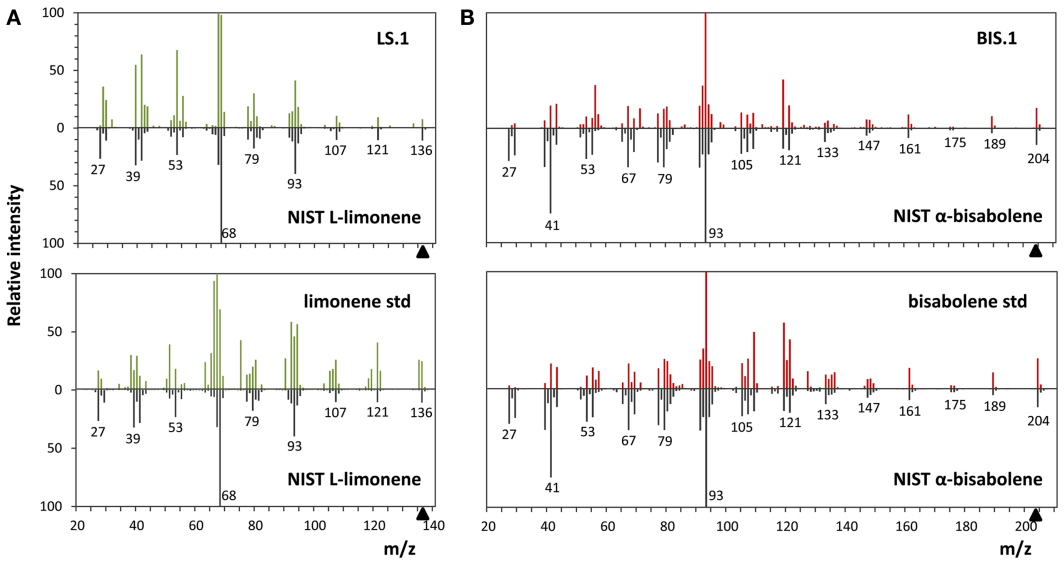

FIGURE 4 | Mass spectra analyses of limonene and α-bisabolene. **(A)** Comparison of the L-limonene reference spectra from the NIST library with the spectra of the putative L-limonene peak in the LS.1 line (upper panel) and the authentic L-limonene standard (lower panel). **(B)** α-Bisabolene NIST reference spectra alignment with the putative α-bisabolene peak in the BIS.1 line (upper panel) and a commercial bisabolene standard (lower panel).

phycobiliproteins were degraded (**Figure 7C**). We confirmed that the reducing carbohydrate content of Δ*glgC* was diminished compared to the wild type, particularly during stationary phase at 48 h in nutrient-replete media, and also during the first 24 h after the onset of nitrogen-deprivation (**Figure 7D**). These data confirmed that Δ*glgC* is defective in glycogen biosynthesis, and that our experimental conditions replicated the phenotypes previously observed in Δ*glgC* mutants of other cyanobacterial species (Carrieri et al., 2012; Grundel et al., 2012; Guerra et al., 2013; Hickman et al., 2013), and in *Synechococcus* sp. PCC 7002 (Guerra et al., 2013).

ORGANIC ACID EXCRETION IN Δ*glgC* AND ITS EFFECT ON TERPENOID YIELDS

Consistent with studies in *Synechocystis* sp. PCC 6803 (Carrieri et al., 2012; Grundel et al., 2012) and *Synechococcus elongatus* PCC 7942 (Hickman et al., 2013), we identified pyruvate, α-ketoglutarate, and succinate in the spent media of nitrogen-deprived *Synechococcus* sp. PCC 7002 Δ*glgC* cultures using HPLC analysis (**Figure 8A**, left panels), through a comparison of elution times with known standards (**Figure 8B**). However, we also detected α-ketoisocaproate and acetate within the same concentration range, metabolites which were not previously reported in the Δ*glgC* strains of the other cyanobacterial species. Acetate is a common fermentation product putatively derived from acetyl-CoA in cyanobacterial metabolism (Xu et al., 2013), while α-ketoisocaproate is the immediate precursor to leucine. This highlights subtle metabolic distinctions in the metabolism of the Δ*glgC* mutants between the model cyanobacterial species. An additional unique observation was the excretion of the same organic acids by Δ*glgC* in nutrient-replete media at the onset of stationary phase, when wild type cells would normally begin to increase glycogen stores for energy reserves (**Figure 8A**, right panels). However, on a per-cell basis, these concentrations were much lower relative to the concentrations of those excreted during nitrogen starvation. NMR was used as a second, independent method to verify the identity of these metabolites (**Figure 8C**). Pyruvate, succinate, and α-ketoisocaproate accumulated at concentrations of ~250 μM, while α-ketoglutarate accumulated at ~200 μM, and acetate ~500 μM under nitrogen starvation.

To assess whether the metabolic flux imbalance in Δ*glgC* redirected metabolites toward terpenoid biosynthesis, we grew the Δ*glgC*:LS and Δ*glgC*:BIS transformants under conditions of nitrogen starvation. The total yields of limonene and α-bisabolene were extremely low relative to the culture volume, which was not surprising given the absence of growth during nitrogen starvation (data not shown). However, terpenoid yield relative to biomass was relatively constant, regardless of whether cultures were grown in the presence or absence of nitrogen. In fact, throughout this study, terpenoid yield as a function of biomass remained relatively constant among all combinations of genetic background (±*glgC*), nutrient availability (±nitrogen), and growth phase (exponential vs. stationary). This indicates that terpenoid biosynthesis is coupled to cell growth and that flux through the terpenoid pathway is tightly regulated, even in the face of metabolic perturbations that increase feedstock availability.

Interestingly, however, we observed a higher extracellular concentration of excreted organic acids in the limonene-producing Δ*glgC*:LS strain, relative to the Δ*glgC* and Δ*glgC*:BIS strains (**Figure 9**). Under nitrogen starvation, Δ*glgC*:LS accumulated ~2.5-fold more α-ketoglutarate, and almost twofold more α-ketoisocaproate, pyruvate, and succinate in the spent media compared with the Δ*glgC* strain, despite no detectable photoautotrophic growth in either strains. It is unclear whether the higher

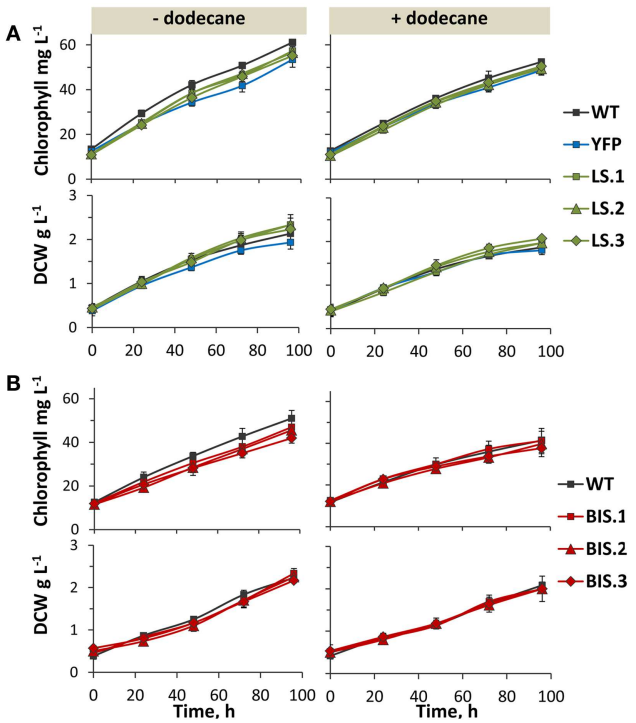

FIGURE 5 | Photoautotrophic growth of transformant lines in the presence and absence of a dodecane overlay. **(A)** Chlorophyll and biomass accumulation [as measured by dry cell weight (DCW)] over 96 h in the YFP control strain, and three independent LS lines (LS.1, LS.2, and LS.3) in comparison to wild type. Left panels show growth in the absence of a solvent overlay, while right panels display growth in the presence of a dodecane overlay. **(B)** Comparison of chlorophyll and biomass accumulation (DCW) between wild type and three independent BIS transformant lines (BIS.1, BIS.2, and BIS.3), in the absence (left panels) and presence (right panels) of a dodecane overlay. Error bars represent standard deviation from at least three biological replicates.

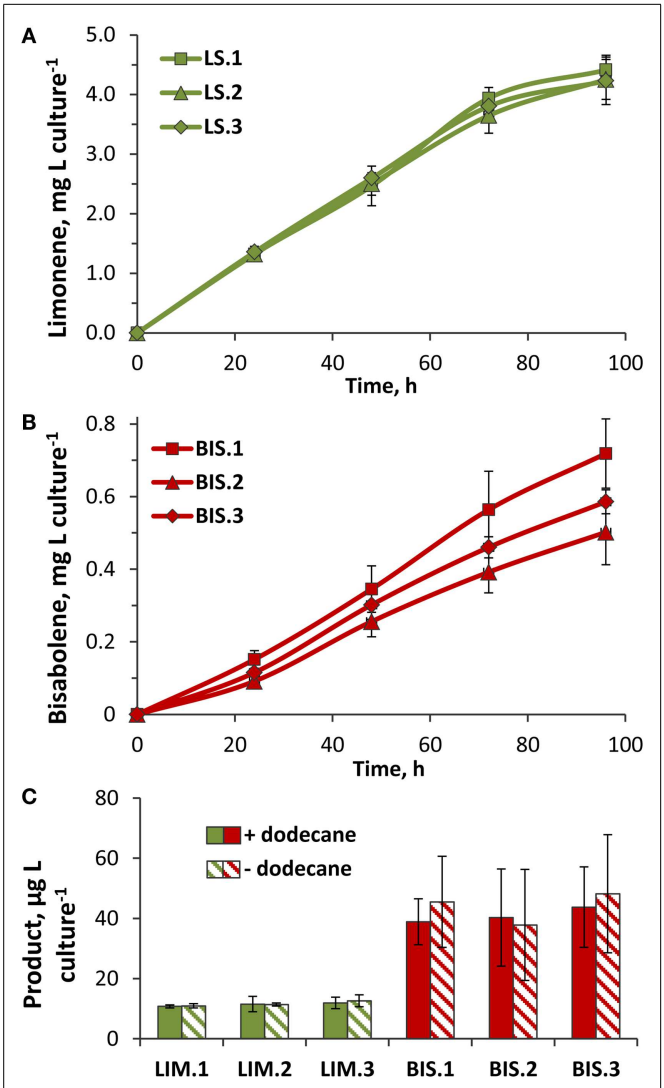

FIGURE 6 | Rates of limonene and α-bisabolene production in transformant lines. **(A)** Limonene accumulation in three independent LS transformant lines (LS.1, LS.2, and LS.3). Yields were quantified by GC–FID analysis of limonene extracted by a dodecane overlay during the 96-h growth phase (**Figure 5A**, +dodecane). **(B)** α-Bisabolene extracted from three independent BIS lines (BIS.1, BIS.2, and BIS.3) over the 96-h growth phase (**Figure 5B**, +dodecane) using a dodecane overlay. **(C)** Yields of limonene and α-bisabolene extracted from cells harvested after 96 h of photoautotrophic growth (**Figure 5**) in the presence (solid bars) or absence (hatched bars) of a dodecane overlay. Terpenoids were extracted from cells using a methanol:chloroform extraction method, and quantified by GC–FID. Error bars represent standard deviation from at least three biological replicates.

concentrations in the Δ*glgC*:LS strain are due to increased metabolism toward these end-products, or if a greater proportion of the organic acids are excreted from the cell, but provides an interesting line of future investigation.

DISCUSSION

We have demonstrated for the first time, the synthesis of limonene in *Synechococcus* sp. PCC 7002, and the first synthesis of α-bisabolene in a photosynthetic microorganism. The choice of *M. spicata* (−)-4*S*-limonene synthase and *A. grandis* (*E*)-α-bisabolene synthase as representative monoterpene and sesquiterpene synthases was based on proven successes in heterotrophic microbial production hosts (Carter et al., 2003; Peralta-Yahya et al., 2011; Alonso-Gutierrez et al., 2013; Ozaydin et al., 2013).

Importantly, (−)-4*S*-limonene synthase and (*E*)-α-bisabolene synthase are known to have high product specificity (Alonso et al., 1992; Rajaonarivony et al., 1992; Bohlmann et al., 1998), which is unusual for TPSs. Most TPSs generate multiple products from the prenyl diphosphate substrate as a result of the highly unstable carbocationic intermediates generated during the electrophilic reaction. Accordingly, these enzymes also produced pure L-limonene and α-bisabolene products in *Synechococcus* sp. PCC 7002; however, the yield of L-limonene was much greater than that of α-bisabolene. This could be explained by the superior enzyme kinetics of the *M. spicata* L-limonene synthase, which has a K_m (GPP) of 1.8 µM and k_{cat} of 0.3 s^{-1} (Alonso et al., 1992; Rajaonarivony et al., 1992), compared to the *A. grandis*

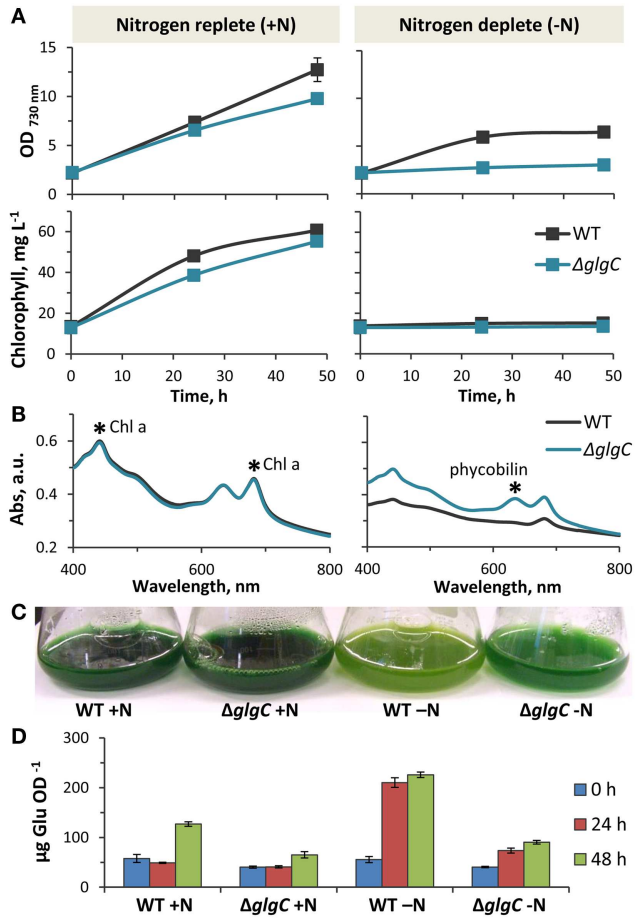

FIGURE 7 | Physiology of *Synechococcus* sp. PCC 7002 Δ*glgC*. **(A)**. Comparison of photoautotrophic growth rates in wild type and Δ*glgC* cultures in nitrogen-replete and nitrogen-deplete media, as measured by changes of optical density (730 nm) and chlorophyll content over 48 h. **(B)** Whole cell absorption spectra of wild type and Δ*glgC* cells after 48 h growth in nitrogen-replete and nitrogen-deplete media. **(C)** Differences in pigmentation of wild type and Δ*glgC* cultures after 48 h growth in the presence or absence of nitrogen in the media. **(D)** Carbohydrate content (reported as glucose equivalents) of wild type and Δ*glgC* cells at the different stages of growth (0, 24, and 48 h) shown in **(A)**. Error bars represent standard deviation from three biological replicates, and are hidden beneath the marker if not apparent.

(E)-α-bisabolene synthase, with a high K_m (FPP) of 49.5 µM and very low k_{cat} of 0.11 min^{-1} (McAndrew et al., 2011). Substrate availability is also likely to play a significant role because monoterpenes and sesquiterpenes are absent in most cyanobacteria, and so GPP and FPP substrates may not accumulate to the levels seen in plants. However, GPP must accumulate in significant quantities as the precursor to GGPP, from which longer chained cyanobacterial terpenoids are synthesized (e.g., carotenoids), and so may also explain the greater L-limonene yield relative to α-bisabolene.

The cyanobacterium *Synechocystis* sp. PCC 6803 has previously been engineered to produce β-phellandrene monoterpenes (Bentley et al., 2013) and β-caryophyllene sesquiterpenes (Reinsvold et al., 2011). The yield of β-phellandrene was 133 µg $gDCW^{-1}$, as measured by a non-continuous heptane-overlay extraction method. Intracellular titers of β-caryophyllene were ~4 µg $gDCW^{-1}$, as measured by a methanol:choloroform extraction method. However, these yields may have been underestimated due to the volatile nature of the terpenoid products. Evaporation was attributed to major losses of the amorphadiene sesquiterpene from *E. coli* liquid cultures, and a dodecane overlay was demonstrated to reduce the loss (Newman et al., 2006). Recently, the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 was engineered for limonene production via heterologous expression of the *Picea sitchensis* limonene synthase (Halfmann et al., 2014). The total yield of limonene (114 µg L^{-1}) was found to partition as a volatile gas in the culture headspace, and titers were improved ~ninefold upon overexpression of three rate-limiting enzymes involved in terpenoid metabolism. Here, we report the yields of 1.7 mg $gDCW^{-1}$ (4 mg L^{-1}) of limonene and 0.3 mg $gDCW^{-1}$ (0.6 mg L^{-1}) of α-bisabolene in *Synechococcus* sp. PCC 7002, upon expression of the single TPS genes, and using a continuous dodecane overlay extraction method that may reduce product loss due to evaporation.

It is also conceivable that dodecane may actively sequester terpenoids from the cell, thereby relieving any negative feedback pressures exerted by the products thus promoting higher end-product yields. Indeed, a 20% (v/v) dodecane overlay was found to alleviate growth inhibition of *Synechocystis* sp. PCC 6803 that was induced by the addition of commercial farnesene at concentrations as low as 0.04% (v/v) (Hellier et al., 2013). Further support for this theory was demonstrated in *E. coli*, where heterologous expression of an export pump in a limonene-producing strain increased limonene yield by ~60% (Dunlop et al., 2011). The microbial toxicity of bisabolene appears to be very low, with concentrations of commercial bisabolene up to 20% (v/v) exerting no effect on cell growth in *E. coli* and yeast (Peralta-Yahya et al., 2011). Although toxicity information is useful in this respect, the product feedback inhibition may not always manifest as growth inhibition. To fully understand the increased limonene and α-bisabolene yields in the presence of dodecane, investigations are required: (i) to probe terpenoid (or other) pathway inhibition by these end-products as well as (ii) to quantify the biologically produced limonene and bisabolene lost via evaporation in the absence of dodecane. It is important to establish whether the product naturally separates from biomass and how the physical properties of the product (mass, boiling point) influence this, or whether the product exerts feedback inhibition so that engineering efforts may be targeted toward this.

Targeted metabolic flux redistribution among the major carbon sinks is the key to producing novel end-products from engineered microbes at commercially available quantities. The goal remains to increase product yield at the expense of biomass accumulation (cell growth and division), without negatively impacting metabolisms associated with the assimilation and direction of carbon to the product. Although disruption of glycogen biosynthesis in *Synechococcus* sp. PCC 7002 produced an oversupply of central metabolites (α-ketoisocaproate, α-ketoglutarate, pyruvate, succinate, and acetate) (**Figure 10**), there was no apparent increase in flux though the MEP terpenoid pathway as conveyed by yields

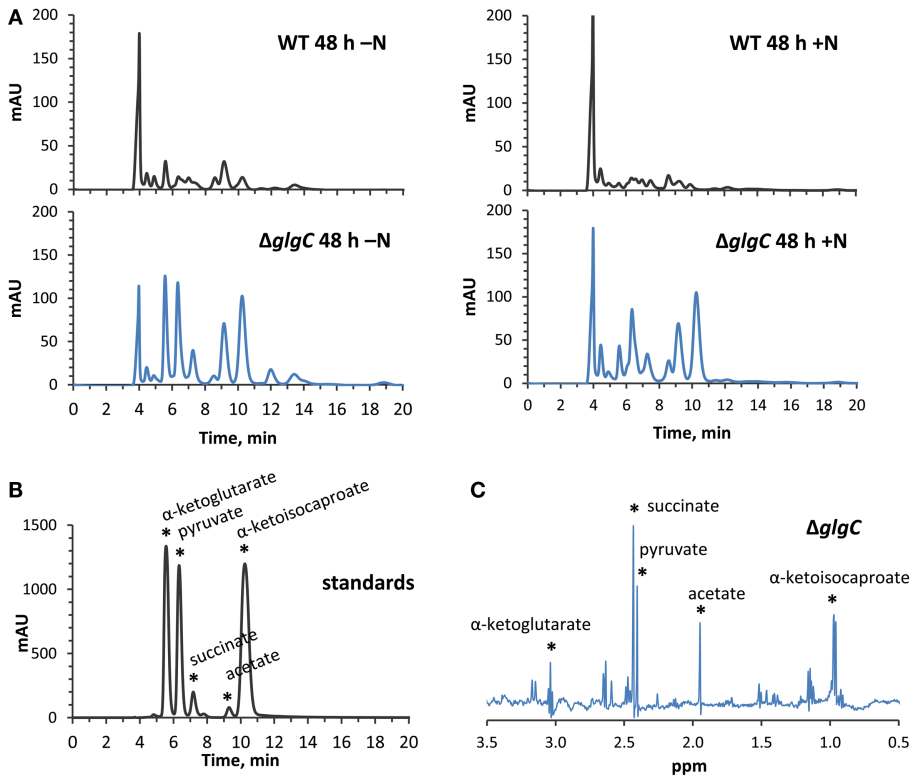

FIGURE 8 | High-performance liquid chromatography and NMR identification of secreted organic acids from Δ*glgC*. **(A)** HPLC-generated chromatograms showing the presence of secreted metabolites in Δ*glgC*, which are absent from the wild type culture media, when grown for 48 h under nitrogen-deplete (left panels) and nitrogen-replete (right panels) conditions. **(B)** The retention times of the metabolites is shown relative to a mixture of 4 mM α-ketoglutarate, pyruvate, succinate, acetate, and α-ketoisocaproate standards. **(C)** Proton NMR spectrum of the secreted metabolites in Δ*glgC*, showing chemical shifts and peak splitting patterns that correspond to α-ketoglutarate, pyruvate, succinate, acetate, and α-ketoisocaproate.

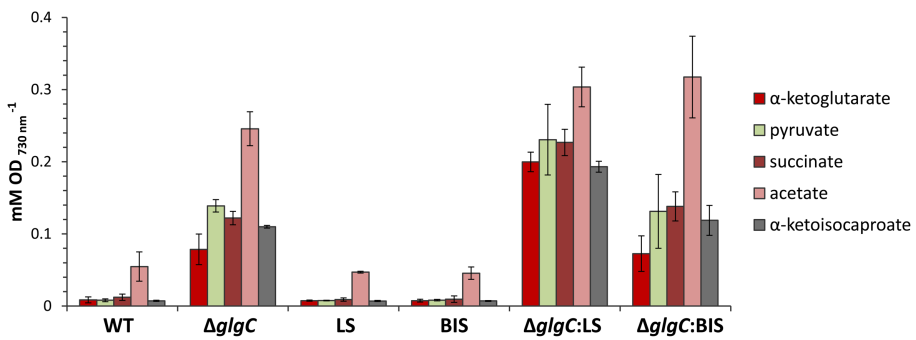

FIGURE 9 | Accumulation of excreted organic acids in the spend media of cells grown under nitrogen starvation for 48 h. Concentrations of organic acids were measured by HPLC analysis and presented relative to cell optical density at 730 nm. Error bars represent standard deviation from three biological replicates.

of limonene and α-bisabolene reporter products. Furthermore, such a severe perturbation of central carbon metabolism had a detrimental effect on the organism fitness as manifested by a complete growth inhibition. To that end, introduction of alternative routes for carbon partitioning have proven successful in cyanobacteria as carbon fixation rates are stimulated by providing alternative sinks for photosynthetically derived NADPH and ATP (Ducat et al., 2012; Li et al., 2014). While the MEP terpenoid pathway utilizes both NADPH and ATP as cofactors, growth and pathway optimization are required so that photosynthesis

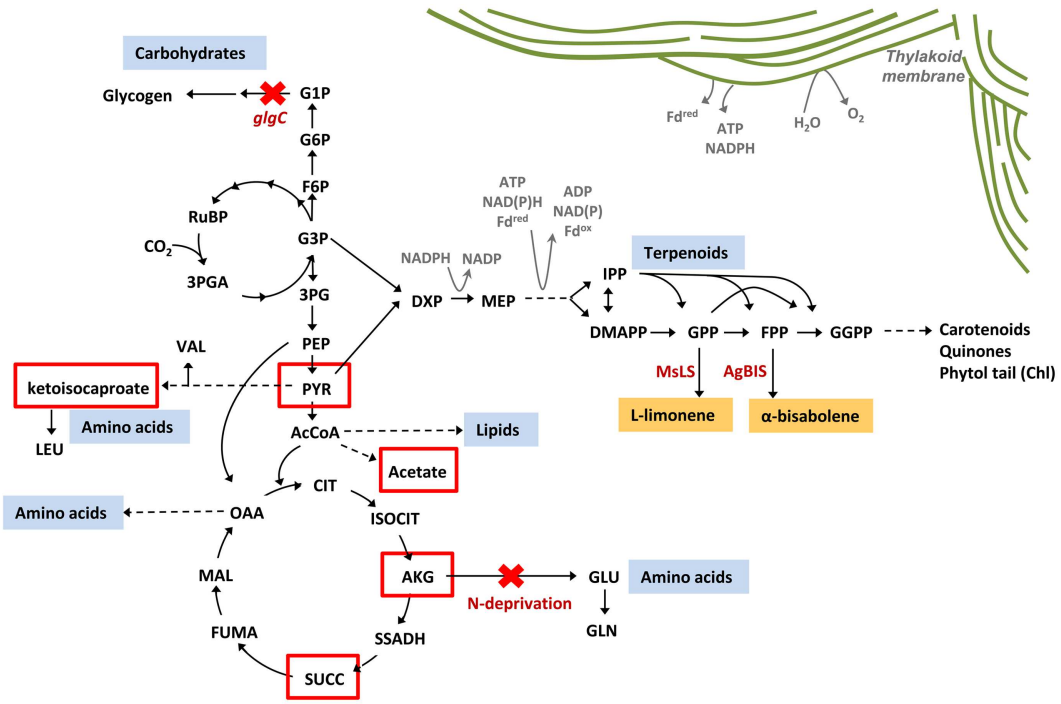

FIGURE 10 | Metabolic redistribution in *Synechococcus* sp. PCC 7002 upon simultaneous blocks to protein and carbohydrate sinks. Protein (amino acids), carbohydrates (mainly glycogen), and lipids represent the major metabolic sinks in *Synechococcus* sp. PCC 7002. When metabolic flux to glycogen and amino acids were simultaneously blocked, through inactivation of Δ*glgC* and elimination of nitrogen from the culture medium (red crosses), a suite of organic acids were secreted from the cell as overflow metabolites from central metabolism (red boxes). There is potential to redirect these spill-over intermediates toward biotechnologically relevant pathways to increase yields of engineered products, such as limonene and α-bisabolene via the terpenoid biosynthetic pathway.

and terpenoid biosynthesis remain active despite major metabolic redistributions.

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Alkane biosynthesis genes in cyanobacteria and their transcriptional organization

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In cyanobacteria, alkanes are synthesized from a fatty acyl-ACP by two enzymes, acyl–acyl carrier protein reductase and aldehyde deformylating oxygenase. Despite the great interest in the exploitation for biofuel production, nothing is known about the transcriptional organization of their genes or the physiological function of alkane synthesis. The comparison of 115 microarray datasets indicates the relatively constitutive expression of *aar* and *ado* genes. The analysis of 181 available genomes showed that in 90% of the genomes both genes are present, likely indicating their physiological relevance. In 61% of them they cluster together with genes encoding acetyl-CoA carboxyl transferase and a short-chain dehydrogenase, strengthening the link to fatty acid metabolism and in 76% of the genomes they are located in tandem, suggesting constraints on the gene arrangement. However, contrary to the expectations for an operon, we found in *Synechocystis* sp. PCC 6803 specific promoters for the two genes, *sll0208* (*ado*) and *sll0209* (*aar*), which give rise to monocistronic transcripts. Moreover, the upstream located *ado* gene is driven by a proximal as well as a second, distal, promoter, from which a third transcript, the ~160 nt sRNA SyR9 is transcribed. Thus, the transcriptional organization of the alkane biosynthesis genes in *Synechocystis* sp. PCC 6803 is of substantial complexity. We verified all three promoters to function independently from each other and show a similar promoter arrangement also in the more distant *Nodularia spumigena*, *Trichodesmium erythraeum*, *Anabaena* sp. PCC 7120, *Prochlorococcus* MIT9313, and MED4. The presence of separate regulatory elements and the dominance of monocistronic mRNAs suggest the possible autonomous regulation of *ado* and *aar*. The complex transcriptional organization of the alkane synthesis gene cluster has possible metabolic implications and should be considered when manipulating the expression of these genes in cyanobacteria.

Keywords: alkane biosynthesis, start sites of transcription, cyanobacteria, operon, promoter, sRNA

INTRODUCTION

The production of liquid fuels and a variety of chemicals indispensable for daily life depends on fossil resources. Due to their limited availability and with respect to environmental concerns the exploitation of alternative, renewable, and sustainable energy sources is gaining in importance. Thus, the conversion of solar energy and carbon dioxide into biofuels and suitable chemicals, e.g., hydrogen, ethanol, ethylene, isobutyraldehyde, or isoprene by genetically modified and improved phototrophic microorganisms such as cyanobacteria is of high interest (Deng and Coleman, 1999; Takahama et al., 2003; Atsumi et al., 2009; Lindberg et al., 2010; McKinlay and Harwood, 2010; Georgianna and Mayfield, 2012; Peralta-Yahya et al., 2012).

Interestingly, many cyanobacteria are naturally able to produce alkanes (Winters et al., 1969), which are the major constituents of gasoline, diesel, and jet fuels. However, there are also strains such as *Synechococcus* sp. PCC 7002 in which no alkanes were detectable, indicating that the responsible synthesis pathway is not present in these strains. Considering this information and using a comparative genomics approach, the according genes were identified (Schirmer et al., 2010). In cyanobacteria, alkanes are synthesized from intermediates of the fatty acid metabolism (Figure S1 in Supplementary Material) by two enzymes: acyl–acyl carrier protein reductase (AAR) and aldehyde deformylating oxygenase (ADO) (Schirmer et al., 2010; Li et al., 2011, 2012; Zhang et al., 2013). These enzymes are encoded by the two adjacent genes *sll0208* (*ado*) and *sll0209* (*aar*) in the model strain *Synechocystis* sp. PCC 6803 (from here: *Synechocystis* 6803). Interestingly, orthologs of these genes have been found so far only in cyanobacteria, suggesting the possible existence of a link to photoautotrophic life style i.e., oxygenic photosynthesis, but the functional relevance of cyanobacterial alkane biosynthesis *in vivo* has remained enigmatic thus far.

Cyanobacteria are morphologically very complex and are represented by unicellular (e.g., *Synechocystis* 6803, *Prochlorococcus* sp. MIT9313) as well as multicellular strains with differentiated cells (e.g., *Anabaena* sp. PCC 7120, *Nodularia spumigena* sp. CCY9414). Moreover, cyanobacteria are found in nearly all light-exposed habitats on earth including extreme environments such as deserts (Cameron, 1962; Garcia-Pichel et al., 2001), hot springs (Miller and Castenholz, 2000), hypersaline water (Reed et al., 1984) as well as Antarctic meltwater ponds (Nadeau and Castenholz,

2000), which underlines also their physiological diversity. Based on morphological properties five subsections were defined. However, due to the insufficient coverage of the cyanobacterial phylum by full genome sequences, truly comprehensive genome analyses have remained impossible for a long time. Recently, 54 additional genomes have been published (Shih et al., 2013), overcoming the lack of genomic data and enabling a comprehensive view on the occurrence and organization of genes important for a particular environmental situation.

For many cyanobacterial strains the expression of *ado* and *aar* orthologs is evident since long-to-medium-chain alkanes were detected (Winters et al., 1969; Schirmer et al., 2010). Though, their physiological function as well as the regulation of expression remains elusive. In *Synechocystis* 6803, alkane synthesis could be abolished by deletion of *ado*/*aar* and thus appears not essential, at least under standard growth conditions (Schirmer et al., 2010). To make an impact on cell physiology, genes need to be expressed. Furthermore, if their function is only needed under certain conditions, their expression should be regulated. The presence of upstream genetic regulatory elements can to a great extent serve as evidence for the functional significance of a gene. Thus, a detailed expression analysis might point to certain environmental conditions under which alkane synthesis is physiologically more relevant or even essential. Global transcriptomic analyses using microarrays are powerful approaches to investigate gene regulation and comparative data for manifold environmental conditions are available also for cyanobacteria (Hernandez-Prieto and Futschik, 2012). Moreover, RNA-seq, especially the differential RNA sequencing approach [dRNAseq, (Sharma et al., 2010)] is often used for the analysis of the primary transcriptome, which provides insight into gene expression changes together with detailed information about transcriptional start sites (TSS) and all promoters active at a given moment. By now, the primary transcriptomes of several cyanobacteria are available, supporting the analysis of the transcriptional organization of alkane synthesis (Mitschke et al., 2011a,b; Voß et al., 2013; Voigt et al., 2014; Pfreundt et al., submitted).

In this work, we present a comprehensive analysis of the genomic arrangement of genes encoding ADO and AAR throughout the cyanobacterial phylum. Moreover, we investigated the transcriptional organization of these genes for *Synechocystis* 6803 but also for *Anabaena* PCC 7120, *N. spumigena* CCY9414, *Prochlorococcus* MIT9313 & MED4, and *Trichodesmium erythraeum* IMS101. Although a dicistronic or polycistronic arrangement appears conserved at the genomic level among almost all cyanobacteria, we found solid evidence for the independent transcription of *ado* and *aar* in all tested strains. Since these strains are rather distantly related, the data indicate that an independent transcription of both genes might be common also for most other cyanobacteria. Additionally, for *Synechocystis* 6803 we compiled available expression data extracted from 115 microarray datasets, which comprise more than 25 environmental stimuli but reveal only modest conditional changes in gene expression.

MATERIALS AND METHODS

CLUSTER ANALYSIS

By using the JGI database and blastP algorithm (threshold *E*-value = $1e^{-5}$), 181 cyanobacterial genomes were screened for orthologs of ADO and acyl-ACP reductase genes. The full list of all genomes included in the study is shown in Table S1 in Supplementary Material. The corresponding protein sequences from *Synechocystis* 6803 were used as reference. For acetyl-CoA carboxylase, short-chain dehydrogenase, and GTP cyclohydrolase I, the respective sequences from *Anabaena* sp. PCC 7120 were used. The phylogenetic tree was generated with MEGA V6.0 (Tamura et al., 2013) by using the neighbor joining algorithm based on 16S rRNA sequences that were extracted from the SILVA database (Quast et al., 2013).

STRAINS AND GROWTH CONDITIONS

The following strains were used: *Synechocystis* 6803, substrain "PCC-M" (Trautmann et al., 2012), *T. erythraeum* IMS101 (obtained from Ilana Berman-Frank, Bar-Ilan University, Tel-Aviv, Israel; originally isolated by Prufert-Bebout et al., 1993) and *Prochlorococcus* MIT9313 & MED4 (courtesy of Sallie W. Chisholm, Massachusetts Institute of Technology, Cambridge, USA). *Synechocystis* was grown in TES-buffered (20 mM, pH 8.0) BG11 medium (Rippka et al., 1979) at 30°C under continuous white light illumination of 50–80 μmol quanta m^{-2} s^{-1} and gentle agitation. To determine the stability of transcripts, cultures were additionally aerated with ambient air through a glass tube and a sterile filter for constant and fast growth. Rifampicin was added as an inhibitor of transcription at a final concentration of 300 μg/ml. Samples were taken before and 3, 5, 10, 15 min after the treatment. *T. erythraeum* cultures were grown in YBCII medium (Chen et al., 1996) at 25°C and 12/12 h light/dark cycles at ~80 μmol photons m^{-2} s^{-1} white light. *Prochlorococcus* cells were grown at 22°C in AMP1 medium (Moore et al., 2007) under 10–30 μmol quanta m^{-2} s^{-1} continuous white cool light. For DNA cloning the *E. coli* strains Top10F′ and DH5α were used and cultivated in LB medium at 37°C.

RNA EXTRACTION, NORTHERN BLOTS, AND MAPPING OF RNA 5′ AND 3′ ENDS

Cyanobacterial cells were harvested in exponential growth phase by vacuum filtration on hydrophilic polyethersulfone filters (Pall Supor-800, 0.8 μm or Supor-450, 0.45 μm), immediately immersed in 1 ml of PGTX solution (Pinto et al., 2009) and frozen in liquid nitrogen. Total RNA was extracted as described (Hein et al., 2013); *T. erythraeum* and *Prochlorococcus* cells were subjected to bead beating (bead diameter 0.1–0.5 mm) for 3 × 20 s at 6500 rpm (Precellys, Peqlab, Germany) immediately prior to extraction. For northern blot analysis, 3 (*Synechocystis*) to 5 μg (*T. erythraeum* and *Prochlorococcus*) of total RNA were separated on 1.5% agarose gels, transferred to Hybond-N^+ nylon membranes (GE Healthcare) by capillary blotting and cross-linked by UV-illumination (125 mJ). Generation of single-stranded radioactively labeled RNA probes and hybridization with the blotted RNA were performed as described before (Steglich et al., 2008). The sequences of oligonucleotides used to amplify the respective probe templates are shown in Table S6 in Supplementary Material. Signals were visualized by using a Personal Molecular Imager FX system and Quantity One software (Bio-Rad). The half-life of transcripts was calculated after densitometric quantification of the corresponding signals. Mapping of RNA 5′ and 3′ ends

was performed by rapid amplification of cDNA ends as described (Argaman et al., 2001). To define precise lengths, RNA molecules were self-ligated, reverse transcribed using gene-specific primers, and amplified by circular PCR as described (Vogel and Hess, 2001). All used RNA and DNA oligonucleotides are listed in Table S6 in Supplementary Material. Reverse transcription was performed at 42°C for 2 h using the Omniscript™ RT system (Qiagen). Prior to sequencing the PCR products were cloned into the pGEM®-T vector (Promega).

GENERATION OF REPORTER STRAINS

The putative promoter elements were fused to a reporter gene by PCR amplification (for oligonucleotides see Table S6 in Supplementary Material), followed by restriction digest with *Fse*I/*Age*I and cloning into the promoter probe vector pILA (Kunert et al., 2000). The pILA plasmid contains a promoterless *luxAB* operon encoding subunits of the luciferase enzyme and sequences for the homologous recombination of the entire promoter probe cassette into the *Synechocystis* 6803 gene *slr0168* on the chromosome. The vector used in this work was modified prior to promoter insertion by introducing the restriction recognition sites *Fse*I and *Age*I upstream of the *luxAB* genes. The sites were introduced by PCR amplification of the original pILA plasmid with the primers "Plux *Age*I fw"/"Plux-bla *Age*I rev" and with the primers "Plux-bla *Fse*I fw"/"Plux *Fse*I rev", resulting in two blunt ended products, which were subsequently ligated. The plasmid derivatives were used for transformation of a *Synechocystis* 6803 strain carrying the *luxCDE* operon in turn encoding enzymes for the synthesis of decanal, the substrate for the luciferase reaction. The expression cassette harboring the genes *luxCDE* under control of the strong promoter of the ncRNA Yfr2a (Voß et al., 2009) and a *cat* gene mediating resistance to chloramphenicol, was integrated into the intergenic region of *sll1691* and *slr1819* (both hypothetical proteins on the chromosome), which is regarded as a neutral site. Transformation was performed as described (Kunert et al., 2000). Genetically modified cells were initially selected on agar-solidified BG11 medium (0.9% KobeI agar, Roth, Germany) containing 10 μg ml^{-1} kanamycin (Km, selection of *luxAB* constructs) and 2 μg ml^{-1} chloramphenicol (Cm, marker for *luxCDE* cassette), but the segregation of clones and subsequent cultivation of mutants was performed in presence of 50 μg ml^{-1} Km and 10 μg ml^{-1} Cm.

LUCIFERASE ASSAYS

Bioluminescence was measured *in vivo* as total light counts per second by using a VICTOR^3 multiplate reader (PerkinElmer). The cells were grown in the presence of 10 mM glucose. Prior to the measurement, cells were diluted to an $OD_{750} = 0.4$ and 200 μl of the suspension were filled into a white 96-well plate (CulturePlate™-96, PerkinElmer). In the multiplate reader the cell suspensions were shaken for 10 s and subsequently total light emission was measured for 1 s. A strain carrying the promotorless *luxAB* genes served as a negative control.

RESULTS

OCCURRENCE AND GENOMIC ORGANIZATION OF ALKANE SYNTHESIS GENES IN CYANOBACTERIA

Today, many cyanobacterial genomes are available in the databases enabling the possibility of comparing genomes with regard to physiological properties such as the production of alkanes. In this study, 181 genomes (Table S1 in Supplementary Material) were screened for orthologs of AAR and ADO (Schirmer et al., 2010; Li et al., 2011, 2012; Zhang et al., 2013). By using the blastP algorithm at an *E*-value cut off 1e^{-5}, orthologs of both enzymes could be identified in 90% (163/181) of the genomes (Tables S2 and S3 in Supplementary Material), which underlines their importance. Notably, their functional connection is highlighted by the fact that we found not a single genome in which one of the two genes, *aar* and *ado*, would have been retained in the absence of the other. Moreover, the genomes were also analyzed for possible synteny at the locus encoding ADO. The different types of arrangements are shown in **Figure 1**. Interestingly, in 76% (138/181) of the genomes tested, the genes encoding AAR and ADO were found adjacent to each other, indicating a possible operon-like organization. Moreover, in 61% (111/181) of the strains both genes clustered together with additional genes encoding the alpha subunit of acetyl-CoA carboxyl transferase (EC 6.4.1.2) and a short-chain dehydrogenase, strengthening the functional connection to fatty acid metabolism. In most genomes, additionally a gene encoding a GTP cyclohydrolase I (EC 3.5.4.16), which is involved in folate biosynthesis, was found downstream of the other four. However, in a few strains *aar* and *ado* are located at different loci as single genes (**Figure 1**). Additionally, in 18 of the tested genomes AAR and ADO genes were not found (for the full list see Table S4 in Supplementary Material), including the previously studied *Synechococcus* sp. PCC 7002 and *Cyanothece* sp. PCC 7424 and in consent with those reports (Schirmer et al., 2010).

TRANSCRIPTIONAL ORGANIZATION OF ALKANE SYNTHESIS GENES IN *SYNECHOCYSTIS* 6803

Data so far available suggest that *ado* and *aar* orthologs form an operon, but a genome-wide mapping of TSS in *Synechocystis* 6803 by using differential RNA sequencing (dRNAseq) revealed that both genes possess their own, specific, TSS (Mitschke et al., 2011a). Thus, they do not seem to be part of an operon since transcription is driven by independent promoters (**Figure 2A**). This unexpected transcriptional organization was further substantiated by northern blots (**Figure 2B**). Hybridization of an RNA probe specific for the *ado* (*sll0208*) ORF, which has a length of 696 bp, yielded a signal of about 900–1000 nt. This size is too short for a dicistronic mRNA, for which a minimal length of >1700 nt would be expected. Moreover, with an *aar*-specific probe, again, a transcript too short for a dicistron was detected but which approximately matches with the length of the *aar* (*sll0209*) open reading frame (1023 bp). Thus, these hybridization signals are consistent with the generation of independent monocistronic mRNAs from two independent promoters. Interestingly, our data uncovered not only two independent TSS for *ado* and *aar* but also a third TSS, which might belong to a putative non-coding RNA, called SyR9, upstream of the two genes (Mitschke et al., 2011a). This finding is supported by the identification of suitable −10 elements within the putative promoter sequences associated with these three TSS (**Figure 2C**). Hybridization using a SyR9-specific RNA probe yielded two signals – the lower band corresponds to the small transcript of SyR9, the upper band, however, was of approximately the same size as found for the *ado* transcript (**Figure 2B**). In this case, the upper

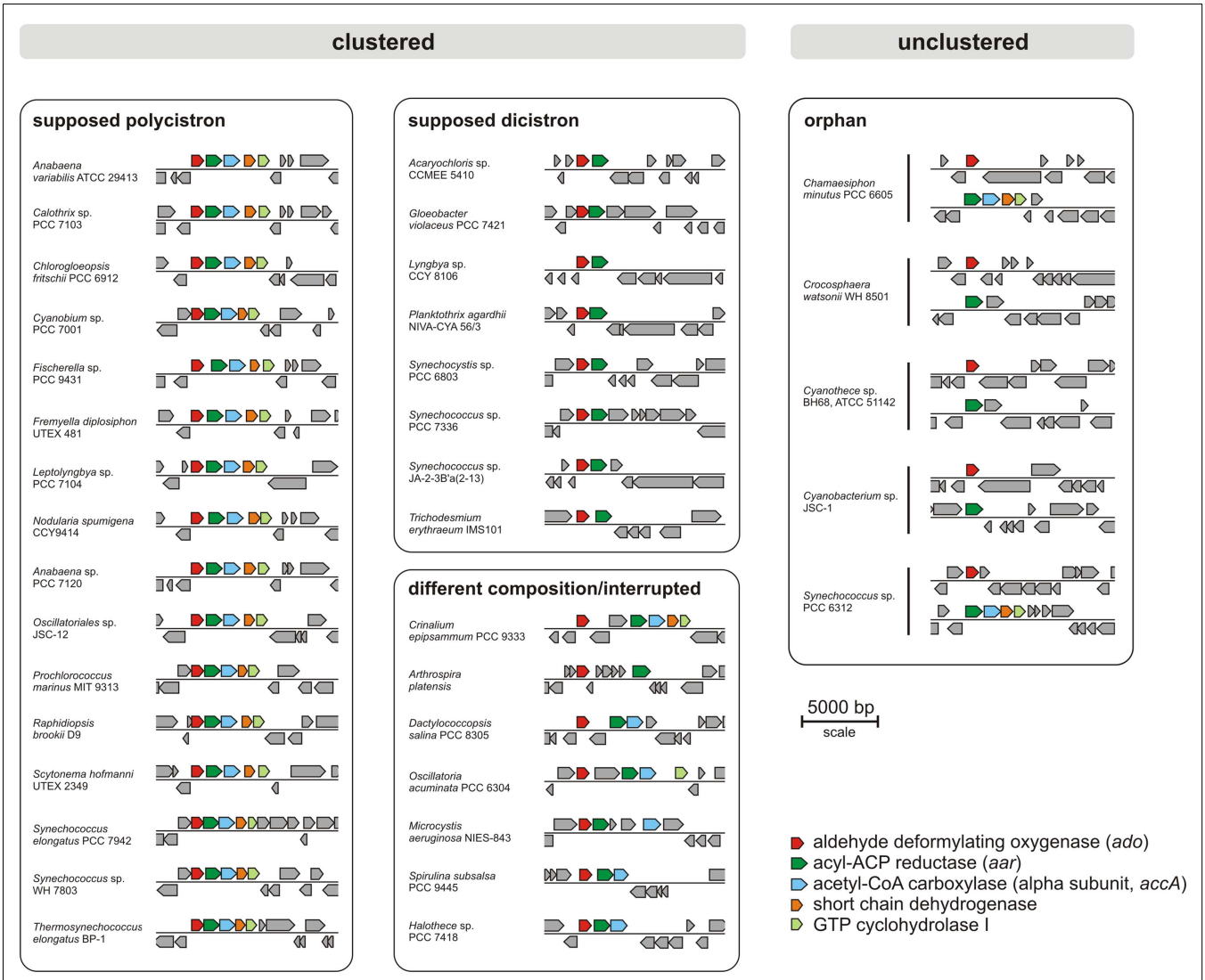

FIGURE 1 | Examples for genomic arrangements of aldehyde deformylating oxygenase orthologs in cyanobacteria. By using JGI database and blastP algorithm (threshold *E*-value = $1e^{-5}$) 181 cyanobacterial genomes were screened for the presence of alkane biosynthesis genes. The corresponding protein sequences from *Synechocystis* sp. PCC 6803 were used as reference.

signal could represent a combined RNA that included SyR9 and the *ado* mRNA. To test this possibility, PCR was carried out on cDNA samples prepared from total RNA circularized by RNA ligase and using primers in outbound orientation. One primer was located within the SyR9 segment and the other at the end of the coding region of *ado*. The obtained fragments were sequenced and revealed the 5′ end mapped for SyR9 to be connected to a sequence finishing 75 nt behind the last nucleotide of the *ado* stop codon [at genomic pos. 2511439 (complement)]. Thus, this analysis confirmed the SyR9-*ado* cotranscript and yielded its precise length of 985 nt, consistent with the sizes estimated by northern analysis. We conclude that in *Synechocystis* expression of *ado* is driven by two promoters, P1 and P2, and that the smaller accumulating transcript SyR9 results from processing of the SyR9–mRNA cotranscript or alternative termination of transcription.

The same approach was taken to determine the precise 5′ and 3′ ends of the *aar* mRNA. Whereas the 5′ end 72 nt upstream of the start codon resulting from initiation of transcription at promoter P3 was confirmed, its 3′ end was mapped 64 nt downstream of the stop codon [at genomic pos. 2510209 (complement)]. Thus, the total length of the *aar* mRNA is 1159 nt, consistent with the major signal obtained in the northern hybridization (**Figure 2B**) and the accumulation of this mRNA as a monocistronic transcript species. This finding receives further strong support from the identification of a putative Rho-independent terminator mapped here at the 3′ end of the *ado* mRNA (**Figure 2D**). We conclude that the *ado* and *aar* orthologs in *Synechocystis* 6803 are not transcriptionally organized as an operon. These results clearly impact approaches to manipulate the expression of these genes, as the activity of a strong promoter upstream of a *ado–aar*

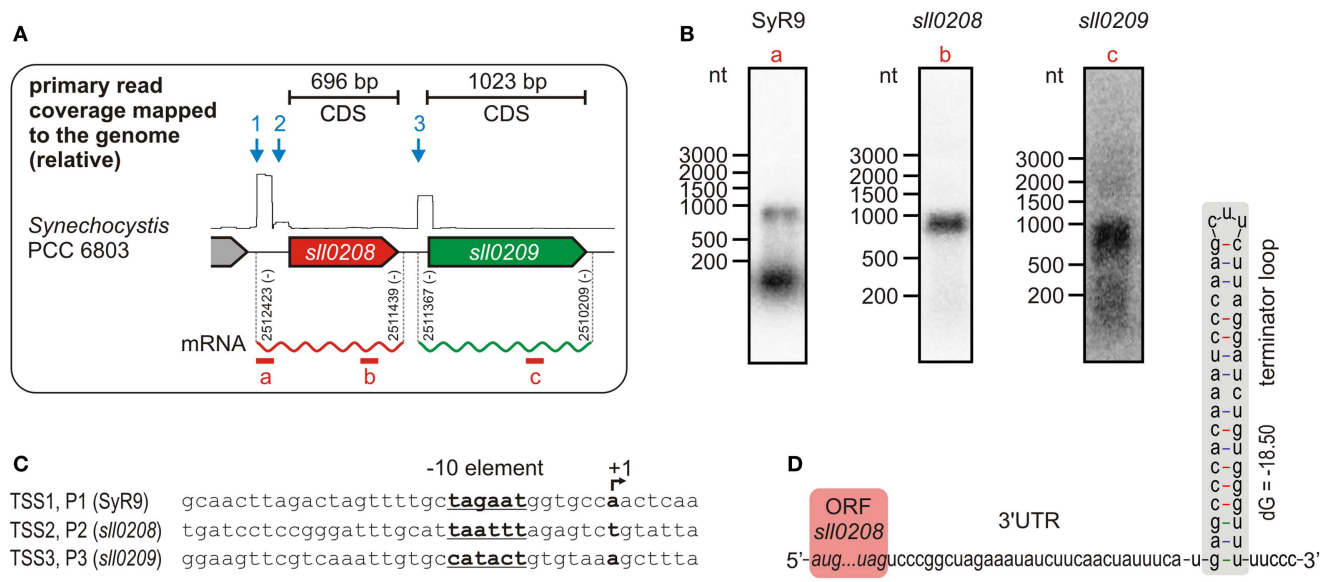

FIGURE 2 | Transcriptional organization of the gene cluster encoding acyl-ACP reductase (*aar, sll0209*) and aldehyde deformylating oxygenase (*ado, sll0208*) in *Synechocystis* sp. PCC 6803. **(A)** Differential RNA sequencing (dRNAseq, data shown as primary read coverage mapped to the corresponding genomic region) of cells grown under standard conditions revealed independent transcriptional start sites (TSS) for both genes indicating for monocistronic mRNAs. The rectangular shape within the graph results from the enrichment of primary 5′-PPP carrying transcripts representing a TSS and the read length (101 nt) of the sequence analysis. Data were extracted from Kopf et al., 2014. Each TSS is represented by an arrow. The experimentally confirmed 5′ and 3′ ends of the main mRNAs are given by the respective genomic positions. **(B)** Northern verification for the monocistronic mRNAs and the small transcript SyR9, which accumulates independently from the *sll0208* mRNA in *Synechocystis* sp. PCC 6803. The part covered by the labeled probe is shown by a red bar in **(A)**. Consistent with the TSS mapping all transcripts detected appeared too short for a dicistronic mRNA. **(C)** Putative promoter elements upstream of the mapped TSS which is designated as +1. **(D)** The mapped 3′ end of the *sll0208* mRNA can be folded into a stem loop secondary structure that is typical for a Rho-independent terminator of transcription.

two-gene-cassette will lead to a high expression of the first, but not the second gene.

TRANSCRIPTIONAL ORGANIZATION OF ALKANE SYNTHESIS GENES IN OTHER CYANOBACTERIA

Synechocystis 6803 has been established as a representative model strain. Nevertheless, cyanobacteria constitute a physiologically and genomically very diverse taxon, hence the independent transcription of *ado* and *aar* found in *Synechocystis* might be different in other cyanobacteria. To test this possibility, we checked the primary transcriptomes that are available for several additional cyanobacteria (Mitschke et al., 2011b; Voß et al., 2013; Voigt et al., 2014; Pfreundt et al., submitted). Interestingly, independent TSS for the two genes exist also in *Anabaena* PCC 7120, *N. spumigena* CCY9414, *Prochlorococcus* MIT9313 & MED4, and *T. erythraeum* IMS101 (**Figure 3A**). The respective transcripts were exemplarily verified for three of these strains. In all cases, the main accumulating RNAs that derive from the TSS upstream of *ado* were shorter than the minimum length of a dicistronic transcript (**Figures 3B–D**). Moreover, the signal patterns that were observed after hybridization with *aar*-specific probes were different from those for the *ado* transcripts. These findings indicate that both genes might be transcriptionally independent from each other also in most other cyanobacteria, which is contrary to their seemingly dicistronic/polycistronic arrangement that appears largely conserved at the genomic level. Interestingly, similar to the arrangement in *Synechocystis* 6803, a second TSS upstream of *ado* is active in *Trichodesmium*, *Nodularia*, and *Prochlorococcus* MIT9313, which suggests that *ado* might be transcribed from two independent promoters also in several other species. For *Trichodesmium*, this arrangement was verified further by using a labeled probe specific for the sequence around the distal TSS (probe a, locus shown in **Figure 3A**). A transcript of ~1600 nt was detected, which demonstrates the existence of a long transcript that encompasses also the coding region of the *ado* gene (**Figure 3B**). Additionally, the two bands that were obtained when using a probe specific for a sequence downstream of the proximal *ado*-TSS (probe b) demonstrated the accumulation of two different mRNAs originating from the two TSS (indicated by black arrows).

EXPERIMENTAL VERIFICATION OF THE MAPPED *ado* AND *aar* PROMOTERS IN *SYNECHOCYSTIS* 6803

In order to experimentally verify the independent TSS for *ado* and *aar*, 5′ RACE experiments were performed with RNA extracted from *Synechocystis* 6803. All three start sites derived from the global mapping of TSS (Mitschke et al., 2011a), could be confirmed [TSS1 at pos. 2512423, TSS2 at pos. 2512315, TSS3 at pos. 2511367; positions are given for the complementary (fwd) strand]. Sequences upstream of the TSS contain putative −10 elements and were assumed as true promoters and therefore designated as P1 (distal *ado* promoter, SyR9 promoter), P2 (proximal

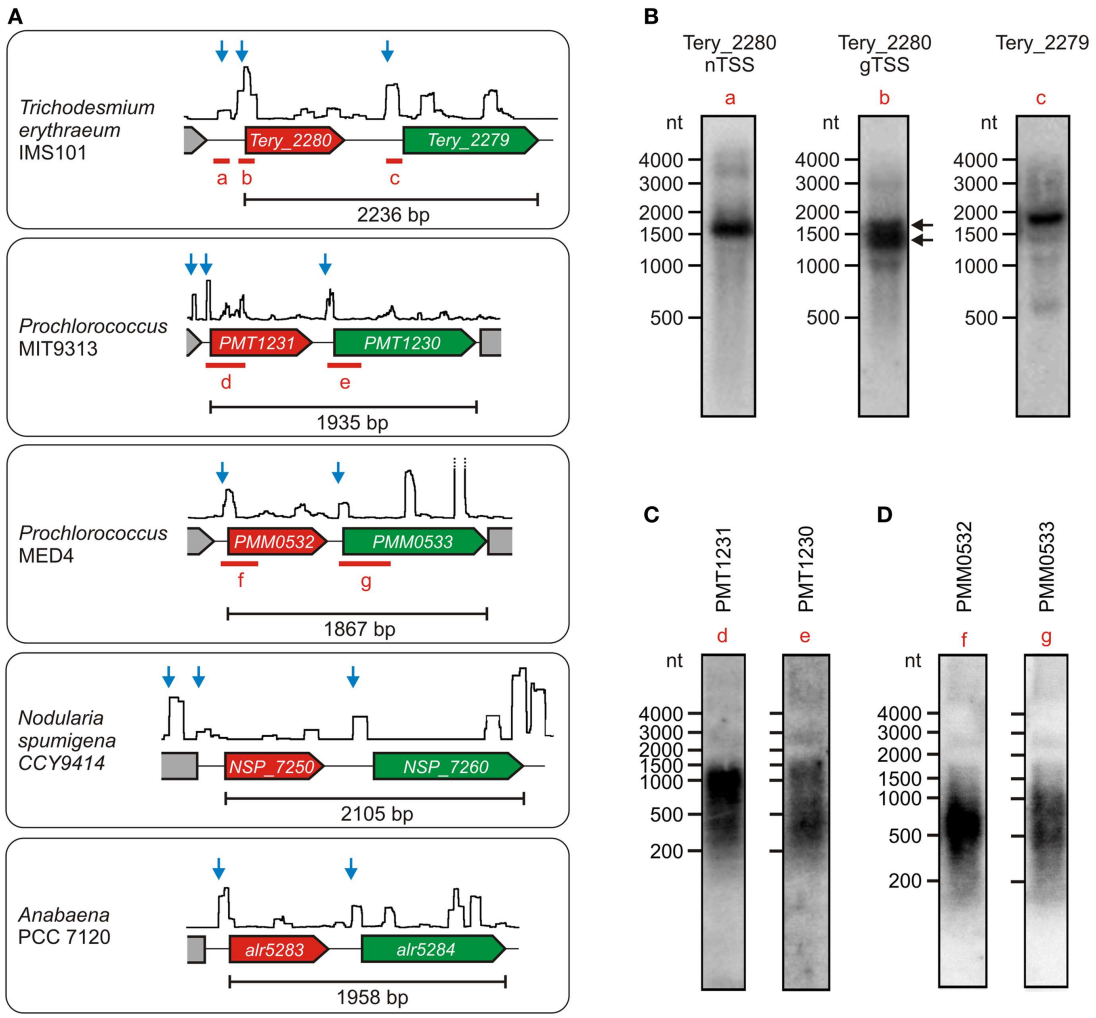

FIGURE 3 | Transcriptional organization of the gene cluster encoding *ado* (red) and *aar* (green) in various cyanobacteria under the respective standard conditions. **(A)** The data are presented analogous to **Figure 2**. Each TSS potentially belonging to the respective *aar*/*ado* mRNA is represented by a blue arrow. Data were extracted from Mitschke et al. (2011b), Voß et al. (2013), Voigt et al. (2014), (Pfreundt et al., submitted). In each case, the minimum length of the dicistronic arrangement is given for the sequence between the start codon of *ado* and the stop codon of *aar*. **(B)** Northern verification of *ado* (Tery_2280) and *aar* (Tery_2279) mRNAs in *Trichodesmium erythraeum* IMS101. The black arrows indicate the two mRNAs originating from the independent TSS upstream of Tery_2280. **(C,D)** Respective northern blots for both mRNAs in *Prochlorococcus* sp. MIT9313 and MED4. The corresponding probe loci are given in **(A)**.

ado promoter), and P3 (*aar* promoter, see **Figure 2**). In order to verify the suggested promoter activities, the sequences upstream and around the corresponding TSS were fused to *luxAB* luciferase genes. With respect to the first transcribed nucleotide (+1) we considered sequence ranges from −116 to +2 for P1, −108 to +106 nt for P2, and −401 to +71 for P3, respectively. Bioluminescence measurements revealed that these sequences indeed contain elements also driving transcription of the reporter gene (**Figure 4**). P1 and P2 showed about one third of the strength measured for the well-characterized promoter of the *petE* gene, which is highly active in the presence of copper ions and has been used for heterologous expression in *Anabaena* as well as *Synechocystis* (Buikema and Haselkorn, 2001; Tan et al., 2011). The data verified that the expression of *ado* and *aar* is regulated independently and that two promoters are found upstream of *ado*. Moreover, luminescence measured for P2 was close to background levels indicating that *luxAB* transcription initiated by this promoter is rather weak, consistent with the much lower read number observed for TSS2 in the dRNAseq analysis (**Figure 2A**).

EXPRESSION OF ALKANE SYNTHESIS GENES IN *SYNECHOCYSTIS* 6803

The described data suggest the autonomous regulation and possibly independent function of *ado* and *aar* genes under certain growth conditions. Thus, a detailed expression analysis could reveal if both are differentially expressed or even contrary regulated. Moreover, it might imply a functional involvement of the genes and possibly alkanes in adapting cyanobacterial physiology according to environmental changes since the function of

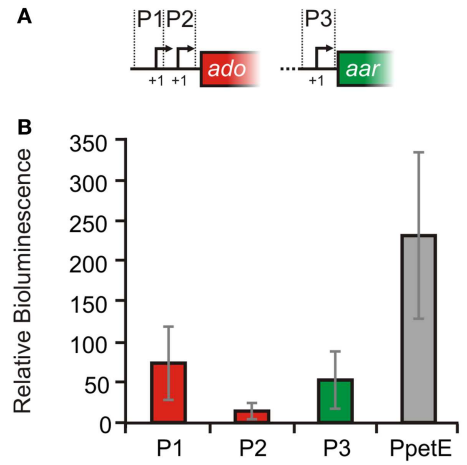

FIGURE 4 | Verification of promoter activities in *Synechocystis* sp. PCC 6803. **(A)** Schematic illustration of the putative promoter regions. For the reporter assays *luxAB* genes were fused with the upstream sequences of either: SyR9 (=P1, −116 to +2), *ado* (=P2, −108 to +106 nt) or *aar* (=P3, −401 to +71 nt), and also *petE* (=P*petE*, −273 to +81, positive control) while +1 represents the first transcribed nucleotide. **(B)** Bioluminescence was measured *in vivo* as total light counts per second. A strain harboring a promoterless *luxAB* operon was used as negative control. The cultures were grown in standard BG11. For the P*petE* activity measurements, however, 2 μM $CuSO_4$ was added. Relative bioluminescence was calculated by subtraction of the values obtained from negative control cultures. Data are the mean ± SD of 60 single measurements obtained with two biological replicates (=independent transformants) in several independent experiments.

Table 1 | Log2 fold changes for *ado* and *aar* in *Synechocystis* 6803 after treatment with novobiocin in addition to heat stress, low temperature, and salt stress.

Condition	*ado* (*sll0208*)	*aar* (*sll0209*)
WT_novobiocin	0.09	−0.16
WT_novobiocin_HS	−2.54	−2.24
WT_novobiocin_LT	−1.57	−0.10
WT_novobiocin_salt_stress	−1.16	−1.13

Data were extracted from the CyanoEXpress database (Hernandez-Prieto and Futschik, 2012).

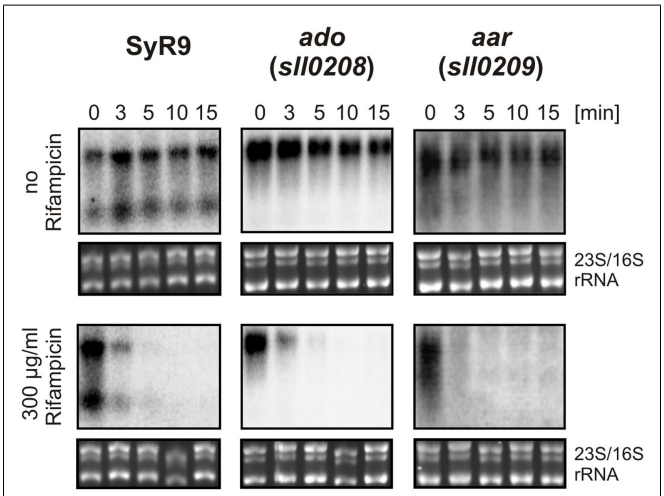

FIGURE 5 | RNA stability measurements. Half-lives of the indicated transcripts were determined in the presence of rifampicin in cultures for the times indicated. For comparison, samples were taken at the identical time points but in the absence of rifampicin. For the control of equal loading, the large rRNA bands stained with ethidium bromide are shown underneath each hybridization.

alkanes in cyanobacteria is still ambiguous. To investigate if *ado* and *aar* are differentially expressed we compiled data extracted from CyanoEXpress, a database for microarray experiments performed with samples from *Synechocystis* (Hernandez-Prieto and Futschik, 2012). Interestingly, there are many conditions for which contrary fold changes for both genes were observed, further verifying their independent transcription. For the full list of fold changes see Table S5 in Supplementary Material. However, for the environmental conditions that are included in the CyanoEXpress database no clear and convincing stimulation of expression was evident. For *ado* the top log2 fold change (1.52) was observed after 4 h of zinc excess, whereas for *aar* it was 12 h of iron depletion (log2 fold change = 1.08). Nevertheless, diminished mRNA levels were observed for both, *ado* and *aar*, when cells were subjected to oxidative stress by H_2O_2 treatment. Furthermore, expression of both genes appear dependent on DNA gyrase since a novobiocin treatment in combination with heat stress, low temperature, or salt stress led to significantly reduced mRNA abundances (**Table 1**).

The actual concentration of an mRNA is the result of two processes, transcription and turnover. Therefore, the stability of an mRNA is of similar importance for gene expression as is the control of transcription. To measure the relative transcript stability, we added the inhibitor of transcription initiation, rifampicin, to our cultures and then followed the disappearance of SyR9 and the mRNAs for *ado* and *aar* in a time course experiment (**Figure 5**). The calculated half-lives of all these transcripts were with <3 min quite short. We observed a transcript stability decreasing from SyR9 with a half-life of 2.2 min over 1.8 min for *ado* (*sll0208*), to only 1.6 min for *aar* (*sll0209*) at the end of this gene cluster in *Synechocystis* 6803.

DISCUSSION

Since the discovery of the cyanobacterial alkane biosynthesis pathway, the major scientific focus has been on the enzymatic properties of ADO and AAR, as well as their potential for biofuel production (Schirmer et al., 2010; Li et al., 2011, 2012; Zhang et al., 2013). Less attention was paid to the expression of the corresponding genes as well as their regulation and possible function in the host organisms. Obviously, in cyanobacteria no other pathways (at least for medium-chain alkanes) exist since a knockout of *ado* and *aar* genes abolished heptadecane synthesis in *Synechocystis* 6803 and no alkanes were detected in *Synechococcus* sp. PCC 7002, a strain that lacks these two genes naturally (Schirmer et al., 2010). Due to the wealth of new genomic information that has become available within the last 2 years, comparative analyses linking phylogeny and the presence of alkane biosynthesis genes

has become straightforward and is yielding new insight. Consistently, orthologs of ADO and AAR were found in all strains for which alkane detection has been reported earlier (see **Figure 6**). Additionally, alkanes were also detected in many other cyanobacterial isolates for which no genome sequence is available, including the genera *Oscillatoria*, *Microcoleus*, *Lyngbya*, *Nostoc*, *Plectonema*, *Chlorogloeopsis*, and *Phormidium* (Han et al., 1968; Winters et al., 1969), indicating that *ado* and *aar* genes are also present in those species. Surprisingly, orthologs of ADO and AAR are not present in other prokaryotes, associating this pathway to cyanobacteria or certain aspects of their phototrophic life style.

MULTIPLE PROMOTERS ASSOCIATED WITH THE ALKANE BIOSYNTHESIS GENES IN CYANOBACTERIA

The clustering of *ado* and *aar* genes is widely conserved throughout the cyanobacterial phylum and frequently three additional genes are found associated with them (**Figure 6**). Despite their close functional connection and co-appearance in most genomes, our data demonstrate that in all tested strains the *ado* and *aar* genes are transcribed from separate, specific promoters. Thus, these two genes can be expressed independently from each other, in turn enabling their different or even divergent regulation. Due to the presence of separate regulatory elements, the dominance of monocistronic mRNAs and additionally the fact that the two genes are split in some species we speculate that physiological situations might exist for which an independent transcription of *ado* and *aar* could be advantageous. Additionally, in some strains *ado* is transcribed from two separate TSS, which increases the transcriptional complexity further. Multiple TSS may indicate different functions. In *Synechocystis,* for instance, two separate TSS were also reported for the *petH* gene that encodes two isoforms of ferredoxin:NADP oxidoreductase, while the decision between both isoforms is triggered by the differential use of these two TSS (Omairi-Nasser et al., 2011). Under standard conditions a proximal promoter is active, resulting in a short 5′UTR whereas under nitrogen depletion a more distally located promoter is active that mediates the transcription of *petH* with a long 5′UTR. Under both conditions only one isoform – a long or a short – is dominant. The different translation initiation sites are dictated by alternative secondary structures only depending on the length of the 5′UTR (Omairi-Nasser et al., 2011). In principle, alternative translation initiation is also imaginable for the *ado* gene. For the transcription of *ado* under standard growth conditions the distal promoter P1 appeared as the dominant regulatory element but situations might exist when the main expression is mediated by P2. Two mRNA species hence offer the possibility of alternative RNA foldings, which could then affect translation initiation or the yield of translated protein.

A highly interesting aspect of the observation that *ado* and *aar* genes are transcribed from separate and distinct promoters in cyanobacteria are the possible metabolic implications. Their monocistronic layout found in this work allows the regulatory autonomy of these two genes. Thus, they may signify also other, unknown, pathways branching off from aldehyde synthesis that would require their separate and non-stoichiometric expression. Such pathways might, e.g., lead to free fatty acid (from aldehyde oxidation) or even fatty alcohols (from aldehyde reduction) for which separate regulation of these two genes would be required. So, it is quite possible that still other unknown aldehyde-derived pathways remain to be discovered in cyanobacteria.

GENOMIC ORGANIZATION OF ALKANE SYNTHESIS GENES IN CYANOBACTERIA

The initial step of alkane synthesis produces a fatty aldehyde by the conversion of a fatty acyl-ACP thus connecting the pathway to fatty acid metabolism. Interestingly, in many cyanobacteria *ado* and *aar* are found adjacent to *accA* encoding the alpha subunit of acetyl-CoA carboxylase (ACCase, EC 6.4.1.2). ACCase catalyzes the synthesis of malonyl-CoA, which is the first and rate-limiting step in fatty acid synthesis (Figure S1 in Supplementary Material). Accordingly, overexpression of ACCase in *Synechocystis* leads to an enhancement of hydrocarbon production especially heptadecane (Tan et al., 2011). Moreover, in *Anabaena* 7120 and also most other strains the gene downstream of *accA* encodes a protein that possibly belongs to the short-chain dehydrogenase/reductase (SDR) family of NAD- or NADP-dependent oxidoreductases (Joernvall et al., 1995). Interestingly, it shows high similarity to the 3-oxoacyl-[acyl-carrier-protein] reductase (EC 1.1.1.100) that also participates in fatty acid biosynthesis. However, despite the clustering and the functional relation, the *accA* gene possesses its own TSS in several strains, similar to the transcriptional separation of *ado* and *aar* (see **Figure 3A**, accumulating primary transcripts at the 3′ end of the *aar* gene), indicating a possibly independent regulation. The functional connection, if any, to the fifth and last gene in this cluster (**Figure 6**), encoding GTP cyclohydrolase I (EC 3.5.4.16), is less obvious. This enzyme is involved in folate biosynthesis from GTP and during the catalysis formate is generated. There is one parallel as during the conversion of the fatty aldehyde to the final alkane catalyzed by ADO, also formate is generated (Li et al., 2011), but if that has any meaning is currently unknown.

REGULATION OF ALKANE SYNTHESIS GENE EXPRESSION

It is interesting to note the short half-lives of <3 min under our standard growth conditions, which we observed for all three major transcripts originating from the *syR9/ado/aar* locus. Consequently, slight changes in transcript stability could impact the mRNA accumulation and therefore the expression of these genes. Theoretically, a differential expression of *ado* and *aar* might point to conditions under which alkanes are physiologically more relevant. However, examining the wealth of expression data available for *Synechocystis* we found no clear evidence for differences in the abundance of *ado* or *aar* mRNA that could be linked to differences in the growth conditions. Expression may well be regulated in a multi-factorial fashion and not exclusively dependent on a single stimuli. Alternatively, the synthesis rate of alkanes could also be regulated by other factors than at the level of transcription or transcript stability, e.g., by modulating enzyme activities. Nevertheless, a regulatory cascade targeting *ado* and *aar* might exist but the stimuli remain ambiguous. Interestingly, most of the strains lacking AAR and ADO orthologs, and most probably alkanes, appear on one branch of the phylogenetic tree (**Figure 6**). Assuming a general importance of alkane synthesis, it is tempting to speculate that their physiological function in some strains of this clade might have been replaced or compensated by other mechanisms or compounds. Indeed, in these strains a likely compensatory polyketide

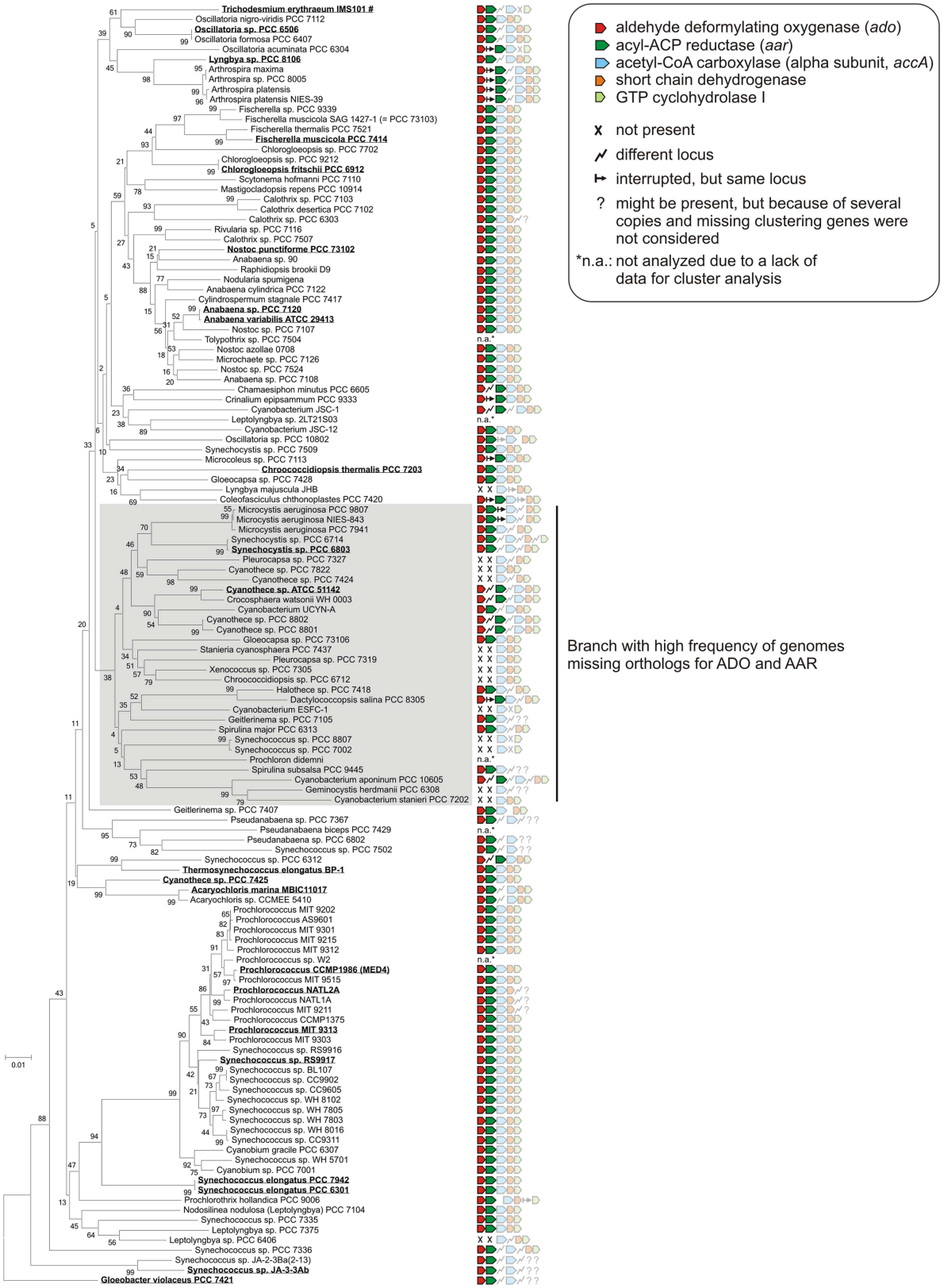

FIGURE 6 | Clustering of alkane biosynthesis genes among the cyanobacterial phylum. The phylogenetic tree was generated by using the neighbor joining algorithm based on 16S rRNA sequences that were extracted from the SILVA database (Quast et al., 2013). Orthologs of aldehyde deformylating oxygenase and acyl-ACP reductase were searched by using

(Continued)

FIGURE 6 | Continued
blastP (threshold $1e^{-5}$) and the protein sequences from *Synechocystis* sp. PCC 6803 as reference. For the other three genes encoding acetyl-CoA carboxylase, short-chain dehydrogenase, GTP cyclohydrolase I the sequences from *Anabaena* sp. PCC 7120 were used. Strains for which alkane biosynthesis has been reported (Schirmer et al., 2010; Coates et al., 2014) are highlighted and underlined. Alkanes were measured either directly in cyanobacterial cell extracts (mainly penta- or hepta-decane) or indirectly by alkane detection in recombinant *E. coli* strains expressing the corresponding *ado* orthologs in combination with the *aar* gene from *S. elongatus* PCC7942. [#]Alkanes were detected in a natural surface bloom of *Trichodesmium erythraeum* near Port Aransas in the Gulf of Mexico (Winters et al., 1969).

synthase pathway which produces 1-alkenes is present (Coates et al., 2014).

PHYSIOLOGICAL FUNCTIONS OF CYANOBACTERIAL ALKANE SYNTHESIS

Although the presence of ADO and AAR appears to be restricted to cyanobacteria, alkanes were also detected in other prokaryotes (Ladygina et al., 2006), where different types of enzymes must be responsible for their biosynthesis. Indeed, alkanes are widely distributed throughout nature, including plants (Cheesbrough and Kolattukudy, 1984; Bernard et al., 2012), green algae (Dennis and Kolattukudy, 1991), and higher animals (Cheesbrough and Kolattukudy, 1988), where they are mainly a component of surface waxes and function as water barrier or in insects where they serve as pheromones (Blomquist and Jackson, 1979; Howard and Blomquist, 2005). Therefore, the physiological function of alkanes is obviously not restricted to cyanobacteria and a more general role can be assumed also for other prokaryotes. Since fatty acid synthesis is mainly necessary for membrane generation, alkanes might also have an influence on membrane composition and fluidity under particular conditions. However, the functional relevance of alkanes under optimal growth conditions might be low since the pathway can be deleted without any obvious growth phenotype (Schirmer et al., 2010) and due to the fact that 10% of all cyanobacterial genomes lack the ADO–AAR pathway altogether.

Cyanobacteria are equipped with several mechanisms to deal with superfluous electrons generated from excess light energy absorbed. Therefore, it might appear as an attractive additional mechanism if newly synthesized alkanes would serve as electron sinks under certain conditions. In such a scenario, one would expect massive accumulation of alkanes under suitable conditions, e.g., high light stress. To the best of our knowledge this has not been observed in wildtype strains thus far, rendering this possibility highly speculative. For instance, the heptadecane content in various cyanobacteria typically ranges between 0.02 and 0.1% of cell dry weight (Coates et al., 2014). For *Synechocystis* 6803, amounts of ~200 μg/L (0.13% of cell dry weight) were reported (Wang et al., 2013), which is relatively low compared to dominant molecules such as chlorophyll a (~8 mg/L), glycogen (~200 mg/L), or the osmoprotectant glucosylglycerol [~100 mg/L, representative values for cells of *Microcystis firma* shocked with 770 mM NaCl, (Erdmann et al., 1992)]. However, for genetically engineered *Synechocystis* strains it was also shown that redirecting the carbon flux to acyl-ACP and overexpressing alkane biosynthetic genes simultaneously can significantly increase the yield of heptadecane to 26 mg/L (1.1% of cell dry weight; Wang et al., 2013). Indeed, these data indicate that alkane accumulation is basically possible.

Nevertheless, the complex transcriptional organization of the alkane synthesis gene cluster needs to be taken into account when manipulating the expression of these genes *in situ*. For example, the insertion of strong or controllable promoters upstream of the *ado* (*sll0208*) gene in *Synechocystis* 6803 is very likely to have an effect on the transcription of this but not of the downstream located *aar* gene due to the presence of the Rho-independent terminator of transcription in between.

AUTHOR CONTRIBUTIONS

Wolfgang R. Hess designed the study. Stephan Klähn and Wolfgang R. Hess supervised the research. Stephan Klähn performed genomic analyses. Claudia Steglich and Desirée Baumgartner performed verification experiments for the promoter mapping. Desirée Baumgartner executed physiological experiments, Northern blots, and promoter analyses for *Synechocystis*. Ulrike Pfreundt and Karsten Voigt performed northern verification for *Trichodesmium* and *Prochlorococcus*, respectively. Verena Schön generated the decanal producing host strain for luciferase reporter assays in *Synechocystis*. Stephan Klähn and Wolfgang R. Hess evaluated and interpreted the data. Stephan Klähn, Desirée Baumgartner, and Wolfgang R. Hess wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fbioe.2014.00024/abstract

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Improved free fatty acid production in cyanobacteria with *Synechococcus* sp. PCC 7002 as host

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Microbial free fatty acids (FFAs) have been proposed as a potential feedstock for renewable energy. The ability to directly convert carbon dioxide into FFAs makes cyanobacteria ideal hosts for renewable FFA production. Previous metabolic engineering efforts using the cyanobacterial hosts *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 have demonstrated this direct conversion of carbon dioxide into FFAs; however, FFA yields in these hosts are limited by the negative impact of FFA production on the host cell physiology. This work investigates the use of *Synechococcus* sp. PCC 7002 as a cyanobacterial host for FFA production. In comparison to *S. elongatus* PCC 7942, *Synechococcus* sp. PCC 7002 strains produced and excreted FFAs at similar concentrations but without the detrimental effects on host physiology. The enhanced tolerance to FFA production with *Synechococcus* sp. PCC 7002 was found to be temperature-dependent, with physiological effects such as reduced photosynthetic yield and decreased photosynthetic pigments observed at higher temperatures. Additional genetic manipulations were targeted for increased FFA production, including thioesterases and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Overexpression of non-native RuBisCO subunits (*rbcLS*) from a *psbAI* promoter resulted in more than a threefold increase in FFA production, with excreted FFA concentrations reaching >130 mg/L. This work illustrates the importance of host strain selection for cyanobacterial biofuel production and demonstrates that the FFA tolerance of *Synechococcus* sp. PCC 7002 can allow for high yields of excreted FFA.

Keywords: cyanobacteria, cyanobacterial biofuels, algal biofuels, free fatty acid, free fatty acid tolerance, *Synechococcus* sp. PCC 7002

INTRODUCTION

Microbial production of free fatty acids (FFAs) has recently garnered much attention as a potential feedstock for renewable energy production (Handke et al., 2011; Lennen and Pfleger, 2012). Engineering efforts for FFA production in *Escherichia coli*, which began nearly four decades ago (Cronan et al., 1975), have increased substantially, leading to FFA yields as high as 5.1 g/L (Liu et al., 2012). However, FFA production from *E. coli* requires a fixed carbon source, such as glucose, for both microbial growth and FFA production. At present, these fixed carbon sources are prohibitively expensive to be used as feedstock for a low-value commodity such as fuel. While technology is currently under development to convert lignocellulosic biomass into a cost-effective carbon feedstock for biofuel production (Himmel et al., 2007), photosynthetic microorganisms offer an alternative means for FFA production, with the ability to directly convert carbon dioxide (CO_2) into FFAs. Cyanobacteria are photosynthetic prokaryotes, which are particularly amenable to genetic manipulation, and as such, several cyanobacterial strains have been engineered for FFA production. *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, two model freshwater strains of cyanobacteria, were engineered for FFA production by gene knockout of the FFA-recycling acyl-acyl carrier protein (ACP) synthetase/long-chain-fatty-acid CoA ligase and expression of a thioesterase for fatty acid (FA) cleavage from the ACP, resulting in extracellular FFA concentrations of 83.6 and 32 mg/L, respectively (Liu et al., 2011; Ruffing and Jones, 2012). In *Synechocystis* sp. PCC 6803, additional metabolic engineering strategies, such as deletion of polyhydroxybutyrate biosynthesis, overexpression of acetyl-CoA carboxylase, weakening of the peptidoglycan layer, and expression of thioesterases from different sources, yielded a maximum of 197 mg/L of excreted FFAs (Liu et al., 2011). Similarly, *S. elongatus* PCC 7942 was further modified by expression of an algal thioesterase, overexpression of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), expression of a chloroplastic acetyl-CoA carboxylase, and improved gene expression using strong native promoters, yet these modifications did not improve the concentration of excreted FFAs in engineered *S. elongatus* PCC 7942 (Ruffing, 2013a). These initial studies of cyanobacterial FFA production illustrate the feasibility of FFA biosynthesis directly from CO_2 and also the difficulties associated with achieving high FFA yields using these cyanobacterial hosts.

In both *E. coli* and cyanobacteria, FFA production had detrimental effects on the cellular physiology of the host strain. These effects include reduced cell viability, compromised membrane integrity, and changes in membrane composition (Lennen et al., 2011), and the cyanobacterial hosts also had increased reactive oxygen species (ROS), reduced photosynthetic yields, and changes in photosynthetic pigments (Liu et al., 2011; Ruffing and Jones, 2012;

Ruffing, 2013b). Interestingly, the change in membrane composition with FFA production varied depending on the host strain; *E. coli* had increased unsaturated membrane fatty acids while *S. elongatus* PCC 7942 showed higher levels of saturated membrane fatty acids during FFA production (Lennen et al., 2011; Ruffing and Jones, 2012). This discrepancy may be due to the differences in chain length of the synthesized FFAs for each host resulting from thioesterase selection, with *E. coli* producing C8-C14 FFAs and *S. elongatus* PCC 7942 producing mostly C16-C18 FFAs. Transcriptomics analyses of FFA production in both *E. coli* and *S. elongatus* PCC 7942 hosts revealed that significant changes in gene expression accompany FFA production. Both hosts had increased expression of genes involved in stress responses and energy production; however, specific gene responses were not conserved among these two hosts. The *E. coli* strains showed significant increases in phage shock response genes and genes involved in aerobic respiration (Lennen et al., 2011), while the *S. elongatus* PCC 7942 strains up-regulated genes involved in heat shock, ROS degradation, photosynthesis, electron transport, and nitrogen metabolism (Ruffing, 2013b). The different responses of *E. coli* and *S. elongatus* PCC 7942 are likely due to the fundamental differences in their carbon and energy metabolisms, i.e., glycolysis and aerobic respiration in *E. coli* vs. the Calvin–Benson–Bassham cycle and photosynthesis in *S. elongatus* PCC 7942. Regardless of the host-specific response, the physiological effects of FFA production are undesirable and likely limit the attainable FFA productivities of each host.

Due to the broad range of effects resulting from FFA biosynthesis and the variability in host cell response, alternative cyanobacterial hosts should be considered for FFA production. In this study, we investigate the use of *Synechococcus* sp. PCC 7002 for FFA production and excretion. Unlike the previous freshwater cyanobacterial strains used for FFA production, *Synechococcus* sp. PCC 7002 is a marine microorganism, capable of surviving salt concentrations as high as 1.7 M (Batterton and Baalen, 1971). As freshwater is an important resource, particularly for the non-arable lands proposed for biofuel production, marine hosts are necessary for economical large-scale biofuel production. Proposed microalgal biofuel production systems will also be exposed to outdoor environmental conditions, including high light intensities during peak sunlight as well as daily and seasonal temperature fluctuations. *Synechococcus* sp. PCC 7002 has desirable traits to address these concerns: *Synechococcus* sp. PCC 7002 displays extreme high light tolerance, surviving under light intensities as high as two times peak sunlight (4.5 mmol photons m^{-2} s^{-1}) (Nomura et al., 2006), and it can survive under a wide range of temperatures, with high growth at 38°C (Ludwig and Bryant, 2012). This thermal tolerance is particularly important for growth in closed photobioreactors (PBRs), where temperatures will reach up to 40°C (Ong et al., 2010). The salt, light, and thermal tolerances of *Synechococcus* sp. PCC 7002 make it an ideal candidate for biofuel production, yet its tolerance for FFA production remains to be tested. This work investigated the FFA production capabilities and tolerances of *Synechococcus* sp. PCC 7002 in comparison to previously engineered strains of *S. elongatus* PCC 7942. FFA production was found to be less toxic in *Synechococcus* sp. PCC 7002 as compared to *S. elongatus* PCC 7942, yet this tolerance was temperature-dependent. Improved carbon fixation from RuBisCO overexpression in *Synechococcus* sp. PCC 7002 led to a threefold increase in FFA production, demonstrating a higher FFA yield than that achieved with comparably engineered strains of *S. elongatus* PCC 7942.

MATERIALS AND METHODS

MATERIALS

Chemicals used in this study were purchased from Acros Organics ($Na_2MoO_4 \cdot 2H_2O$), BD (agar), MP Biomedicals ($CuSO_4 \cdot 5H_2O$), Fisher Scientific (NaCl, $MgSO_4 \cdot 7H_2O$, KCl, $NaNO_3$, tris base, H_3BO_3, and kanamycin monosulfate), and Sigma-Aldrich (Na_2EDTA, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, vitamin B12, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $CoCl_2 \cdot 6H_2O$, and spectinomycin dihydrochloride pentahydrate). Primers were synthesized by Integrated DNA Technologies (IDT). DNA purifications were performed using the Plasmid Miniprep, DNA Clean and Concentrator, and Gel DNA Recovery kits from Zymo Research. Restriction enzymes, DNA polymerases, and ligases were purchased from New England Biolabs. Suppliers of all other materials used in this study are described below.

STRAIN CONSTRUCTION

Strains used and constructed in this study are listed in **Table 1**. The first step in engineering *Synechococcus* sp. PCC 7002 for FFA production was gene knockout of the long-chain-fatty-acid CoA ligase (*fadD*, SYNPCC7002_A0675), responsible for FFA recycling (Kaczmarzyk and Fulda, 2010). Homologous regions (~500 bp) upstream and downstream of *fadD* were cloned using the primers in Table S2 in Supplementary Material and integrated into pSE15 (Ruffing and Jones, 2012) at the *Afl*II/*Spe*I (5′ fragment) and *Bgl*II/*Xho*I (3′ fragment) restriction enzyme sites, generating the *fadD* knockout plasmid, pS12. Transformation of pS12 into *Synechococcus* sp. PCC 7002, followed by spectinomycin selection and PCR screening of *fadD*, resulted in the construction of strain S01. Transformation of *Synechococcus* sp. PCC 7002 was performed using a modified protocol based on that proposed by Stevens and Porter (1980). *Synechococcus* sp. PCC 7002 was grown in medium A^+ until $OD_{730} = 1.0$ was reached. Approximately, 0.1 μg of linearized plasmid DNA was added to 1 mL of culture ($OD_{730} = 1.0$) in a 16 mm glass test tube with ventilated cap. The transformation culture was incubated at 30°C with 150 rpm and 60 μmol photons m^{-2} s^{-1}. After 24 h of incubation, the transformation culture was concentrated to 100 μL using centrifugation (5000 × *g*, 5 min) and spread on medium A^+/agar plates with 1 mM of sodium thiosulfate and 40 μg/mL of spectinomycin dihydrochloride pentahydrate and 50 μg/mL of kanamycin monosulfate, as required. Colonies were re-streaked a minimum of three times to obtain complete segregants. After antibiotic selection and PCR screening, two positive transformants were tested for FFA production in 400 mL cultures (see Culture Conditions).

For S02 construction, the truncated *E. coli* thioesterase, *'tesA*, from pSE16 (Ruffing and Jones, 2012) was cloned using primers in Table S2 in Supplementary Material and integrated into pS12 at the *Eco*RI and *Bam*HI sites following the inducible trc promoter to form pS13. Transformation of pS13 into *Synechococcus* sp. PCC 7002 resulted in gene knockout of *fadD* and integration of *'tesA*. Gene knockout of the desaturase gene, *desB*, was achieved by transforming pSB into S02. pSB was constructed by

Table 1 | Strains used and constructed in this study.

Strain	Description	Reference
Escherichia coli DH5α	*E. coli* strain used for cloning and plasmid construction	New England Biolabs
Synechococcus elongatus PCC 7942	Model freshwater cyanobacterium (ATCC 33912); previously used as host for FFA biosynthesis (Ruffing and Jones, 2012; Ruffing, 2013a,b)	American type culture collection
Synechococcus sp. PCC 7002	Model marine cyanobacterium (ATCC 27264); host for FFA biosynthesis	American type culture collection
SE01	*S. elongatus* PCC 7942 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*)	Ruffing and Jones (2012)
SE02	*S. elongatus* PCC 7942 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*) and expression of truncated *E. coli* thioesterase (*'tesA*)	Ruffing and Jones (2012)
SE03	*S. elongatus* PCC 7942 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*) and expression of *C. reinhardtii* acyl-ACP thioesterase (*fat1*)	Ruffing (2013a)
SE04	*S. elongatus* PCC 7942 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*), expression of *C. reinhardtii* acyl-ACP thioesterase (*fat1*), and expression of *rbcLS* (P_{trc}-*fat1*-*rbcLS*)	Ruffing (2013a)
SE06	*S. elongatus* PCC 7942 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*), expression of *C. reinhardtii* acyl-ACP thioesterase (*fat1*), and expression of RuBisCO (*rbcLS*) from the *psbAI* promoter of *S. elongatus* PCC 7942 (P_{trc}-*fat1*-P_{psbAI}-*rbcLS*)	Ruffing (2013a)
S01	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*)	This study
S02	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*) and expression of truncated *E. coli* thioesterase (*'tesA*)	This study
S02Δ*desB*	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*) and desaturase B (*desB*), along with expression of truncated *E. coli* thioesterase (*'tesA*)	This study
S03	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*) and expression of *C. reinhardtii* acyl-ACP thioesterase (*fat1*)	This study
S05	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*) and expression of a truncated *C. reinhardtii* acyl-ACP thioesterase (*tfat1*)	This study
S06	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*), expression of truncated *E. coli* thioesterase (*'tesA*), and expression of RuBisCO (*rbcLS*) (P_{trc}-*'tesA*-*rbcLS*)	This study
S07	*Synechococcus* sp. PCC 7002 with gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*), expression of truncated *E. coli* thioesterase (*'tesA*), and expression of RuBisCO (*rbcLS*) from the *psbAI* promoter of *S. elongatus* PCC 7942 (P_{trc}-*'tesA*-P_{psbAI}-*rbcLS*)	This study

replacing the NSII homologous regions of pSA (Ruffing, 2013a) with 951 and 936 bp fragments homologous to the sequences upstream and downstream of *desB*. The *fat1* thioesterase from *Chlamydomonas reinhardtii* was cloned from pSE20 and integrated at the *Eco*RI and *Bam*HI sites of pS12 to form pS14, using primers listed in Table S2 in Supplementary Material. Strain S03 was constructed by transformation and integration of pS14 in *Synechococcus* sp. PCC 7002. A truncated *fat1* thioesterase (*tfat1*) was constructed by redesigning the forward primer to eliminate the predicted chloroplast-targeting signal, determined using ChloroP 1.1 (Emanuelsson et al., 1999). Integration of *tfat1* into the *Eco*RI and *Bam*HI sites of pS12 yielded pS17, and subsequent transformation and integration of pS17 into *Synechococcus* sp. PCC 7002 produced strain S05.

To insert another restriction enzyme site for *rbcLS* integration, *'tesA* was re-cloned using primer 'tesAR2, yielding pS18. The small and large subunits of RuBisCO (*rbcLS*) from *S. elongatus* PCC 7942 were cloned from pSE18 (Ruffing, 2013a) (primers rbcLSF1/rbcLSR1 in Table S2 in Supplementary Material) and inserted downstream of *'tesA* at the *Aat*II site in pS18 to produce pS19. Transformation of pS19 into *Synechococcus* sp. PCC 7002 yielded strain S06. To insert the *psbA1* promoter from *S. elongatus* PCC 7942 upstream of *rbcLS*, the *psbA1* promoter was cloned from pSE20 and inserted into pS18 to yield pS20. Subsequent

cloning of *rbcLS* from SE20 using the second *rbcLS* primer set (rbcLSF2/rbcLSR2) and insertion into pS20 at the *Bsr*GI site produced pS21. Transformation of pS21 into *Synechococcus* sp. PCC 7002 yielded strain S07.

Escherichia coli DH5α was used for plasmid construction; competent cells were prepared as described in Sambrook and Russell (2001). All *E. coli* DH5α strains used for plasmid maintenance were grown in LB medium at 37°C and 350 rpm in a VWR incubating mini-shaker. After overnight growth in a test tube containing 4 mL of LB media, the plasmid-containing *E. coli* strains were harvested for plasmid isolation using the plasmid mini-prep kit from Zymo Research. Plasmids derived from pS12 were linearized using *Nde*I digestion, and plasmids derived from pSA were linearized using *Spe*I digestion.

CULTURE CONDITIONS

Wild-type and engineered strains of *Synechococcus* sp. PCC 7002 were grown in medium A^+ with antibiotic supplementation as required (40 μg/mL spectinomycin dihydrochloride pentahydrate for S01, S02, S02Δ*desB*, S03, S05, S06, and S07 and 50 μg/mL of kanamycin monosulfate for S02Δ*desB*). Agar plates of medium A^+ contained 1 mM of sodium thiosulfate. Single transformed colonies were re-streaked at least three times or until full segregants were obtained, as confirmed by PCR. *Synechococcus* sp. PCC 7002 strains grown on solid media were inoculated into 4 mL of medium A^+ with antibiotics as necessary and grown at 30 or 38°C and 150 rpm in a New Brunswick Innova 42R shaking incubator with photosynthetic light bank. Alternating cool white and plant fluorescent lights provided ~60 μmol photons m^{-2} s^{-1} of continuous illumination. After 5–7 days of growth, 1 mL of the test tube inoculum was transferred to a 500 mL baffled Erlenmeyer flask containing 100 mL of medium A^+ with appropriate antibiotics. After 4 days of growth under the aforementioned conditions, the 100 mL culture was used to inoculate 400 mL of medium A^+ in a 1 L glass media bottle with a three-port lid for sampling, bubbling of 1% CO_2 in air, and ventilation through a 0.22 μm filter. The initial cell concentration of the 400 mL culture vessels was approximately an OD_{730} of 0.1. No antibiotics were added to the large culture vessels to eliminate any potential effects associated with antibiotic supplementation. The large cultures were sampled every 2 days for ~3 weeks. IPTG was added at 100 h to induce expression from the trc and LlacO-1 promoters.

PHYSIOLOGICAL MEASUREMENTS

Cell growth was measured using optical density (OD) readings at 730 nm from a PerkinElmer Lambda Bio spectrophotometer. Photosynthetic yield measurements were obtained using a Waltz mini-PAM photosynthetic yield analyzer; for each sample, triplicate technical measurements were averaged due to variability associated with these measurements. Photosynthetic yield or efficiency measurements $\left(F_V'/F_m'\right)$ are a fluorescence-based measurement of the photochemical efficiency of photosystem II (PSII) in higher plants. Variable fluorescence (F_v) is the difference between the maximum fluorescence (F_m) under saturated light and the initial fluorescence (F_0) under actinic light; the apostrophe indicates that the samples were not dark adapted prior to measurement. Photosynthetic yield $\left(F_V'/F_m'\right)$ measurements in cyanobacteria are complicated by fluorescence contributions from phycobiliproteins and the low fraction of total chlorophyll associated with PSII; however, $\left(F_V'/F_m'\right)$ measurements in cyanobacteria have been shown to correlate well with changes in the rate of oxygen evolution from PSII (Campbell et al., 1998). In this study, photosynthetic yield measurements are used as a general indicator of photosynthetic efficiency and overall cell health. FFA measurements were performed as described previously (Ruffing and Jones, 2012) using the FFA Quantification Kit from Biovision. Cell growth, photosynthetic yield, and FFA measurements were taken every 2 days. At day 16, the absorbance spectrum of each culture was measured using a Beckman Coulter DU-800 UV-Vis Spectrophotometer to determine changes in photosynthetic pigment concentrations. Each data point is the average of three biological replicates with error bars indicating the standard deviation.

RESULTS

REDUCED FFA TOXICITY IN *SYNECHOCOCCUS* sp. PCC 7002 COMPARED TO *S. ELONGATUS* PCC 7942

Synechococcus sp. PCC 7002 was genetically engineered for FFA production and excretion by targeting the long-chain-fatty-acid CoA ligase (*fadD*, SYNPCC7002_A0675) for gene knockout and overexpression of a truncated thioesterase from *E. coli* (*'tesA*). The resulting engineered strains, S01 (Δ*fadD*) and S02 (Δ*fadD*, *'tesA*$^+$), are analogous to the previously constructed, FFA-producing strains of *S. elongatus* PCC 7942, SE01 (Δ*aas*) and SE02 (Δ*aas*, *'tesA*$^+$). A schematic of the engineered pathway for FFA production can be found in the Supplemental Material (Figure S1). The FFA concentrations, cell growth profiles, and photosynthetic yields of *Synechococcus* sp. PCC 7002, S01, and S02 are shown in **Figure 1**, along with the previous results from *S. elongatus* PCC 7942, SE01, and SE02 (Ruffing and Jones, 2012). As expected, no FFAs are excreted by the wild-type (7002), yet both S01 and S02 synthesized and excreted FFAs. The FFA concentrations produced by *fadD* knockout in S01 (**Figure 1A**) are very low (<6 mg/L), particularly in comparison to the comparably engineered *S. elongatus* PCC 7942 strain, SE01, which produced up to 43 mg/L of excreted FFAs. Expression of the truncated *E. coli* thioesterase in S02, however, increased the level of excreted FFAs, producing concentrations similar to SE01 and higher levels than the analogous *S. elongatus* PCC 7942 strain, SE02 (**Figure 1A**). While the FFA-producing strains of *Synechococcus* sp. PCC 7002 (S01 and S02) did not show improved FFA production in comparison to the *S. elongatus* PCC 7942 strains (SE01 and SE02), there were notable differences in the physiological responses of the hosts. The late exponential growth rates of S01 and S02 showed a slight decrease in comparison to the wild-type 7002 after induction at 100 h (4.17 days) (**Figure 1B**); this small reduction is expected as FFA production reduces the available pool of acyl-ACP for cell membrane biosynthesis. On the other hand, SE01 and SE02 showed a severe reduction in final cell concentrations, 21 and 59% lower than wild-type (**Figure 1B**), much greater than expected due to FFA production. Additionally, the photosynthetic yields of S01 and S02 are very similar to the wild-type 7002, while the photosynthetic yields of SE01 and SE02 were significantly reduced compared to the wild-type 7942 strain (**Figure 1C**). These results suggest that FFA production in *Synechococcus* sp. PCC 7002 does

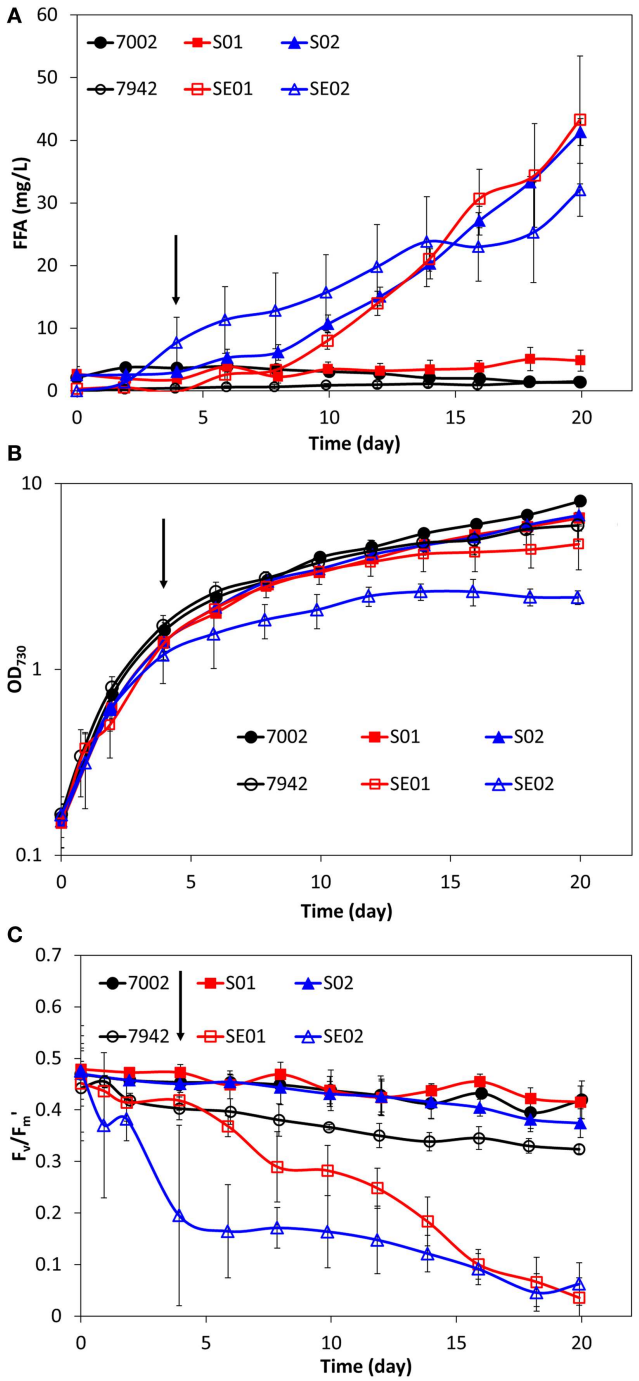

FIGURE 1 | Comparison of extracellular FFA concentration (A), cell concentration (B), and photosynthetic yields (C) during FFA production in two cyanobacterial hosts: *S. elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002. Wild-type strains are illustrated with black circles (7942 – open, 7002 – filled). Strains with gene knockout of the FFA-recycling acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*aas*/*fadD*) are illustrated with red squares (SE01 – open, S01 – filled). Strains with gene knockout of the FFA-recycling gene and *'tesA* expression are illustrated with blue triangles (SE02 – open, S02 – filled). Addition of IPTG is indicated by the arrows (100 h). All data are averages of at least three biological replicates with error bars indicating the standard deviation.

not compromise the cellular physiology of the host strain, unlike the engineered strains of *S. elongatus* PCC 7942, which exhibited a stress response to FFA production and excretion.

FFA TOLERANCE IN *SYNECHOCOCCUS* sp. PCC 7002 IS TEMPERATURE-DEPENDENT

The FFA production experiments described in the previous section and the results reported in **Figure 1** were conducted at a temperature of 30°C for comparison with the engineered *S. elongatus* PCC 7942 strains, which have an optimal growth temperature of 30°C (Golden and Sherman, 1984). However, the reported optimal growth temperature for *Synechococcus* sp. PCC 7002 is 38°C (Sakamoto and Bryant, 1997). Therefore, FFA production experiments were also conducted at this higher temperature for 7002, S01, and S02 (**Figure 2**). As expected, the higher cultivation temperature yielded a slight improvement in FFA concentrations excreted by S01 and S02 (**Figure 2A**), likely due to the increased thermal kinetics associated with the higher temperature. Unexpectedly, cell growth was reduced at the higher temperature for all three strains, including the wild-type (**Figure 2B**). To confirm the culture temperature, the aqueous temperature of the culture vessels was measured using a standard glass thermometer. The aqueous temperature readings indicated that the culture temperatures were slightly higher than the setpoints, 32°C for the 30°C setting and 39°C for the 38°C setting. The lower range light intensities used in this experiment (60 μmol photons m^{-2} s^{-1}) may also influence the optimal growth temperature, and a more rigorous, multi-factorial investigation of *Synechococcus* sp. PCC 7002 growth is required to evaluate the optimal growth temperature of this strain. In addition to this unexpected growth response, there were significant changes in photosynthetic yields at 38°C in comparison to 30°C. At the higher temperature, the wild-type (7002) had reduced photosynthetic yields with values up to 50% lower compared to the 30°C temperature condition (**Figure 2C**). The FFA-producing strains showed an additional decrease in photosynthetic yield, with S02, the highest FFA producer, having the most reduced photosynthetic yields. To investigate whether these reduced photosynthetic yields were accompanied by changes in photosynthetic pigments, the absorbance spectra of the wild-type (7002) and engineered strains (S01 and S02) were measured for cultures at 38 and 30°C after significantly reduced photosynthetic yields were detected (day 16). While the absorbance spectrum for the wild-type (7002) did not show significant differences at the two temperatures, the spectrum of the S02 cultures showed reduced peaks for both chlorophyll-*a* (680 nm) and the phycobiliproteins (635 nm) at 38°C (**Figure 3**). The reduced growth, photosynthetic yields, and photosynthetic pigments that accompany FFA production at 38°C suggest that the FFA tolerance of *Synechococcus* sp. PCC 7002 is temperature-dependent.

At high temperatures, bacteria have been shown to change the degree of saturation of their cell membrane lipids, with increasing saturation accompanying increasing temperatures (Koga, 2012). The increased saturation of membrane FAs in *Synechococcus* sp. PCC 7002 at 38°C may affect the ability of FFAs to cross the cell membrane. Alternatively, the removal of saturated FAs by the recombinant thioesterase may compromise the cell's ability to grow at this higher temperature. To investigate this hypothesis, the

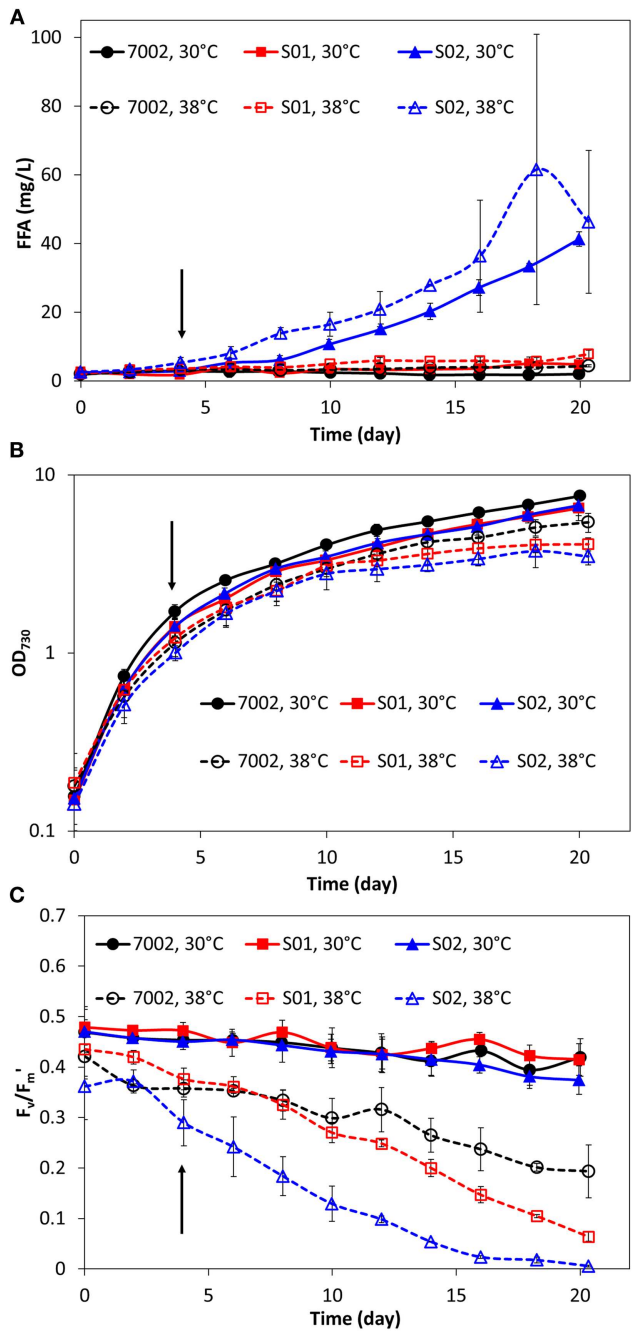

FIGURE 2 | Comparison of extracellular FFA concentration (A), cell concentration (B), and photosynthetic yields (C) during FFA production in *Synechococcus* sp. PCC 7002 strains at two temperatures: 30°C (solid lines, filled markers) and 38°C (dashed lines, open markers). Addition of IPTG is indicated by the arrows (100 h). All data are averages of at least three biological replicates with error bars indicating the standard deviation.

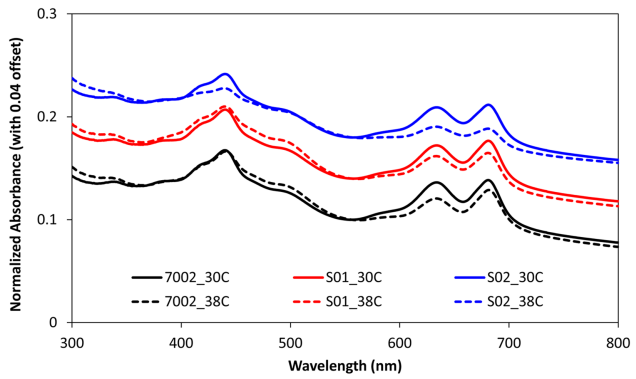

FIGURE 3 | Absorbance spectra of *Synechococcus* sp. PCC 7002 strains at day 16 for 30°C (solid lines) and 38°C (dashed lines) conditions. OD readings were normalized with respect to OD_{556} to account for variations in cell density, and spectra for each strain were offset by 0.04 to aid in visualization. The phycobiliproteins, phycocyanin, and allophycocyanin, have a peak at 635 nm, and chlorophyll-*a* pigments have a peak at 680 nm with Soret bands at 440 nm.

S02 strain was modified via gene knockout of the *desB* gene, encoding a desaturase involved in cold temperature tolerance in *Synechococcus* sp. PCC 7002 (Sakamoto et al., 1997). At 30°C, the *desB* mutant did not show any significant changes in FFA production, growth, or photosynthetic yield in comparison to S02 (data not shown). This suggests that DesB is not involved in FFA tolerance at this temperature, but it does not rule out a role for membrane saturation in the mechanism of temperature-dependent FFA tolerance in *Synechococcus* sp. PCC 7002.

GENETIC TARGETS FOR IMPROVED FFA PRODUCTION

To determine if higher yields of FFA can be produced in *Synechococcus* sp. PCC 7002 compared to *S. elongatus* PCC 7942, additional strategies for improving FFA biosynthesis were investigated. The acyl-ACP thioesterase from *C. reinhardtii* (*fat1*) was previously shown to have activity similar to *'tesA* in *S. elongatus* PCC 7942 (Ruffing, 2013a). Therefore, *fat1* was expressed in *Synechococcus* sp. PCC 7002, along with *fadD* knockout, to form strain S03. Unexpectedly, S03 showed only a slight increase in excreted FFA concentration compared to S01, which has only the *fadD* knockout (**Figure 4A**). The *fat1* gene was previously cloned from the associated *C. reinhardtii* mRNA transcript (Ruffing, 2013a), which likely includes a chloroplast-targeting signal for translocation of Fat1, a nuclear-encoded protein, into the chloroplast. Inclusion of the chloroplast-targeting signal may lead to either low enzyme activity or secretion of the enzyme, as the cyanobacterial membrane is evolutionarily similar to the chloroplast membrane (Giovannoni et al., 1988). To eliminate this potential source of low activity, a putative chloroplast-targeting signal was predicted using ChloroP 1.1 (Emanuelsson et al., 1999), and a new forward primer was designed to exclude this 5′ signal sequence, yielding a truncated *fat1* (*tfat1*). Expression of *tfat1* in S05 led to a nearly threefold increase FFA production and excretion compared to the *fat1*-expressing S03 strain (**Figure 4A**). However, the amount of FFA produced by S05 was still lower than that produced by the *'tesA*-expressing S02 strain. Additionally, S05 had lower growth and photosynthetic yield compared to S02 (**Figures 4B,C**); therefore, further strain development utilized the *'tesA* thioesterase rather than *fat1* or *tfat1* acyl-ACP thioesterases.

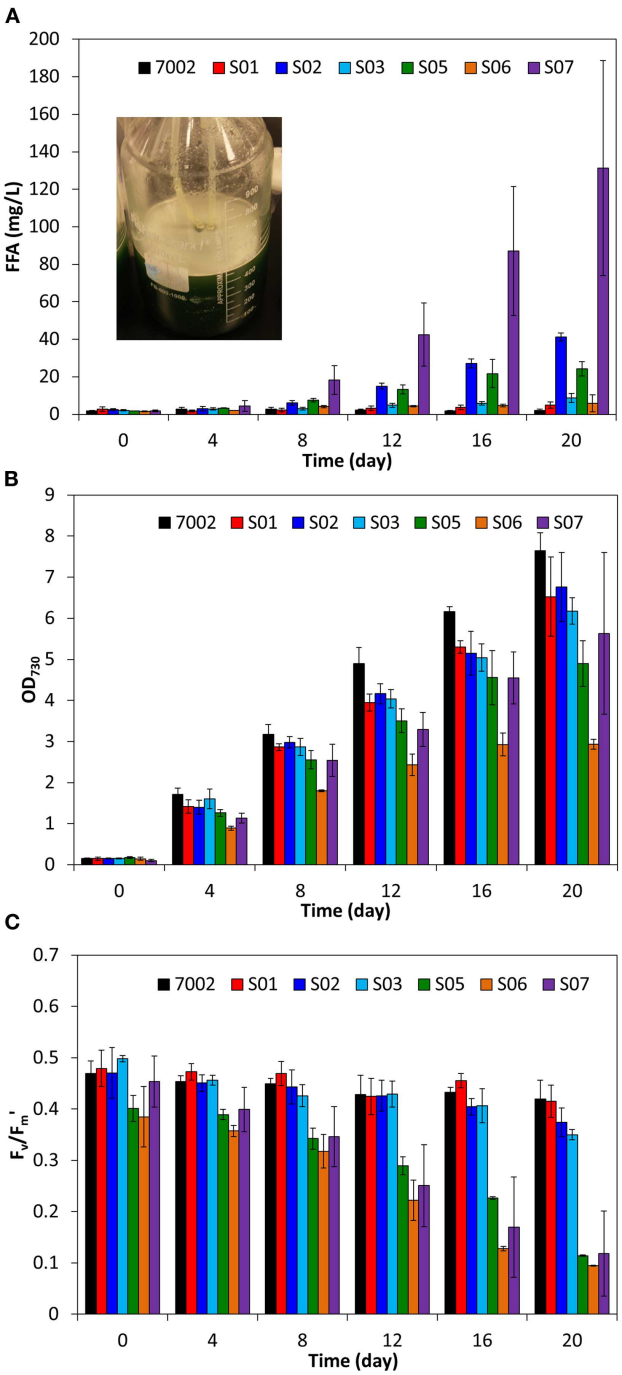

FIGURE 4 | Comparison of extracellular FFA concentration (A), cell concentration (B), and photosynthetic yields (C) during FFA production in engineered *Synechococcus* sp. PCC 7002 strains. The inset image in plot A shows FFA precipitation and floatation (white precipitate) in S07 cultures after 20 days. All data are averages of at least three biological replicates with error bars indicating the standard deviation.

In addition to thioesterase expression, carbon fixation by RuBisCO is an important step for high carbon flux to support both growth and FFA biosynthesis. Similar to RuBisCO overexpression in *S. elongatus* PCC 7942, expression of the large and small subunits of RuBisCO from *S. elongatus* PCC 7942 (*rbcLS*) did not yield an improvement in FFA production in the *Synechococcus* sp. PCC 7002 strain S06 [(Ruffing, 2013a), **Figure 4A**]. Previous quantitative PCR studies in engineered *S. elongatus* PCC 7942 indicated that *rbcLS* expression is not significantly improved by integration of the P_{trc}-*fat1-rbcLS* synthetic operon (Ruffing, 2013a). Therefore, an additional promoter was inserted into the synthetic operon to drive *rbcLS* expression. The *psbA1* promoter from *S. elongatus* PCC 7942 was cloned and inserted after *'tesA* to form the synthetic operon: P_{trc}-*'tesA*-P_{psbA1}-*rbcLS* in S07, an engineered strain of *Synechococcus* sp. PCC 7002. Contrary to the results obtained in the *S. elongatus* PCC 7492 host (Ruffing, 2013a), *rbcLS* overexpression in S07 resulted in more than a threefold increase in excreted FFA concentrations (**Figure 4A**), suggesting that carbon fixation is in fact a rate-limiting step in FFA biosynthesis for *Synechococcus* sp. PCC 7002. The extracellular FFA concentrations in the S07 cultures had high variability, as illustrated by the error bars in **Figure 4A**, which represent the standard deviation of the three biological replicates. This measurement variability was due to FFA precipitation, illustrated by the inset image in **Figure 4A**, which made homogenous sampling difficult. The higher FFA production in S07 also resulted in reduced cell growth and photosynthetic yields (**Figures 4B,C**), indicating that high levels of FFA production may affect cellular physiology in *Synechococcus* sp. PCC 7002.

DISCUSSION

The results presented in this work demonstrate that *Synechococcus* sp. PCC 7002 has a higher tolerance for FFA production compared to the freshwater cyanobacterium, *S. elongatus* PCC 7942. As such, *Synechococcus* sp. PCC 7002 is a beneficial host for photosynthetic FFA biosynthesis. The FFA tolerance of *Synechococcus* sp. PCC 7002 was shown to be dependent on both temperature and FFA concentration, as high temperature or high FFA production compromised the physiology of the host cell. Through gene knockout of the acyl-ACP synthetase/long-chain-fatty-acid CoA ligase (*fadD*), overexpression of the truncated *E. coli* thioesterase (*'tesA*), and overexpression of a heterologous RuBisCO (*rbcLS*), an engineered strain of *Synechococcus* sp. PCC 7002 was constructed, which produced FFAs at concentrations as high as 131 mg/L. The high levels of FFA achieved in this study demonstrate not only a potential for large-scale production applications, but also a potential separation mechanism, as the FFAs precipitated from the culture medium and floated to the surface upon reaching saturation (**Figure 4A**, inset).

Surprisingly, *Synechococcus* sp. PCC 7002 showed higher FFA tolerance at a low growth temperature of 30°C rather than its reported optimum of 38°C. Temperature-induced changes in the chemical composition of the cell membrane may be responsible for the low FFA tolerance of *Synechococcus* sp. PCC 7002 at higher temperatures. The increase in saturated membrane FAs at higher temperatures may influence the ability of FFAs to pass through the cell membrane, ultimately leading to intercalation of the FFAs and membrane damage. Alternatively, expression of the recombinant thioesterase may lead to a reduced level of available saturated FAs for membrane biosynthesis, compromising the cell's

ability to grow at the elevated temperature. High temperatures will also influence the solubility of FFAs, with a positive correlation between FFA solubility and temperature (Oliveira et al., 2009). Thus, the enhanced FFA solubility at 38°C will increase the soluble FFA fraction, leading to higher effective FFA concentrations in the culture. In this study as well as previous work, we have shown that high concentrations of FFAs will lead to detrimental effects on cell growth and physiology. The FFAs may compromise cellular integrity via integration into the cellular membranes and disruption of the activity of membrane-associated enzymes, such as those involved in photosynthesis. While the mechanism of this temperature-dependent FFA tolerance remains to be elucidated, the physiological effects of FFA production can be alleviated in *Synechococcus* sp. PCC 7002 by using lower temperatures (near 30°C) which are more desirable, as these temperatures are readily achieved in open outdoor cultivation systems without additional heating (Moheimani and Borowitzka, 2007).

Cyanobacterial tolerance to exogenous FFAs was previously investigated (Ruffing and Trahan, submitted). When a cytotoxic unsaturated FFA, α-linolenic acid, was exogenously added to cultures of *S. elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002, *S. elongatus* PCC 7942 demonstrated a higher tolerance. At 5 μM of α-linolenic acid, *S. elongatus* PCC 7942 had minimal growth inhibition while *Synechococcus* sp. PCC 7002 growth was reduced by nearly 95%. Unexpectedly, cyanobacterial tolerance to intracellular FFA production led to inverse results, with *Synechococcus* sp. PCC 7002 having improved tolerance over *S. elongatus* PCC 7942. The improved FFA production tolerance of *Synechococcus* sp. PCC 7002 may be related to differences in membrane composition between these two strains. *Synechococcus* sp. PCC 7002 has an elevated amount of polyunsaturated membrane FAs as compared to *S. elongatus* PCC 7942 (unpublished data). The enhanced membrane viscosity associated with a lower degree of membrane saturation may allow for increased FFA diffusion across the cell membrane and therefore higher extracellular FFA concentrations. This proposed mechanism of FFA excretion also supports the aforementioned hypothesis for improved FFA tolerance at lower temperatures. The change in photosynthetic pigments in response to high FFA production also differed for the *Synechococcus* sp. PCC 7002 strains in comparison with the *S. elongatus* PCC 7942 strains. FFA-producing strains of *S. elongatus* PCC 7942 showed selective degradation of chlorophyll-*a* pigment in response to FFA production (Ruffing and Jones, 2012), while *Synechococcus* sp. PCC 7002 strains had reduced levels of both chlorophyll-*a* and the light-harvesting phycobiliproteins, phycocyanin, and allophycocyanin, during FFA production at 38°C. The degradation of phycobiliproteins is a common stress response among cyanobacteria (Collier and Grossman, 1994); however, the selective degradation of chlorophyll-*a* in FFA-producing strains of *S. elongatus* PCC 7942 appears to be an unconserved response.

In addition to differences in FFA stress response, *Synechococcus* sp. PCC 7002 also displayed different responses to the genetic manipulations of *aas/fadD* knockout, thioesterase expression, and RuBisCO expression. While Aas from *S. elongatus* PCC 7942 and FadD from *Synechococcus* sp. PCC 7002 are homologs (58.4% amino acid identity), *aas* knockout in SE01 led to the accumulation of 43 mg/L of excreted FFAs, yet *fadD* knockout in S01 yielded only 5 mg/L of excreted FFAs. These results may indicate that *S. elongatus* PCC 7942 has a naturally higher rate of membrane degradation and FFA recycling compared to *Synechococcus* sp. PCC 7002. In the *S. elongatus* PCC 7942 strains SE02 and SE03, both 'TesA' and 'Fat1' thioesterases had similar activities, generating nearly equivalent levels of excreted FFA (Ruffing, 2013a). In the analogous *Synechococcus* sp. PCC 7002 strains S02 and S03, however, only 'TesA' showed significant activity. Some improvement in Fat1 activity was achieved by using a truncated form, tFat1, with deletion of the predicted chloroplast-targeting signal. The overall low activity of 'Fat1' and 'tFat1' in S03 and S05 may be caused by reduced expression levels or possibly unidentified regulatory mechanisms. Lastly, no improvement in FFA production was achieved with RuBisCO expression in the FFA-producing strains of *S. elongatus* PCC 7942, while *Synechococcus* sp. PCC 7002 strains showed more than a threefold improvement in FFA production with heterologous *rbcLS* expression from the *psbA1* promoter of *S. elongatus* PCC 7942. This difference may indicate that carbon fixation is rate-limiting for *Synechococcus* sp. PCC 7002 but not for *S. elongatus* PCC 7942. Another possible explanation is that amino acid differences in RbcLS from *S. elongatus* PCC 7942 compared to that of *Synechococcus* sp. PCC 7002 led to improved activity in *Synechococcus* sp. PCC 7002. RbcL and RbcS from these two cyanobacteria show 86.2 and 68.81% identity in their amino acid sequences, indicating that these are highly similar proteins but not identical. Lastly, native *rbcLS* activity is post-transcriptionally regulated; thus, overexpression of native *rbcLS* in *S. elongatus* PCC 7942 did not improve carbon fixation, but the activity of *rbcLS* from *S. elongatus* PCC 7942 in the *Synechococcus* sp. PCC 7002 host was not regulated and therefore able to improve carbon flux and FFA biosynthesis. Heterologous RuBisCO expression was also shown to improve biofuel production by twofold in an isobutyraldehyde-producing strain of *S. elongatus* PCC 7942 (Atsumi et al., 2009). Codon usage in *S. elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002 is very similar, with an average difference of 0.418 in percent usage for each codon sequence; this difference is slightly higher for the codons used in *rbcLS* (a difference of 13.3), suggesting that further improvement may be achieved with codon optimization. Regardless of the underlying mechanisms for improved FFA tolerance and production, *Synechococcus* sp. PCC 7002 was shown to be a superior host for FFA biosynthesis compared to *S. elongatus* PCC 7942. This work highlights the importance of host selection in metabolic engineering efforts, and since host responses are difficult to predict *a priori*, screening and testing of potential hosts remains a critical step in cyanobacterial strain development.

To date, three model cyanobacterial strains have been genetically engineered for FFA production: *Synechococcus* sp. PCC 7002 (this study), *S. elongatus* PCC 7942 (Ruffing and Jones, 2012; Ruffing, 2013a), and *Synechocystis* sp. PCC 6803 (Liu et al., 2011). These three strains are model hosts for biofuel production, as they have available genome sequences and established genetic tools and protocols for genetic manipulation. A comparison of these three strains with regard to their FFA tolerance, FFA production, and other desirable strain traits is presented in **Table 2**. While engineered *Synechocystis* sp. PCC 6803 strains yielded the

Table 2 | Comparison of FFA production and host strain traits of three model cyanobacterial strains.

Property	*Synechococcus* sp. PCC 7002	*S. elongatus* PCC 7942	*Synechocystis* sp. PCC 6803
FFA productivity (mg/L/h)[a]	0.273 (This study)	0.103 (Ruffing and Jones, 2012)	0.438 (Liu et al., 2011)
Final FFA concentration (mg/L)	131 (This study)	49.3 (Ruffing and Jones, 2012)	197 (Liu et al., 2011)
Exogenous FFA tolerance	Saturated FFA: no growth inhibition, polyunsaturated FFA: tolerant to $<1\,\mu M$ before growth is inhibited (Ruffing and Trahan, submitted)	Saturated FFA: no growth inhibition, polyunsaturated FFA: tolerant to $5\,\mu M$ before growth is inhibited (Ruffing and Trahan, submitted)	Saturated FFA: no growth inhibition, polyunsaturated FFA: tolerant to $25\,\mu M$ before growth is inhibited (Ruffing and Trahan, submitted)
Doubling time (h)	2.6–4 (Sakamoto and Bryant, 1997; Ludwig and Bryant, 2012)	5–6 (Kondo et al., 1997)	6 (Tu et al., 2004)
Light tolerance	Can grow under 4,500 μmol photons $m^{-2}\,s^{-1}$ (Nomura et al., 2006)	Significant photodamage occurs with light intensities of 500–1000 μmol photons $m^{-2}\,s^{-1}$ (Clarke et al., 1995; Kulkarni and Golden, 1995)	At 900 μmol photons $m^{-2}\,s^{-1}$, rate of photodamage = rate of repair (Allakhverdiev and Murata, 2004)
Salt tolerance	1.7 M NaCl (Batterton and Baalen, 1971)	0.5 M NaCl (Fulda et al., 1999)	1.2 M NaCl (Fulda et al., 1999)
Temperature tolerance	Optimum = 34–38°C [this study (Ludwig and Bryant, 2012)], maximum growth temperature not determined	Optimum = 30–35°C (Mori et al., 2002), maximum growth temperature not determined	Optimum = 30°C, cannot grow above 43°C (Inoue et al., 2001)

[a] *FFA productivity calculated by taking the final concentration of excreted FFA concentration and dividing by the total time of cultivation.*

highest FFA productivity and final FFA concentration, the genetic modifications in these strains (i.e., deletion of surface layer proteins in the cell wall) may weaken the integrity of the host strain and its ability to survive under adverse conditions, which may be experienced in large-scale production systems. Moreover, as the genetic manipulations differ between the engineered strains of *Synechocystis* sp. PCC 6803 and those of *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942, this direct comparison must be viewed with caution. *Synechocystis* sp. PCC 6803 also has the highest tolerance of exogenous FFA, yet the results of this study clearly demonstrate that exogenous FFA tolerance does not directly correlate with tolerance to intracellular FFA biosynthesis. When comparing inherent strain properties of doubling time, high light tolerance, high salt tolerance, and temperature tolerance, *Synechococcus* sp. PCC 7002 is superior to the other two cyanobacteria. These basic strain traits, which can be difficult to engineer into a host, make *Synechococcus* sp. PCC 7002 a beneficial host for cyanobacterial biofuel production, and this study provides direct evidence of the advantages afforded by this host, encouraging further investigation and development of *Synechococcus* sp. PCC 7002 for biofuel production.

AUTHOR CONTRIBUTIONS

Anne M. Ruffing conceived the study, conducted all experimental work and data analysis, and drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fbioe.2014.00017/abstract

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Lauric acid production in a glycogen-less strain of *Synechococcus* sp. PCC 7002

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The cyanobacterium *Synechococcus* sp. Pasteur culture collection 7002 was genetically engineered to synthesize biofuel-compatible medium-chain fatty acids (FAs) during photoautotrophic growth. Expression of a heterologous lauroyl-acyl carrier protein (C12:0-ACP) thioesterase with concurrent deletion of the endogenous putative acyl-ACP synthetase led to secretion of transesterifiable C12:0 FA in CO_2-supplemented batch cultures. When grown at steady state over a range of light intensities in a light-emitting diode turbidostat photobioreactor, the C12-secreting mutant exhibited a modest reduction in growth rate and increased O_2 evolution relative to the wild-type (WT). Inhibition of (i) glycogen synthesis by deletion of the *glgC*-encoded ADP-glucose pyrophosphorylase (AGPase) and (ii) protein synthesis by nitrogen deprivation were investigated as potential mechanisms for metabolite redistribution to increase FA synthesis. Deletion of AGPase led to a 10-fold decrease in reducing carbohydrates and secretion of organic acids during nitrogen deprivation consistent with an energy spilling phenotype. When the carbohydrate-deficient background (Δ*glgC*) was modified for C12 secretion, no increase in C12 was achieved during nutrient replete growth, and no C12 was recovered from any strain upon nitrogen deprivation under the conditions used. At steady state, the growth rate of the Δ*glgC* strain saturated at a lower light intensity than the WT, but O_2 evolution was not compromised and became increasingly decoupled from growth rate with rising irradiance. Photophysiological properties of the Δ*glgC* strain suggest energy dissipation from photosystem II and reconfiguration of electron flow at the level of the plastoquinone pool.

Keywords: cyanobacteria, turbidostat photobioreactor, fatty acid secretion, metabolic overflow, organic acids, *glgC*, nitrogen deprivation, dodecanoic acid

INTRODUCTION

Photosynthetic metabolism generates a wide range of biomolecules fundamental to energy, agriculture, and health (Durrett et al., 2008; Hu et al., 2008; Atsumi et al., 2009; Lubner et al., 2009; Lindberg et al., 2010; Lu, 2010; Niederholtmeyer et al., 2010; Kilian et al., 2011; Wahlen et al., 2011; Ducat et al., 2012; Elliott et al., 2012; Work et al., 2013; Sorek et al., 2014). Having rapid growth rates, efficient energy conversion, and metabolic adaptability, photosynthetic microorganisms (PSMs) including genetically tractable unicellular algae and cyanobacteria have received substantial attention for synthesizing biofuel precursors via native or transgenic processes (Ferrari et al., 1971; Cascon and Gilbert, 2000; Heifetz, 2000; Lee, 2001; Schmer et al., 2008; Rodolfi et al., 2009; Elliott et al., 2011; Fore et al., 2011; Liu et al., 2011; Radakovits et al., 2011; Soratana and Landis, 2011; Rosgaard et al., 2012; Bentley et al., 2013; Gronenberg et al., 2013; Leite et al., 2013; Möllers et al., 2014; Davies et al., 2015).

Biodiesel can be derived from biological fatty acids (FAs) extracted from photosynthetic organisms (Ma and Hanna, 1999; Durrett et al., 2008; Hu et al., 2008). While oil content and quality differs between species, the composition of FAs typically includes the predominant 16- and 18-carbon FAs (C16 and C18), as well as varying levels of shorter (C8–C14) and longer (≥C20) FAs (Gopinath et al., 2010). Currently, vegetable oil is the main source of the approximately 20 billion liters (L) of biodiesel produced yearly worldwide (Hoekman et al., 2012; Kopetz, 2013). However, recent efforts in genetic engineering seek to utilize microorganisms for FA production, a concept that would transition fuel production from cropland to bioreactors or pond systems (Lee, 2001; Hu et al., 2008; Li et al., 2008; Rodolfi et al., 2009; Leite et al., 2013). Lauric acid (C12:0) is naturally synthesized by coconut, palm, and bay trees (Litchfield et al., 1967; Denke and Grundy, 1992) and, when esterified, exhibits qualities comparable to modern diesel fuel, with better cold-flow properties relative to longer chain FAs (Gopinath et al., 2010; Hoekman et al., 2012). Microbial C12 synthesis has been achieved via transgenics in both heterotrophic and photoautotrophic hosts (Ohlrogge et al., 1995; Lu et al., 2008; Liu et al., 2011; Radakovits et al., 2011; Lennen and Pfleger, 2012), offering diverse opportunities in production platforms.

Abbreviations: ADP, adenosine diphosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LED, light-emitting diode; PCC, Pasteur culture collection.

In photosynthetic eukaryotes, FAs of specific chain lengths are hydrolyzed from acyl carrier protein (ACP) by thioesterase enzymes, and the released FAs move out of the chloroplast into the cytoplasm where they are activated by coenzyme A (CoA) to facilitate transfer into higher lipids (Radakovits et al., 2010; Li et al., 2013). It has been found that many bacteria, including cyanobacteria, typically bypass the free FA (FFA) intermediate when assembling newly synthesized FA into membrane lipids (Sato and Wada, 2010; Jansson, 2012). When heterologous thioesterases are expressed in certain bacteria, most of the hydrolyzed FFA is found either in the culture medium or associated with the outside of the cell (Voelker and Davies, 1994; Ohlrogge et al., 1995; Liu et al., 2011; Zhang et al., 2011; Ruffing and Jones, 2012; Ruffing, 2014). Though the mechanism of secretion is not established, it is known that extracellular FFA can move back across the membrane and be reincorporated into metabolism by an acyl–acyl carrier protein synthetase (AAS) (Kaczmarzyk and Fulda, 2010). If this enzyme is disrupted, FFAs remain in the medium and can separate from the aqueous cultures. In the present study, carbon distribution in the cyanobacterium *Synechococcus* sp. Pasteur culture collection (PCC) 7002 was modified for C12 FFA synthesis by heterologous thioesterase expression (and AAS deletion) in both wild-type (WT) and carbohydrate-deficient genetic backgrounds.

MATERIALS AND METHODS

GENETIC ENGINEERING OF *SYNECHOCOCCUS* sp. PCC 7002

Synechococcus sp. PCC 7002 (*Synechococcus* sp. 7002 hereafter) was genetically modified using previously described protocols (Frigaard et al., 2004; Xu et al., 2011). The pAQ1Ex plasmid containing the *Synechocystis* sp. 6803 promoter *cpcBA* (Xu et al., 2011) and spectinomycin antibiotic-resistance gene *aadA* (Frigaard et al., 2004); and the kanamycin-resistant Δ*glgC* mutant (Guerra et al., 2013) harboring the gene *aphII* (Frigaard et al., 2004) were kindly provided from the laboratory of Donald A. Bryant. In *Synechococcus* sp. 7002, the gene *glgC* (NC_010475.1) encodes ADP-glucose pyrophosphorylase (AGPase), which activates glucose for polymerization. The gene *fadD* (NC_010475.1) encodes a putative AAS with homology to the *Synechocystis* sp. PCC 6803 gene *slr1609* (NC_000911.1) (Kaczmarzyk and Fulda, 2010; Gao et al., 2012). A thioesterase derived from *Umbellularia californica* encoded by the gene *fatB1* (GenBank M94159) hydrolyzes 12-carbon FA chains from ACP during FA synthesis yielding lauric acid (C12), and a version of this gene codon optimized for expression in *Synechocystis* sp. 6803 was generously provided from the laboratory of Roy Curtiss III (Liu et al., 2011). Lauric acid and C12 in the text designate transesterifiable 12-carbon saturated fatty acyl chains.

The pAQ1Ex vector was modified for knockin expression of *fatB1* concurrent with deletion of the putative AAS. To construct the lauric acid secretion (LAS) module, *fatB1* was placed between the vector's promoter and antibiotic selection marker via *Nco*I/*Bam*HI restriction sites. Flanking sequences of the *fadD* gene were inserted to target the cassette for homologous recombination using F||R primer pairs (5′–3′) 1:gttcacATGCATggctaggttcgtaatctttgggggtaTgtatagGAATTCgccgaaatcatggctacaatcctacttt, 2:catactGTCGACgatccgaatggcggaatcttcgTgttcacGCATGCgtgctggctttttgt cacaatcttcttg (restriction enzyme recognition sequences used in plasmid construction are capitalized). Transformation was accomplished following an established protocol for homologous recombination in this organism (Xu et al., 2011). Integration of the LAS module into all genome copies was achieved by increasing spectinomycin pressure and confirmed by PCR (not shown) for complete allele segregation using the 1F and 2R primers listed above. The strain SA01 contains the LAS module in a WT background, and the strain SA13 contains this module in a carbohydrate-deficient background (**Table 1**).

BATCH CULTIVATION

The saltwater medium A+ used in batch experiments contained, per liter, 18 g NaCl, 5 g $MgSO_4 \cdot 7H_2O$, 1 g $NaNO_3$, 0.6 g KCl, 0.05 g KH_2PO_4, 0.03 g Na_2-EDTA, 0.27 g $CaCl_2$, 1 g Trizma base (Tris), 1 mL L^{-1} of 3.89 g L^{-1} $FeCl_3 \cdot 6H_2O$ stock in 0.1 N HCl, and 1 mL L^{-1} of P1 metals micronutrient solution. The P1 stock solution contained, per liter, 34.26 g H_3BO_3, 4.32 g $MnCl_2 \cdot 4H_2O$, 0.315 g ZnCl, 0.03 g MoO_3 (85%), 12.15 mg $CoCl_2 \cdot 6H_2O$, and 3 mg $CuSO_4 \cdot 5H_2O$. For A+ medium without nitrogen (−N), $NaNO_3$ was replaced by an equimolar amount of NaCl.

Liquid cell cultures were grown using a rotary shaker under constant illumination in an atmosphere of 1% CO_2, 34°C, and 160 μmol photons m^{-2} s^{-1} (μmol m^{-2} s^{-1} hereafter) photosynthetically active radiation (PAR) in 250-mL Erlenmeyer flasks with soft caps to facilitate gas exchange (VWR, Radnor, PA, USA). Batch flask cultures were grown in quadruplicate and standardized to 2.5 mg L^{-1} chlorophyll *a* at the beginning of each experiment. Pre-cultures were similarly normalized and grown to mid-linear phase (15–25 μg mL^{-1} chlorophyll *a*), whereupon cells were concentrated by centrifugation and resuspended in fresh medium for experimental replicates, which were sampled over a time course.

CONTINUOUS CULTURE

Steady-state physiology was assayed in a photobioreactor (PBR) that maintains constant optical density (turbidostasis) over a range of light intensities delivered by 630 and 680 nm light-emitting diodes (LEDs), as described previously (Melnicki et al., 2013). For maximal light penetration and steady-state illumination, cell cultures were maintained at 0.08 OD_{730} in A+ medium containing 0.9 g L^{-1} NH_4Cl as the nitrogen source, and Tris was omitted as pH 7.5 was maintained independently. Cultures were held at

Table 1 | *Synechococcus* sp. 7002 strains used in this study.

Strain	Description	Genotype
WT	Wild-type *Synechococcus* sp. 7002	
SA01	Secretes lauric acid[a]	Δ*fadD*::P_{cpcBA}-*fatB1-aadA*[b]
Δ*glgC*	RC-deficient (AGPase disrupted)	Δ*glgC::aphII*
SA13	RC-deficient and secretes lauric acid	Δ*glgC::aphII*, Δ*fadD*::P_{cpcBA}-*fatB1-aadA*

Antibiotic-resistance markers confer resistance to spectinomycin (aadA) or kanamycin (aphII) (Frigaard et al., 2004). The cpcBA promoter drives fatB1 expression. RC, glucose-equivalent reducing carbohydrate.

[a] *Transesterifiable C12:0.*

[b] *LAS module.*

30°C and constantly sparged with N_2 gas containing 1.3% CO_2 at 4.1 L min^{-1}. Doubling times were calculated by ln(2)/dilution rate, O_2 evolution by percent air saturation, photophysiology by pulse amplitude modulation (PAM) fluorometry, and biochemical composition were measured in WT, SA01, and Δ*glgC* strains at light intensities of 5/5 (33), 10/10 (66), 15/15 (99), 20/20 (132), 25/25 (165), 40/40 (264), 60/60 (396), 70/70 (462), 125/60 (610), and 170/60 (759) as incident 630/680 nm light each and (in parentheses) total spherical μmol m^{-2} s^{-1}. A linear 2π incident sensor was used to measure individual wavelengths, and total spherical illumination was reported by a 4π sensor. Absorbance scans of total cell culture were measured over a 350–900 nm spectral range using a Shimadzu BioSpec 1601 spectrophotometer (Shimadzu, Kyoto, Japan). Non-transmitted fractions of 630 and 680 nm light were calculated using transmitted light values obtained *in situ* from the linear sensor and normalized to total spherical irradiance as described previously (Melnicki et al., 2013).

BIOCHEMICAL ANALYSES

Chlorophyll *a* was measured by absolute methanol extraction of a 1-mL cell pellet and calculated as described previously (Meeks and Castenholz, 1971; Porra et al., 1989). Reducing carbohydrates (RCs) were measured as glucose equivalents by a colorimetric anthrone–sulfuric acid assay described previously (Meuser et al., 2012).

Dry cell weight (DCW) of batch cultures was measured from 2 mL of liquid culture concentrated by centrifugation. The cell pellet was washed once in 1 g L^{-1} Tris buffer (TB), resuspended in 1 mL TB, thoroughly dried at 80°C, and the dry weight of 1 mL TB subtracted to give DCW. From PBR cultures, DCW is represented as ash-free weight from 400 mL steady-state culture concentrated by centrifugation, resuspended in distilled water, dried at 105°C, and burned at 550°C for 1 h. Ash-free weight was calculated as mass lost between drying and burning.

Organic acids (OAs) were quantified by HPLC (Surveyor Plus, Thermo Scientific, Waltham, MA, USA) using 0.45-μm filtered supernatant from −N cultures. A 25-μL sample was injected onto a 150 mm × 7.8 mm fermentation monitoring column (BioRad, Hercules, CA, USA) at 0.5 mL min^{-1} 8 mM H_2SO_4 eluent, 45°C column operating temperature, and 50°C refractive index (RI) detector operating temperature, in parallel with a photodiode array detector for absorbance at 210 nm. A standard mix of acetate, pyruvate, succinate, α-ketoglutarate, and α-ketoisocaproate was used for quantification, and all samples were held at 10°C in a thermostated sample tray before injection.

Fatty acyl content was measured as transesterifiable fatty acid methyl esters (FAMEs) using an adapted method (Radakovits et al., 2011). Briefly, 0.5 mL of liquid culture was hydrolyzed and lipids saponified at 100°C for 2 h in 1 mL 95:5% v/v absolute methanol:0.8 g L^{-1} KOH (in H_2O), after which 1.5 mL 94.2:5.8% v/v methanol:12N HCl was added for acid-catalyzed methylation at 80°C for 5 h. FAMEs were extracted into 1 mL *n*-hexane and the extract was analyzed using an Agilent 7890A gas chromatograph (GC) and DB-5ms column with flame ionization detection (Agilent Technologies, Santa Clara, CA, USA). A flow rate of 1.15 mL min^{-1} H_2 carrier gas was used to separate FAMEs at 20°C min^{-1} to 230°C, held for 1 min, then 20°C min^{-1} to 310°C and held for 5 min. A standard mix of FAMEs was used for quantification and retention time correlation (37-component FAME mix, Supelco, Bellefonte, PA, USA). Due to insufficient resolution between unsaturated C18 FAs, the combined contents of 18:1, 18:2, and 18:3 are reported as 18:*n*. The unknown (unk) compound that elutes prior to C16:1 was not included in FAME tabulations. A two-tailed *t*-test was performed to determine statistical significance (*p*-value). Lauric acid methyl ester (C12 FAME) was identified via mass spectral analysis conducted using a Varian 3800 GC and Varian 1200 quadrupole MS/MS (Agilent Technologies, Santa Clara, CA, USA) equipped with a Rxi-5ms column (30 mm × 0.25 mm; 0.25 μm film thickness) (Restek Corporation, Bellefonte, PA, USA). A flow rate of 1.2 mL min^{-1} He carrier gas was used to separate FAMEs at 20°C min^{-1} from 70 to 230°C for a 1-min hold, then 20°C min^{-1} to 310°C for a 5-min hold. Mass spectra were obtained after electron ionization at 70 eV. Results were compared to the known mass spectrum of C12 FAME (NIST Mass Spec Data Center, and Stein, 2015).

PULSE AMPLITUDE MODULATION FLUOROMETRY

Variable chlorophyll fluorescence was measured using PAM fluorometry in a DUAL-PAM-100 system (Walz GmbH, Effeltrich, Germany) with a photodiode detector and RG665 filter (Schreiber, 1986). Red measuring light (620 nm) at the lowest power was pulsed at 1000 Hz during the dark and at 10,000 Hz during 635 nm actinic illumination at 98 μmol m^{-2} s^{-1}. From PBR cultures, 3 mL was immediately transferred to a cuvette and fluorescence induction was measured with a programed script consisting of 15 s darkness, 30 s actinic illumination (O), application of a saturating pulse at 2000 μmol m^{-2} s^{-1} for 200 ms (J), 5 s of only far-red light (730 nm) (I), another 15 s of actinic light (P), and 30 s of darkness (S). Variable fluorescence observed during the O-J-I-P-S induction provided the basis to compare changes in the electron transport processes downstream of PSII. The effective quantum yield of PSII (YII') was measured by transient fluorescence changes between "J" and "I" states. The estimated redox status of the plastoquinone (PQ) pool was determined by the rise from "I" to "P" level, normalized to the total variable fluorescence observed over this period, and subtracted from 1 (Chylla and Whitmarsh, 1989). Relative changes in electron transport downstream of the PQ pool were measured by P >> S quenching as the drop from "P" to "S" states relative to the variable fluorescence (Serrano et al., 1981). The relative dark rate of PQ oxidation was obtained from the declining slope of post-illumination fluorescence, calculated from between 10 and 20 s after the level had peaked (Ryu et al., 2003). Dark-adapted measurements were taken after cells were held in the dark for 20 min and then acclimatized in actinic light for 90 s before induction.

RESULTS

BATCH CULTURE PRODUCTIVITY

Nitrogen replete and nitrogen deplete batch cultures of WT, SA01, Δ*glgC*, and SA13 were analyzed over 48 h for chlorophyll *a*, DCW, FAME, RC, and OA. Cultures of C12-secreting strains developed a layer of surfactant bubbles (**Figure 1**).

Chlorophyll a and dry cell weight

Bulk biomass accumulation in nutrient replete batch cultures yielded an increase in chlorophyll *a* content of 12- to 15-fold over 48 h (Figure S1A in Supplementary Material), and DCW accumulated 4- to 5-fold (Figure S1C in Supplementary Material). In nitrogen-deplete cultures, growth attenuation was suggested by unchanging chlorophyll *a* content and DCWs that were within the range of error relative to inoculum DCWs over the time course (Figures S1B,D in Supplementary Material).

Fatty acids

Secretion conferred by the LAS modification of transesterifiable C12 FAs into batch culture medium is demonstrated in **Figures 2A–D**. The identity of transesterified C12 was confirmed by GC–MS (**Figure 2E**). Lauric acid was detected neither during nitrogen starvation nor when *fatB1* was expressed at a neutral locus without concurrent deletion of the putative AAS *fadD* (not shown). After 48 h, total FAME recovered from nutrient replete batch cultures reached 85.0 ± 0.7 (WT), 83.6 ± 4.5 (SA01), 95.3 ± 5.0 ($\Delta glgC$), and 93.6 ± 6.1 (SA13) mg L^{-1} (**Figure 3A**), representing 3–5% of DCW (**Figure 3B**). Over 48 h, SA01 and SA13 generated, respectively, 9.1 ± 0.4 and 8.7 ± 0.6 mg L^{-1} C12, accounting for ~10% of total FAME (**Table 2**). C12 was recovered from culture medium of SA01 and SA13, respectively, at concentrations of 4.4 ± 0.4 and 3.5 ± 0.2 mg L^{-1} after the first 24 h, and 6.5 ± 0.6 and 5.9 ± 0.2 mg L^{-1} after 48 h (**Figure 3C**), an ~70% secretion level in both strains.

Distribution of FAME in 48-h nutrient replete cultures (**Table 2**) is consistent with previous studies of *Synechococcus* sp. 7002, including cumulative levels of unsaturated C18 FA (Kenyon, 1972; Sakamoto et al., 1997; Sakamoto and Bryant, 2002). Strains with the LAS modification showed diminished contents of 16:0 ($p < 0.02$), 16:1 ($p < 0.001$), and 18:*n* ($p < 0.01$). All three mutants contained twofold more 18:0 than WT. The $\Delta glgC$ mutant exhibited less 16:1 than WT ($p < 0.02$), while 16:0 and 18:*n* occurred at WT levels. In the AGPase-disrupted background, the LAS modification conferred a lower fraction

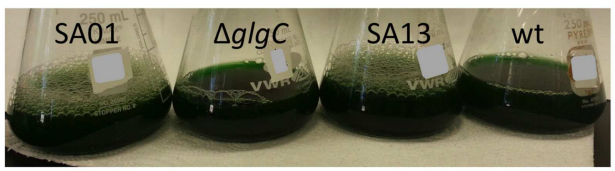

FIGURE 1 | Foaming is visible atop culture medium of the lauric acid-secreting *Synechococcus* sp. PCC 7002 strains SA01 and SA13.

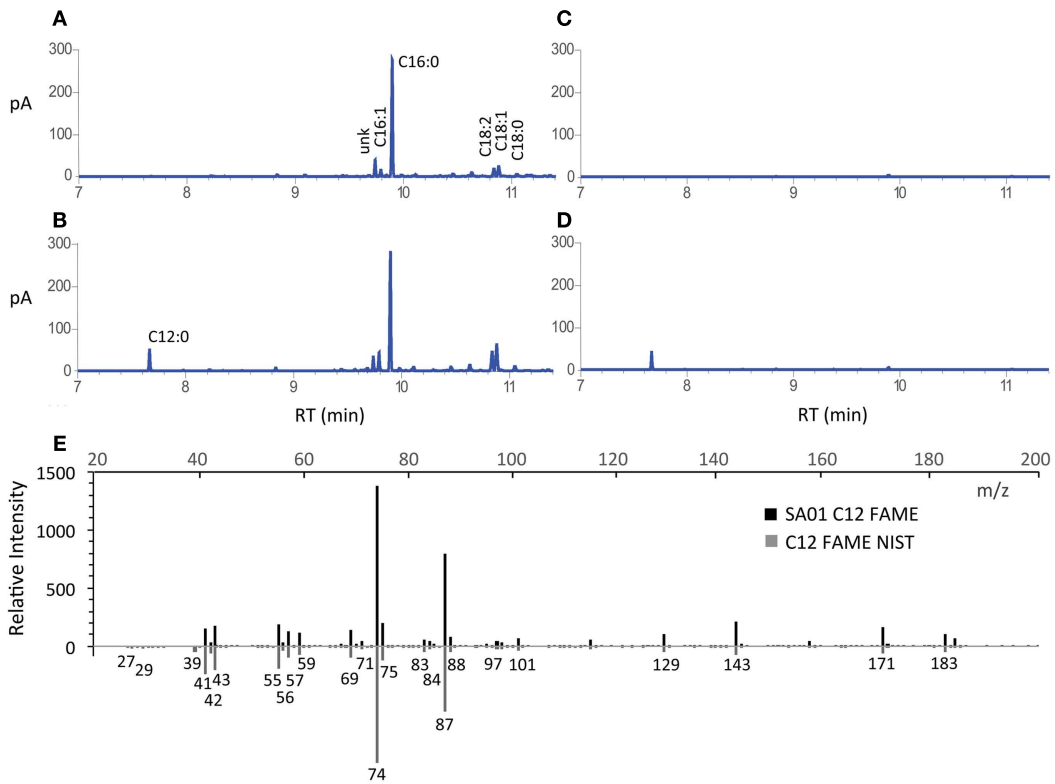

FIGURE 2 | FAME profiles and identification of secreted FA from batch cultivation of WT and SA01 strains of *Synechococcus* sp. 7002. GC-FID chromatograms of FAME from representative **(A)** WT and **(B)** SA01 cell culture, and **(C)** WT and **(D)** SA01 cell-free supernatant. **(E)** Mass spectrum from GC–MS of SA01 FAME matching lauric acid methyl ester from the NIST database (C12 FAME NIST) (NIST Mass Spec Data Center, and Stein, 2015). The labeled standard is shown on the mirror axis. m/z, mass-to-charge ratio; pA, picoamps (detection current); RT, retention time.

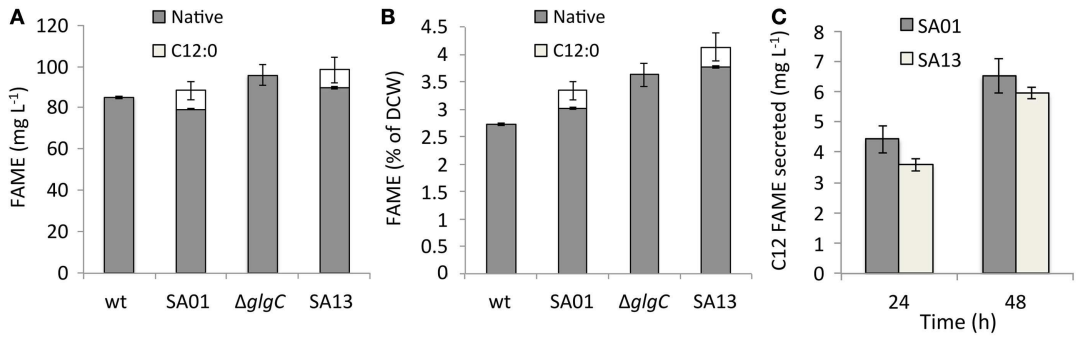

FIGURE 3 | FAME content of nutrient replete batch cultures after 48 h represented (A) by volume and (B) as a function of average dry cell weight (DCW). Native, endogenous fatty acyls; C12:0, heterologous lauric acid. **(C)** C12 FAME recovered from the medium of nutrient replete SA01 and SA13 cultures after 24 and 48 h. Error bars represent SD over four biological replicates.

Table 2 | Percent of total FAME by chain length from 48-h nutrient replete cultures, corresponding to Figure 3.

	% of total FAME				
Strain	12:0	16:0	16:1	18:0	18:*n*
wt	nd	53.1 ± 0.8	11.8 ± 0.3	1.7 ± 0.1	33.4 ± 0.6
SA01	10.9 ± 0.4[a] 7.8 ± 0.4[b]	50.2 ± 0.9	7.3 ± 0.3	3.1 ± 0.4	28.4 ± 0.6
Δ*glgC*	nd	53.5 ± 1.1	10.2 ± 0.6	3.6 ± 0.7	32.6 ± 0.7
SA13	9.0 ± 0.5[a] 6.3 ± 0.2[b]	48.6 ± 0.2	8.3 ± 0.5	3.2 ± 1.1	30.8 ± 0.5

The two values for C12:0 represent total (a) and secreted (b) FAs and are not additive. FAMEs are derived from lauric (12:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic (C18:3) FAs. Unsaturated C18 FA content is grouped (18:n). Error is represented as SD over four biological replicates.

[a] Cell culture.

[b] Cell-free supernatant of [a].

nd, none detected.

($p < 0.02$) of C12 relative to total FAME under the culturing conditions used.

Carbohydrates and organic acids

During nitrogen deprivation (−N), AGPase-disrupted strains accumulated substantially less RC than the WT background: over 24–48 h, RC on a culture volume basis reached 7–11% of WT levels in Δ*glgC* and 6–13% in SA13 (**Figure 4A**). Under the same conditions, RC comprised 30% of DCW in the WT background after 24 h and remained at this percentage over the next 24 h; whereas RC was accumulated to 4–5% of DCW in Δ*glgC* and 4–7% in SA13. The carbohydrate-deficient background secreted OA during −N to a total of 15% of DCW in both Δ*glgC* and SA13 after 24 and 48 h. Neither WT nor SA01 secreted detectable amounts of OA over the −N time course, and OAs were not detected in nutrient replete growth media (not shown). Combined, RC and OA (RC + OA) in the AGPase-disrupted strains accumulated under −N conditions to 19% of DCW. Relative to WT RC levels, RC + OA in Δ*glgC* cultures reached 31% after 24 h and 39% after 48 h, and SA13 reached 28 and 38% of WT, respectively (**Figure 4A**). While WT and SA01 cultures developed yellow coloration during −N, the AGPase-disrupted cultures did not (**Figure 4B**). Under the culturing conditions used, acetate was the most abundant OA secreted from the carbohydrate-deficient strains during −N, followed by succinate and α-ketoisocaproate (**Figures 4C–E**). Lesser concentrations of pyruvate and α-ketoglutarate were also observed (**Figures 4F,G**). Levels of OA secretion were not affected by the LAS modification.

STEADY-STATE PHYSIOLOGY

At stable growth rate for each indicated light intensity in the LED-PBR, cultures of *Synechococcus* sp. 7002 WT, SA01, and Δ*glgC* were analyzed for RC, DCW, FAME, O_2 evolution, doubling rate, and photophysiological characteristics. Growth rate and O_2 production measurements for the same conditions were made previously using a separate cultivar of WT *Synechococcus* sp. 7002 (not shown), which demonstrate the reproducibility of PBR measurements (Work, 2014).

Biomass profiles

Disruption of AGPase inhibited RC accumulation while RC levels in the WT background increased with light intensity (**Figure 5**). At 610 μmol m^{-2} s^{-1}, RC represented 51% of DCW in WT and 43% in SA01, but Δ*glgC* reached only 10% of DCW which occurred at 264 μmol m^{-2} s^{-1}. Due to the dilute concentration of PBR cultures, FAMEs of C16:0 and C12:0 (C12 hereafter) were detectable but not quantifiable (<1 mg L^{-1}). In representative PBR cultures at 396 μmol m^{-2} s^{-1}, all observed C12 was recoverable from SA01 cell-free filtrate, and C12 was not detected in WT or Δ*glgC* (not shown) (Work, 2014).

Growth rates and O_2 evolution

Minimum stable doubling times of 3.5 h (WT), 3.8 h (SA01), and 4.6 h (Δ*glgC*) were observed at 759 μmol m^{-2} s^{-1} in the WT background and at 462 μmol m^{-2} s^{-1} in Δ*glgC* (**Figure 6A**). Bulk O_2 evolved by SA01 exceeded both WT and Δ*glgC* over the majority of light intensities tested (**Figure 6B**). On a per-doubling basis, Δ*glgC* produced O_2 at levels similar to WT and in fact surpassed WT at 396 μmol m^{-2} s^{-1} and above (**Figure 6C**) despite

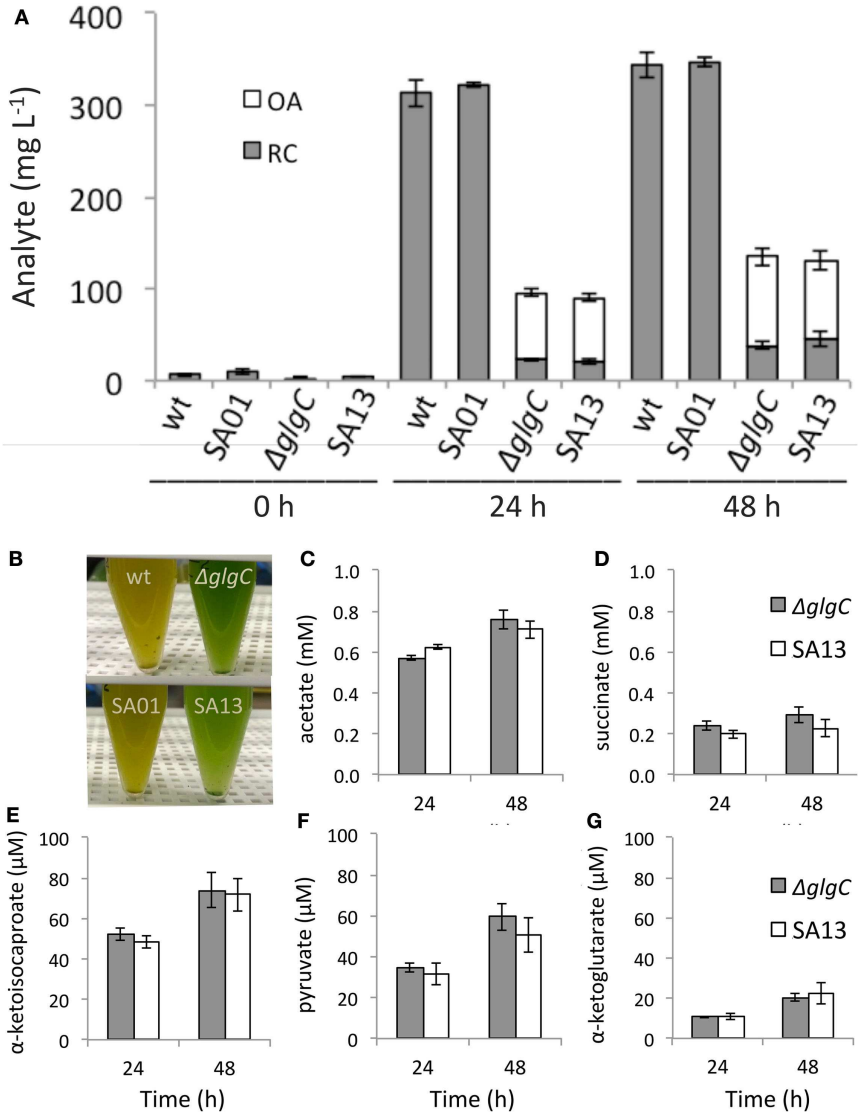

FIGURE 4 | (A) Reducing carbohydrate (RC) content and secreted organic acids (OAs) by volume in batch cultures of WT, SA01, Δ*glgC*, and SA13 during nitrogen deprivation at inoculation (0 h) and after 24 and 48 h. **(B)** Pigmentation differences between wild-type and carbohydrate-deficient backgrounds during nitrogen deprivation. **(C–G)** Individual OA secreted from Δ*glgC* and SA13 strains after 48 h of nitrogen deprivation. Error bars represent SD over four biological replicates.

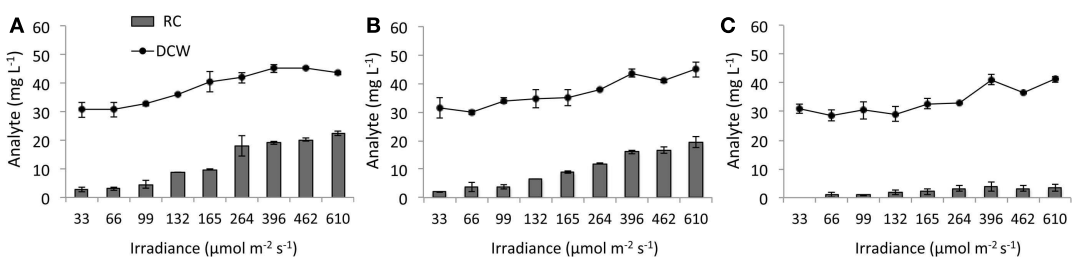

FIGURE 5 | Dry cell weight (DCW) and reducing carbohydrates (RCs) in steady-state PBR cultures. **(A)** WT, **(B)** SA01, and **(C)** Δ*glgC* strains of *Synechococcus* sp. 7002 with increasing 630/680 nm light intensity reported as spherical irradiance. The 33 μmol m^{-2} s^{-1} RC value for Δ*glgC* and 759 μmol m^{-2} s^{-1} biomass values were not available. Error bars represent SD over three samplings.

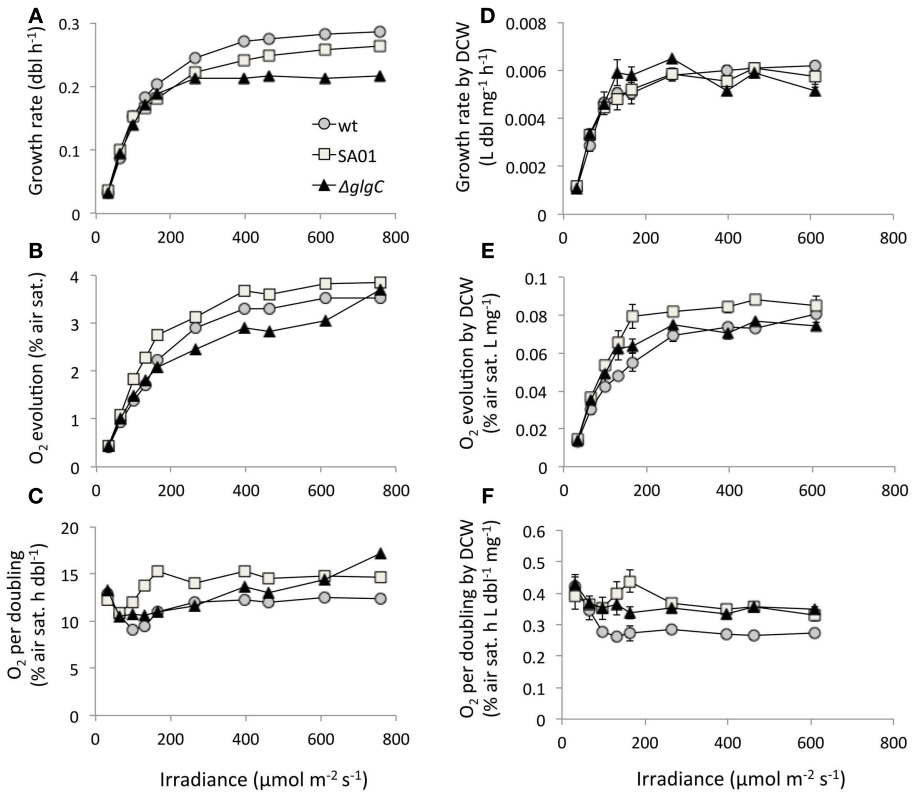

FIGURE 6 | Growth rates, O_2 evolution, and dry cell weight (DCW) normalization of steady-state PBR cultures by light intensity. **(A)** Doubling rate, **(B)** bulk O_2 evolution, **(C)** O_2 production normalized to growth rate, **(D)** doubling rate per unit DCW, **(E)** O_2 evolved on the basis of DCW, and **(F)** per-doubling O_2 evolution normalized to DCW. Values for DCW at 759 μmol m^{-2} s^{-1} were not available. Light intensity is reported as spherical irradiance. Error bars represent SD over three samplings at each light intensity.

diminished growth rates (**Figure 6A**). The doubling rate required per unit DCW was similar between all strains (**Figure 6D**). O_2 evolved by Δ*glgC* was comparable to WT by bulk DCW (**Figure 6E**) but greater on the basis of DCW-normalized growth rate (**Figure 6F**). The uncoupling of O_2 evolution from growth rate in Δ*glgC* at high irradiance was not observed when further normalized to DCW (**Figure 6F**). Despite attaining lower growth rates than WT at 264 μmol m^{-2} s^{-1} and above, SA01 exhibited consistently elevated O_2 evolution by volume (**Figure 6B**), growth rate (**Figure 6C**), bulk DCW (**Figure 6E**), and DCW-normalized growth rate (**Figure 6F**).

Photophysiology

Photosynthetic electron transport appears to be altered by AGPase disruption (**Figure 7**). With increasing irradiance of the Δ*glgC* culture, a higher quantum yield of PSII was observed (**Figure 7A**), and the PQ pool became more reduced (**Figure 7B**) than the WT background. The rate of electron transport downstream of the PQ pool was also adversely affected by *glgC* disruption (**Figure 7C**), as less P >> S quenching occurred in this background with higher light. After dark adaptation, Δ*glgC* cultures exposed to 165 μmol m^{-2} s^{-1} and above exhibited more rapid rates of PQ oxidation in the dark (**Figure 7D**). The transmittance of 630 nm light by cell cultures was unaffected between strains (**Figure 7E**), but Δ*glgC* transmitted less 680 nm light than the WT background (**Figure 7F**) indicating more absorption or scattering by the strain at this wavelength.

DISCUSSION

Derived from photosynthetically fixed CO_2, FAs secreted by genetically engineered cyanobacteria have yielded up to 197 mg L^{-1} FFA by *Synechocystis* sp. 6803 and 131 mg L^{-1} FFA (6.5 mg L^{-1} d^{-1}) by *Synechococcus* sp. 7002 (Liu et al., 2011; Ruffing and Jones, 2012; Ruffing, 2014). Secretion of 4.4 mg L^{-1} day^{-1} transesterifiable lauric acid (C12) from modified strains of *Synechococcus* sp. 7002 was achieved in batch cultures that grew at a similar rate to WT. Sodium lauryl sulfate is a common ingredient in soap, and the foam layer atop cultures secreting C12 suggests detergent activity. Under these conditions, C12 may accumulate in surface bubbles. Phase separation may be a consideration in applying photosynthetic FA secretion on an industrial scale, and actively removing C12 from cultures, for example by hexane overlay (Davies et al., 2014) or solid-state methods (Léonard et al., 2011), may create more favorable conditions for productivity.

Attenuating the synthesis of polymeric carbohydrates did not augment C12 production during normal growth, and attempts

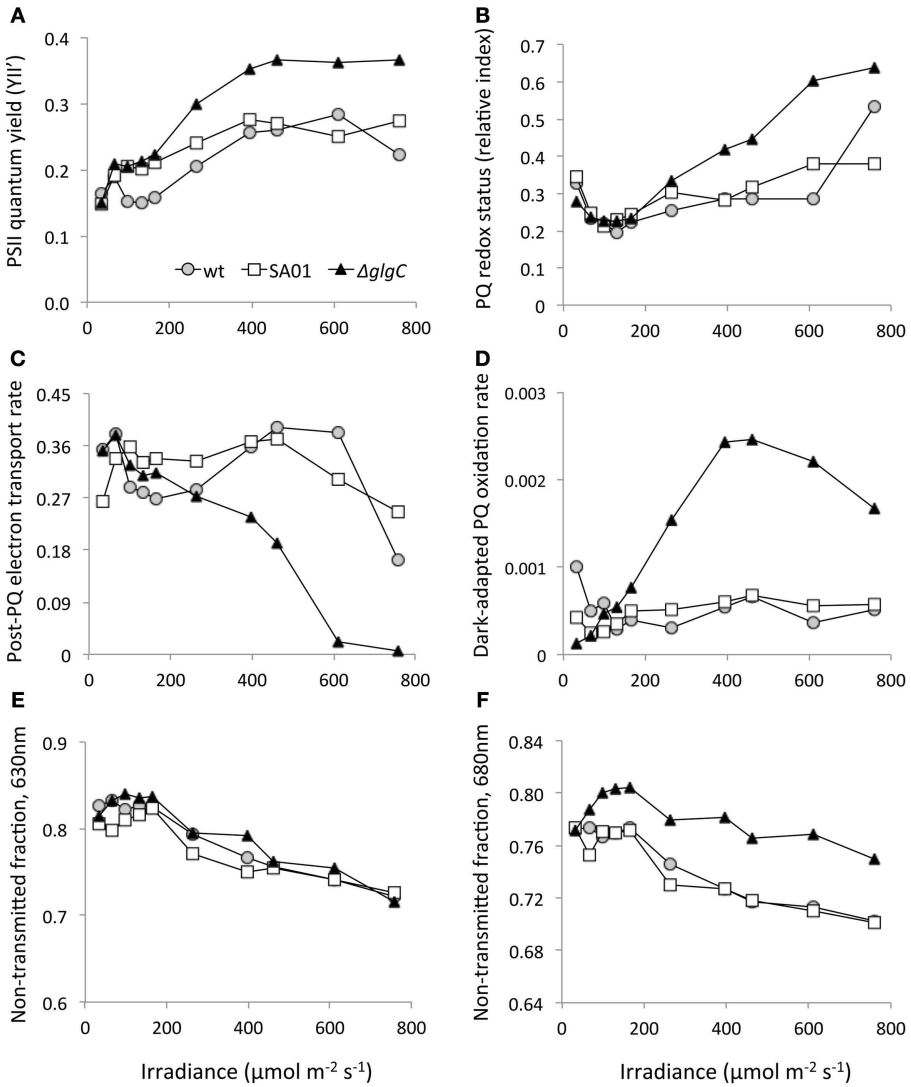

FIGURE 7 | Pulse amplitude modulation (PAM) fluorometry from steady-state PBR cultures of WT, SA01, and Δ*glgC* over a range of light intensities. **(A)** PSII quantum yield (YII'). **(B)** Relative redox status of the PQ pool (more positive is more reduced). **(C)** Relative P >> S electron transport rates downstream of PQ. **(D)** Dark PQ oxidation rates in dark-adapted cultures. Non-transmitted fractions of **(E)** 630 nm and **(F)** 680 nm light by cell culture.

to direct metabolism to FAs by nitrogen starvation instead eliminated C12 altogether. The absence of C12 may be due to cessation of protein and/or lipid synthesis under these conditions, or, since a phycocyanin-related promoter is responsible for *fatB1* expression, the gene may be downregulated in times of nitrogen stress, as phycobiliproteins can be degraded as an intracellular nutrient source (Sauer et al., 1999; Richaud et al., 2001). Additionally, the AGPase-disrupted batch cultures exhibited a non-bleaching phenotype when nitrogen-deprived, and as previously reported, higher absorbances in the 580–650 nm phycobilin range suggest that these proteins are not deconstructed for nutrients in this background as they are in WT (Guerra et al., 2013; Davies et al., 2014). Similar characteristics were described in carbohydrate-deficient mutants of *Synechocystis* sp. 6803 and *Synechococcus elongatus* 7942 (Carrieri et al., 2012; Gründel et al., 2012; Hickman et al., 2013).

Intracellular carbohydrate accumulation during nitrogen stress requires AGPase for glucose polymerization in *Synechococcus* sp. 7002 (Davies et al., 2014), *S. elongatus* PCC 7942 (Hickman et al., 2013), and *Synechocystis* sp. 6803 (Carrieri et al., 2012; Gründel et al., 2012), and energy spilling in the form of OA secretion was observed upon disruption of this function; and a similar outcome occurred with glycogen synthase deletions (Xu et al., 2013). Of the OA secreted by nitrogen-deprived Δ*glgC* and SA13 strains, pyruvate (C_3), α-ketoglutarate (C_5), and succinate (C_4) are also gluconeogenic metabolites (Zhang and Bryant, 2011; Steinhauser et al., 2012). In *S. elongatus* 7942, α-ketoglutarate has been demonstrated as an effector of the nitrogen regulator NtcA (Vázquez-Bermúdez et al., 2001; Tanigawa et al., 2002). Possibly derived from protein degradation or metabolite redistribution, α-ketoisocaproate (C_6) is a biosynthetic intermediate of the amino

acid leucine and can be converted to acetyl-CoA and acetoacetate, which, along with pyruvate and acetate (C_2), are direct precursors of FAs, terpenoids, higher alcohols, and reduced storage polymers such as poly-3-hydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA). Though biosynthetic enzymes for the latter two have not been identified in *Synechococcus* sp. 7002 (McNeely et al., 2010), secreted OA could be supplied to a capable organism by medium exchange (Niederholtmeyer et al., 2010) or co-cultivation (Contag, 2012; Therien et al., 2014).

Steady-state photoautotrophic doubling times of 3.5 h (WT), 3.8 h (SA01), and 4.6 h (Δ*glgC*) are close to the fastest observed in *Synechococcus* sp. 7002 (Ludwig and Bryant, 2012). Increased O_2 production on the basis of DCW-normalized growth rate in both SA01 and Δ*glgC* may represent unidentified photosynthetic energy sinks (Badger et al., 2000; Nomura et al., 2006; Suzuki et al., 2010; Zhu et al., 2010; Xu et al., 2013), which in SA01 could be related to the synthesis of secreted FA. Diminished photosynthetic productivity caused by AGPase disruption was evident, as Δ*glgC* reached maximum growth rate at a lower light intensity than WT. After growth rate saturation, dissipation of excess radiant energy appears to be accomplished in part by RC storage in the WT background. Restricting RC by AGPase disruption resulted in a more reduced, less oxidizable PQ pool indicating overreduction of the photosynthetic electron transport chain and/or the inability to utilize photosynthetic reductant. However, high rates of PQ oxidation by Δ*glgC* in the dark possibly demonstrate a respiratory or other continuous quenching function (Joët et al., 2002; Bailey et al., 2008; McDonald et al., 2011). The severity of these redox alterations may lead to the protection of PSII in Δ*glgC* under irradiances at which WT and SA01 accumulated RC, as evidenced by O_2 evolution decoupling, elevated PSII quantum yields, and more scattered or absorbed 680 nm light, perhaps owing in part to increased content of PSII or a phycobiliprotein such as phycocyanobilin that can absorb at 680 nm and is involved in free radical scavenging (Alvey et al., 2011; Ge et al., 2013). Demonstrating a robust capacity to manage excess light energy, *Synechococcus* sp. 7002 could be a promising organism in scaled systems (Dong et al., 2009; Zhu et al., 2010; Ludwig and Bryant, 2012), and efforts to reroute metabolic flux may identify enzyme targets through further investigation of carbon partitioning at high light in the present genetic backgrounds.

The planktonic cyanobacterium *Synechococcus* sp. 7002 was engineered to convert photosynthate into biofuel precursors, which were naturally secreted from the cell. Lauric acid and OAs can be processed into diesel and alcohols or used as a carbon source for other organisms, and their recovery from culture filtrate avoids costly cell harvesting and lysis. Though redirection of carbohydrate-deficient metabolism toward FA synthesis was not effective under the present conditions, central metabolites for FA, terpenoid, and glucan biosynthesis were generated that potentially could be captured with further metabolic adjustments for redistribution into desired pathways.

AUTHOR CONTRIBUTIONS

The LAS module was constructed by VW, who also transformed and developed the LAS strains, performed batch cultivations and biochemical assays, and compiled this manuscript. MM performed PAM fluorometry measurements and contributed to PBR research design. EH designed, built, and operated the LED-PBR to generate O_2 and dilution rate measurements, and assisted with dry weight measurements. FD performed mass spectral analysis, identified and helped quantify OAs, and assisted with quantitation of RCs. LK contributed to PAM fluorometry data collection. AB and MP were responsible for the project's conception and provided laboratory resources. All authors revised the manuscript for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fbioe.2015.00048/abstract

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