The Synthetic Biology Toolkit for Photosynthetic Microorganisms[1][OPEN]

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Cyanobacteria and algae are photosynthetic microorganisms that are emerging as attractive hosts for synthetic biology applications, ranging from metabolic engineering for the production of industrial biochemicals to microbial energy storage. Photosynthetic microbes have a unique combination of characteristics that drives their consideration for synthetic biology, including relatively simple physiology, fast photosynthetic growth, an availability of model systems, and useful subcellular structures. However, their development as synthetic biology hosts/chassis has been relatively slow compared to that of model heterotrophs. Here we review current synthetic biology tools and applications in photosynthetic microbes and identify opportunities and challenges moving into the future. We focus on cyanobacteria as model photosynthetic prokaryotes, specifically the freshwater unicellular species *Synechocystis* sp PCC 6803 and *Synechococcus elongatus* PCC 7942, the marine unicellular species *Synechococcus* sp PCC 7002, and the nitrogen fixing filamentous species *Nostoc* sp PCC 7120. Moreover, we present algae as model photosynthetic eukaryotes, specifically the unicellular green alga *Chlamydomonas reinhardtii* and the diatom *Phaeodactylum tricornutum*. Finally, we include an examination of some emerging systems. For each organism, the toolbox of genetic parts, high-throughput assembly systems, and other methods that are key components to facilitate synthetic biology are described. Challenges and future directions

ADVANCES

- New synthetic biology advances in photosynthetic microorganisms are aimed at making genetic engineering more facile and offer more options for applications. A key factor for the successful establishment of photosynthetic microbes as synthetic biology hosts is the development of standardized circuits and techniques.
- New tools such as artificial circular chromosomes for more controlled assembly and expression, modular cloning kits, component libraries, and genome editing tools are accelerating development of applications in photosynthetic microorganisms
- The increasing availability of genome sequences and genome scale models can be exploited for identification and harvesting of useful genetic components

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for synthetic biology and its applications in photosynthetic microorganisms are also discussed.

PHOTOSYNTHETIC MICROORGANISMS: ATTRACTIVE TARGETS FOR SYNTHETIC BIOLOGY

The appearance of microorganisms capable of oxygenic photosynthesis was a turning point in the history of life: They shaped evolution by oxygenating the oceans and the atmosphere (Catling et al., 2005; Buick, 2008). A few billion years after the first cyanobacterial-like microbe started splitting water into oxygen and hydrogen, photosynthetic microbes were found in almost every terrestrial ecosystem, from hot springs to arctic ice. Moreover, a cyanobacterium was the likely ancestor of chloroplasts (Jensen and Leister, 2014), an endosymbiosis that was deemed crucial for the colonization of land by plants (Cavalier-Smith, 2010).

Photosynthetic microorganisms—specifically, cyanobacteria and algae—offer novel characteristics as synthetic biology hosts. Their unique combination of characteristics, including relatively fast photosynthetic growth, availability of redox power, a plethora of internal membranes, and subcellular microcompartments, opens up a completely new palette of synthetic biology strategies that is not possible in other well-studied heterotrophic host organisms. The photosynthetic machinery is full of interesting targets for synthetic biology strategies (Leister, 2019), such as enhancing photosynthesis for increased biomass production and yield (Zhu et al., 2010), direct coupling of metabolic pathways to photosynthetic reducing power (Mellor et al., 2017), and creation of bio-nano hybrids where photosynthetic modules are used as a source of electrons for nonbiological processes by linking them to abiotic catalysts or electrode nanomaterials (Saar et al., 2018) or by derivatization of photosynthetic electrons by redox mediators (Longatte et al., 2015; Fu et al., 2017). Although in principle such applications can be hosted in plants, photosynthetic microorganisms offer distinct advantages: the combination of photosynthesis with simple unicellular organization, facile genetic manipulation strategies, quick growth in liquid cultures, relative ease of scale-up, and, if grown in contained facilities, potentially fewer regulatory challenges.

However, synthetic biology applications in photosynthetic microbes lags behind when compared to model heterotrophs (e.g. *Escherichia coli* and yeast). One reason is the lack of synthetic biology tools and enabling technologies, which are crucial to achieve their full potential. Fortunately, in recent years, significant advances have rendered synthetic biology applications in algae and cyanobacteria using classical design–build–test cycles more feasible (Fig. 1). In its most advanced form, synthetic biology relies on standardization of genetic parts and high-throughput assembly and transformation, the latter of which is far more achievable in photosynthetic microbes than in higher

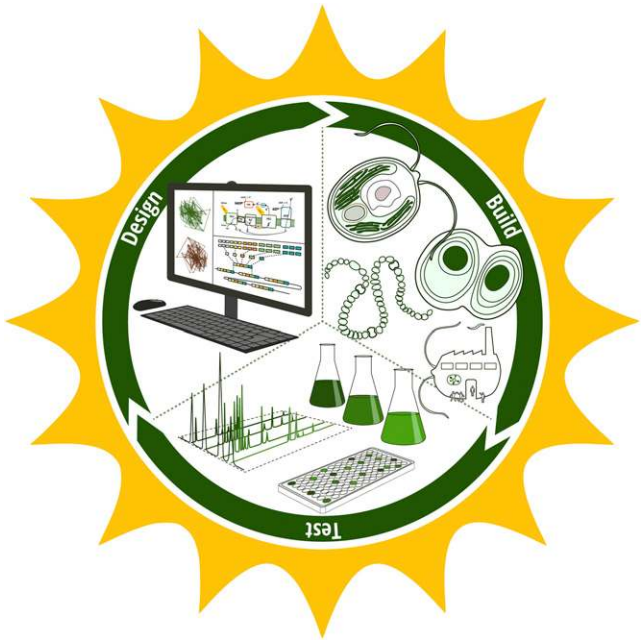

Figure 1. Design–Build–Test cycle using photosynthetic microorganisms. Recent technologies have expanded the capabilities and throughput of this cycle by providing improved tools and design options, greater build capacity, and more efficient and informative strain characterization. Overview of modular cloning system based on that in Weber et al. (2011).

photosynthetic organisms. This perspective provides an overview of recent developments in these areas and identifies some of the challenges facing synthetic biology applications in photosynthetic microbes.

SYNTHETIC BIOLOGY IN CYANOBACTERIA

Cyanobacteria are photoautotrophic prokaryotes with fossil records dating back 3.5 billion years, making them the oldest known microbes (Giordano et al., 2005). The cyanobacterial clade is quite diverse; despite the apparent morphological and physiological similarity between different species, extant cyanobacteria may have branched evolutionarily from their last common ancestor more than a billion years ago (Tomitani et al., 2006). Now, in the era of synthetic biology, cyanobacteria have been identified as excellent chassis for microbial cell factories due to their fast growth, small genome size, genetic amenability, and natural transformability. The recently identified *Synechococcus elongatus* UTEX 2973 strain has one of the fastest growth rates reported for any photosynthetic microbe (Yu et al., 2015; Ungerer et al., 2018); high rates of photosynthesis to produce photosynthates and biomass are required to support this growth. Cyanobacteria are also known for their metabolic flexibility (Xiong et al., 2017), meaning they can thrive in conditions where dramatic fluctuations in nutrient availability, salinity, pH, irradiance, temperature, and moisture occur. Thus, cyanobacteria can rapidly acclimate to environmental fluctuations,

which is an important characteristic of a robust industrial strain. They can also grow at environmental extremes. For example, *Arthrospira* sp (‘*Spirulina*’) are grown successfully in large-scale open ponds at high pH and salt concentrations, conditions that resist invasion from predating and contaminating species (Jiménez et al., 2003).

Cyanobacteria have carbon fixation and photosynthesis characteristics that may lead to unique synthetic biology applications. Firstly, carbon fixation takes place in dedicated organelles called “carboxysomes.” Carboxysomes can be manipulated to host heterologous enzymes, thus generating artificial metabolic microcompartments with a variety of uses (Gonzalez-Esquer et al., 2016). Secondly, photosynthesis does not take place in specialized photosynthetic organelles. This means that their photosynthetic membranes and photosystems therein are more accessible to heterologous proteins. This has enabled the direct linkage of photosynthesis with heterologous metabolic reactions (direct light-driven synthesis) by expression of electron-consuming enzymes (cytochromes P450) in the thylakoid membranes (Berepiki et al., 2016; Wlodarczyk et al., 2016; Mellor et al., 2017). A drawback of the extensive membrane system in cyanobacteria is that our understanding of metabolic regulation through their intermingling respiratory and photosynthetic electron transport chains is incomplete.

The most commonly utilized cyanobacteria for basic and applied research are the freshwater unicellular species *Synechocystis* sp PCC 6803 and *S. elongatus* PCC 7942, the marine unicellular species *Synechococcus* sp PCC 7002, and the nitrogen-fixing filamentous species *Nostoc* sp PCC 7120. Synthetic biology applications in cyanobacteria have principally been directed toward metabolic engineering to redistribute photosynthetically fixed carbon toward chemicals of commercial significance. In 1999, the first synthetic metabolic pathway was constructed in *S. elongatus* PCC 7942 by introducing *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase II genes to allow ethanol production in this species (Deng and Coleman, 1999). A wealth of subsequent literature describes synthetic biology applications in cyanobacterial metabolic engineering (Nielsen et al., 2016; Matson and Atsumi, 2018; Sengupta et al., 2018). Synthetic biology is also being used to deliver applications other than industrial biochemical production in cyanobacteria. For example, it was recently shown that 3D printed cyanobacteria could generate and store electricity from light and subsequently power small devices for a limited time (McCormick et al., 2015; Sawa et al., 2017).

Genetic Parts

Many cyanobacteria (including all of the model species) are naturally transformable and can integrate transgenes into their chromosomes via homologous recombination. Whereas current resources are not as extensive as in heterotrophic model microbes, numerous synthetic components have been characterized for use in cyanobacteria. Vectors and genome editing tools are described in subsequent sections.

Promoters and Other Control Elements

Promoters are key synthetic biology components in cyanobacteria (Camsund and Lindblad, 2014; Stensjö et al., 2018); a large selection is available, including constitutive, inducible, and repressible promoters and riboswitches (Ma et al., 2014; Ohbayashi et al., 2016; Videau et al., 2016; Immethun and Moon, 2018; Qiao et al., 2018; Wegelius et al., 2018).A detailed description of the promoter landscape is provided in Box 1, and promoter characteristics are summarized in Table 1 for ease of reference. In addition, ribosome binding sites (Englund et al., 2016; Thiel et al., 2018) and riboregulators, which are RNA sequences that respond to signal nucleic acids to modify expression (Abe et al., 2014b), have been characterized.

Selectable Markers

Genetic engineering of cyanobacteria is facilitated by antibiotic resistance cassettes. Thus far, resistance to spectinomycin, kanamycin, chloramphenicol, gentamycin, and erythromycin has been used successfully (Eaton-Rye, 2010; Heidorn et al., 2011). Another selection technique that has been applied results in unmarked mutants, and it is based in two consecutive recombination events: the first introduces an antibiotic resistance marker and a Sucsensitivity gene (selection on antibiotic), and the second replaces the selection markers with the native sequence, maintaining the transgene (selection on Suc; Lea-Smith et al., 2016). This technique is applicable only to cyanobacteria that can grow mixotrophically. For example, *Synechocystis* sp PCC 6803 and *Synechocystis* sp PCC 7002 are naturally mixotrophic, but *S. elongatus* UTEX 2973 and *S. elongatus* PCC 7942 are not. However, mixotrophy can also be engineered, as has been demonstrated in *S. elongatus* PCC 7942 (Kanno and Atsumi, 2017).

Reporter Genes

Reporter genes are used to illustrate the behavior of genetic parts in a variety of different contexts and are essential to characterize synthetic biology circuits. Bacterial luciferase and fluorescent proteins are most commonly used (reviewed in Heidorn et al. [2011] and Berla et al. [2013]).

Promoters, characterized using reporter genes, are used for transcriptional regulation. Numerous studies have identified and characterized promoters in cyanobacteria. Cyanobacteria have been classically studied as model photosynthetic organisms and, as a result, several key parts of the synthetic biology toolbox are

Table 1. *Overview of cyanobacterial promoters*

Characteristics	Example	References
Constitutive	P*cpc560*	(Huang et al., 2010; Zhou et al., 2014; Englund et al., 2016; Ruffing et al., 2016; Ferreira et al., 2018; Li et al., 2018; Liu and Pakrasi, 2018)
Inducible		
CO_2	P*cpcB*	(Sengupta et al., 2019)
Cobalt	P*coaT*	(Peca et al., 2008; Guerrero et al., 2012; Englund et al., 2016)
Copper	P*petE*	(Briggs et al., 1990; Guerrero et al., 2012; Englund et al., 2016)
Green-light	P*cpcG2*	(Abe et al., 2014a)
Heavy metals	P*smt*	(Guerrero et al., 2012)
Light	P*psbA2*	(Englund et al., 2016; Li et al., 2018)
Nickel	P*nrsB*	(Peca et al., 2008; Englund et al., 2016)
Nitrite	P*nirA*	(Qi et al., 2005)
Rhamnose	P*rhaBAD*	(Kelly et al., 2018)
UV-B	P*psbA3*	(Máté et al., 1998)
Zinc	P*zia*A	(Peca et al., 2008; Englund et al., 2016)
Repressible		
CO_2	P*rbc*	(Sengupta et al., 2019)
High light intensity	P*cpcB*	(Sengupta et al., 2019)
LacI (IPTG Inducible)	P*trc*	(Huang et al., 2010; Guerrero et al., 2012; Oliver et al., 2013; Markley et al., 2015; Ferreira et al., 2018; Li et al., 2018)
TetR (aTc Inducible)	L03	(Huang and Lindblad, 2013; Zess et al., 2016; Ferreira et al., 2018)

sourced from photosynthesis genes. The light-inducible promoter driving expression of the D1 subunit of PSII is commonly used, due to strong expression in the light and repression under limited UV (Máté et al., 1998).

Heterologous promoters, such as broadly *Lac*I-repressible and metal-inducible promoters, have also been characterized in cyanobacteria. *Lac*I-inducible promoters, such as p_{tac} and p_{trc}, are well characterized in eubacteria. However, cyanobacteria have fundamental differences in their transcription machinery (Stensjö et al., 2018); consequently, the same elements behave differently than in other eubacteria (cyanobacteria are part of the eubacteria; Cavalier-Smith, 2010). The main distinction is reduced efficiency of repression by *Lac*I, rendering the construction of tightly-controlled on/off transcriptional circuits practically impossible in cyanobacteria (Guerrero et al., 2012; Huang and Lindblad, 2013; Oliver et al., 2013; Camsund and Lindblad, 2014).

Inducible promoters are widely used in synthetic biology for intermittent controlled expression. These include promoters activated by increased metal concentration, such as copper, iron, zinc, and nickel, in the growth medium (Briggs et al., 1990; Peca et al., 2008). These promoters show good induction ratios and are useful for experimentation, but are not suitable for large-scale applications due to the cost and the toxicity of the metal ions. A rhamnose-inducible expression system has been developed by Kelly et al. (2018), which combines strong expression and good induction, suitable for synthetic biology applications. Several riboswitches also provide tightly regulated, highly tunable expression systems in cyanobacteria (Ma et al., 2014; Ohbayashi et al., 2016; Immethun and Moon, 2018).

A very strong, constitutive, synthetic promoter (cpc560) was constructed by combining the high-level expression phycocyanin C promoter with 14 transcription factor recognition sequences (Zhou et al., 2014).

For expression characteristics of promoters in specific species, see the following references:

- *Synechocystis* sp PCC 6803 (Huang et al., 2010; Huang and Lindblad, 2013; Englund et al., 2016; Ferreira et al., 2018; Liu and Pakrasi, 2018)
- *Synechococcus* sp PCC 7002 (Markley et al., 2015; Ruffing et al., 2016; Zess et al., 2016)
- *S. elongatus* PCC 7942 (Ma et al., 2014; Qiao et al., 2018)
- *S. elongatus* UTEX 2973 (Li et al., 2018)
- *Nostoc* sp PCC 712mix0 (Videau et al., 2016; Wegelius et al., 2018)

Vectors and Sandardized Assembly

The first shuttle vector that enabled assembly of genetic constructs and expression in several cyanobacteria (using the then-popular BioBricks standard; Shetty et al., 2008) was reported in 2010 (Huang et al., 2010). Standardized genetic assembly paved the way for large-scale development and characterization of parts. The first broad-host cyanobacterial shuttle vector pPMQAK1 (Huang et al., 2010), as well as the pDF-trc vector (Guerrero et al., 2012), enabled more streamlined construct generation and strain characterization using restriction-based and isothermal cloning techniques. There are several advantages to using extrachromosomal (ectopic) self-replicating vectors: (1) more

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- *Nostoc* sp. PCC 712mix0 (Videau et al., 2016; Wegelius et al., 2018)

efficient transformation using conjugation and electroporation (compared to homologous recombination); (2) avoidance of the need for time-consuming segregation to achieve incorporation of the transgenic sequence in all copies of the genome; and (3) increased orthogonality of the heterologous sequences, meaning no insertion in genomic loci that may not be neutral. A hybrid strategy that combines the advantages of using a shuttle vector and the stability of genomic insertion involves using native cyanobacterial plasmids, either as insertion sites (Ng et al., 2015) or as true shuttle vectors after isolation and modification (Jin et al., 2018).

BioBricks and isothermal assembly techniques are adequate for the generation of a limited number of

constructs. However, the automated generation of very large numbers of plasmids and the subsequent streamlined strain generation and characterization requires an alternative approach for genetic assembly. Golden Gate-based techniques, which rely on Type II restriction enzymes (Engler et al., 2008), are more compatible with high-throughput applications. So far, there are two reported Golden Gate toolboxes for cyanobacteria. The first is a streamlined approach for generating constructs either for insertion in the genome or to be incorporated into self-replicating vectors (Taton et al., 2014). This toolkit works in the frequently used model species (including *Synechocystis* sp PCC6803, *S. elongatus* PCC7942, and *Synechocystis* sp. PCC7003, and *Nostoc* sp. PCC 7120) as well as the Californian species *Leptolyngbya* sp BL0902, showing that tools can function across the cyanobacterial phylum. A more recent Golden Gate kit is based on the modular cloning (MoClo; Weber et al., 2011) assembly standard (Vasudevan et al., 2019). This kit contains more than 100 parts for transforming four model cyanobacterial species, including a broad selection of native, heterologous, and synthetic promoters, terminators, linkers, fluorescent makers, antibiotic resistance genes, and more. It also uses the same assembly syntax as the plant and algal MoClo kits, thus making the exchange of parts straightforward. To display the suitability of this kit for high-throughput applications and to characterize promoter/terminator combinations, Vasudevan et al. (2019) used automated assembly to generate more than 200 constructs.

Genome Editing Tools

A significant development was the implementation of clustered regularly interspaced short palindromic repeats (CRISPR)-mediated modification systems, including transformation using CRISPR/CRISPR-associated protein 9 (Cas9) and CRISPR-associated endonuclease in Prevotella and Fancisella 1 (Cpf1; Li et al., 2016a; Ungerer and Pakrasi, 2016), and CRISPR-interference, the latter of which has the potential to provide simultaneous knockdown of multiple genes (Gordon et al., 2016; Wendt et al., 2016; Yao et al., 2016).

Challenges and Future Directions

Despite the recent advances, there are several challenges that still limit the synthetic biology potential of cyanobacteria. Cyanobacteria harbor multiple genome copies per cell (Watanabe et al., 2015), making the generation of stable transformant lines a slow process that requires extensive segregation to achieve genomic homogeneity. Although homologous recombination is very efficient (requiring as little as 100 bp of overlap for successful transformation; Vogel et al., 2017), it is difficult to ensure that multiple genes have been edited on the same genome copy. This makes the use of elaborate synthetic biology methods challenging, and sometimes impossible. For example, multiplex automated genome engineering (Wang et al., 2009) and its counterpart yeast (*Saccharomyces cerevisiae*) oligo-mediated genome engineering (DiCarlo et al., 2013), which are very useful in *E. coli* and yeast for genomic editing, and Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution, used for accelerated evolution in yeast artificial chromosomes (Dymond and Boeke, 2012), face significant challenges in cyanobacteria due to their genomic redundancy.

Whereas CRISPR systems are available (see "Genome Editing Tools"), CRISPR-mediated marker-free and multiplex transformation have not yet been achieved in cyanobacteria. A marker-free transformation system has been reported, based on SacB toxicity in the presence of Suc; however, this system relies on two rounds of segregation—thus obtaining unmarked colonies after a couple of months—and works only on cyanobacteria that can grow mixotrophically (Lea-Smith et al., 2016).

Success in synthetic biology applications often requires reasonably solid understanding of the target biological system. However, the metabolism of cyanobacteria remains only partly elucidated. This is particularly problematic for modeling, as it interferes with the predictive capability of models. More reactions and major metabolic routes are being discovered even in model species (Xiong et al., 2015; Chen et al., 2016; Zhang et al., 2018), whereas key regulation mechanisms remain more or less unknown (Vavitsas et al., 2017; Stensjö et al., 2018). Pioneering work in understanding cyanobacterial metabolism, such as the use of transposon libraries to determine essential and beneficial genes (Rubin et al., 2015), as well as advances in metabolic modeling (Nogales et al., 2012; Knoop et al., 2013; Broddrick et al., 2016; Abernathy et al., 2017) provide essential tools for studying metabolism. Good knowledge and careful manipulation of metabolism have been proven essential for metabolic engineering applications, as displayed in the production of isoprene (titer: 1.26 g/L) and 2,3-butanediol (titer: 12.6 g/L) in *S. elongatus* PCC 7942 (Gao et al., 2016; Kanno et al., 2017).

SYNTHETIC BIOLOGY IN EUKARYOTIC ALGAE

Eukaryotes present distinct advantages compared to prokaryotes for synthetic biology. This includes the existence of membrane-enclosed organelles that allow exploitation of separated cellular compartments (Polka et al., 2016), thus permitting the engineering of channeling and compartmentalization of metabolism to enhance overall production rates and limit side reactions for metabolic engineering applications. The existence of multiple genetic systems in eukaryotes (nucleus, mitochondria, and chloroplasts) can also offer new design possibilities and allows exploration of the coordination between the compartments, which plays a

major role in numerous fundamental cellular processes and is especially crucial for photosynthesis and respiration. The main eukaryotic chassis are *Saccharomyces cerevisiae* and mammalian cells (Adams, 2016). In the case of photosynthetic eukaryotes, synthetic biology tools have been developed for Arabidopsis (*Arabidopsis thaliana*) and *Marchantia polymorpha* (Boehm et al., 2017); however, unicellular chassis have only emerged recently. Many eukaryotic algal genomes have been sequenced (data from the National Center for Biotechnology Information Genome, https://www.ncbi.nlm.nih.gov/): 64 green algae, nine red algae, nine diatoms, five dinoflagellates, and three brown algae. Several industrial hosts of different phyla (Brodie et al., 2017) are used for biotechnology or metabolic engineering applications, including *Nannochloropsis sp* (Poliner et al., 2018) and diverse green algae (Scaife and Smith, 2016), such as *Dunaliella salina* (Feng et al., 2014) and *Chlorella sp* (Yuan et al., 2018). Nevertheless, most of these organisms are not yet amenable to advanced synthetic biology approaches, which require robust genetic tools, reliable parts, standardized high-throughput construction, and advanced molecular biology techniques (Hlavova et al., 2015). Two model organisms are therefore rising as interesting chassis: the unicellular green alga *C. reinhardtii* (herein referred to as "*Chlamydomonas*") and the diatom *P. tricornutum* (Nguyen et al., 2016). Both are easily cultivated even at moderate to large scale (Posten, 2009) and can be transformed efficiently, with known physiology and available systems-wide datasets (genome, transcriptomics, proteomics, metabolomics; Scaife and Smith, 2016). Synthetic biology may allow these two model organisms to become industrial hosts (Scaife et al., 2015), and the knowledge and tools developed might also be applicable to other algal species (Doron et al., 2016).

Genetic Parts

Many genetic elements have been characterized in, or adapted to, microalgae. Several recent reviews have been published on algal synthetic biology and chloroplast genome modification (Doron et al., 2016; Scaife and Smith, 2016; Dyo and Purton, 2018; Poliner et al., 2018). Therefore, we will focus here on new developments and on nuclear genetic elements.

Promoters and Terminators

Numerous genetic parts are available for the control of gene expression including diverse inducible, repressible and constitutive promoters and multiple 3′untranslated region (3′UTR)/terminators (Ohresser et al., 1997; Schroda et al., 2000; Fischer and Rochaix, 2001; Ferrante et al., 2008; Helliwell et al., 2014; Sawyer et al., 2015; Baek et al., 2016; Lee et al., 2018; Watanabe et al., 2018; Beltran-Aguilar et al., 2019). The promoters are detailed in Box 2. Available nuclear 3′UTR/terminators are derived from various genes such as photosystem I reaction centre subunit II, ribulose bisphosphate carboxylase small chain 2 (RBCS2), ribosomal protein S29, carbonic anhydrase 1, β-tubulin 2, or ribosomal protein L23 (Crozet et al., 2018). Interestingly, in the context of a small transgene expressing the nanoluciferase reporter, the 3′UTR/terminator was found to have a stronger impact than the promoter on global expression, when comparing *PSAD* and heat shock protein 70A (*HSP70A*)/RBCS2 promoter and terminator elements (Crozet et al., 2018). Further studies will be required to precisely assess the impact of multiple combinations of genetic elements on gene expression.

Posttranscriptional Control Tools

Coordinated transcription of multiple genes can be achieved through use of the foot-and-mouth-disease-virus 2A peptide, which allows cistron-like gene expression in eukaryotes and was found to be functional in *Chlamydomonas* (Rasala et al., 2012; Plucinak et al., 2015; López-Paz et al., 2017). To control gene expression posttranscriptionally, two other versatile genetic parts were characterized: riboswitches and artificial microRNA (miRNA). Riboswitches are RNA sensors that bind small molecules and in turn regulate gene expression (Nguyen et al., 2016). These regulatory elements are particularly interesting for synthetic biology as they are modular with an aptamer part that recognizes the ligand and an expression platform that controls the expression. This modularity and the knowledge of the design rules of riboswitches make them primed for engineering (Hallberg et al., 2017). The *THI4* riboswitch identified in *Chlamydomonas* responds to vitamin B1 and can be used to control the expression of any gene (Croft et al., 2007; Moulin et al., 2013; Crozet et al., 2018). Artificial miRNA is based on the endogenous miRNA machinery that controls gene expression posttranscriptionally (Rogers and Chen, 2013). This complex machinery was identified in *Chlamydomonas* (Molnár et al., 2007) and now it is possible to design artificial miRNA that can repress the expression of any gene in a sequence-dependent manner (Molnár et al., 2009).

Selection Systems

Several antibiotic resistance genes are available in *Chlamydomonas*, including the newly developed sulfadiazine resistance gene (Tabatabaei et al., 2019) and the well-established selectable marker genes conferring resistance to paromomycin, hygromycin, spectinomycin, kanamycin, and zeocin. Most of these genes are functional in numerous other algal species (Doron et al., 2016; Poliner et al., 2018). In addition, the Nourseothricin resistance gene, *Nat*, is routinely used in *P. tricornutum* (Zaslavskaia et al., 2001), and very recently a novel selection marker, namely the Blasticidin-s

BOX 2. Promoters for eukaryotic algae synthetic biology

Promoters are usually seen as the key genetic element controlling gene expression. Despite the absolute requirement of a promoter to recruit RNA polymerase, other genes parts, including the untranslated regions (UTRs) and terminators, also contribute to the regulation of gene expression, ultimately leading to fine-tuning of protein abundance *in vivo*.

In Chlamydomonas, these other gene parts used to influence expression include regulatory sequences from the PSAD gene (Fischer and Rochaix, 2001) and the chimeric promoter HSP70A-RBCS2 and the RBCS2 terminator (Schroda et al., 2000) for strong constitutive expression, as well as the NIT1 (Ohresser et al., 1997) or METE (Helliwell et al., 2014) promoters, which are repressible by ammonium and vitamin B_{12}, respectively. Three inducible promoters are known: the CYC6 promoter induced by nickel and also repressed by copper (Ferrante et al., 2008), the sulfur starvation-induced promoter of LHCBM9 (Sawyer et al., 2015), and the *Dunaliella* LIP promoter that is light-inducible and functional in Chlamydomonas (Baek et al., 2016). Recent reports added two new inducible promoters to the toolbox: a salt-inducible promoter from CrGPDH3 (Beltran-Aguilar et al., 2019) and the alcohol-inducible promoter recently adapted from the fungus *Aspergillus nidulans* (Lee et al., 2018). The latter requires the specific transcription factor alcR and may be coupled to another input to generate a logic gate, which should enable more complex design of genetic circuits. In diatoms, several promoters were developed including constitutive or inducible ones (Huang and Daboussi, 2017). Very recently, a new promoter, namely the endogenous V-ATPase C promoter, was isolated for driving high level expression of transgenes in *Phaeodactylum tricornutum* (Watanabe et al., 2018).

resistance gene (encoding Blasticidin-s-deaminase), was added to the diatom toolbox (Buck et al., 2018).

Reporter Genes

A set of reporter genes are also available, including fluorescent proteins and diverse luciferases (Lauersen et al., 2015; Huang and Daboussi, 2017), which can be targeted in *Chlamydomonas* to most compartments thanks to a collection of targeting peptides (Lauersen et al., 2015). Recently, the very sensitive and highly stable Nanoluciferase (Hall et al., 2012) was adapted to *Chlamydomonas* (Crozet et al., 2018).

Parts for Other Eukaryotic Microalgae

Although less diverse, genetic parts including promoters, reporters and targeting peptides have been developed for other diatoms (Apt et al., 1996; Rosenwasser et al., 2014; Chu et al., 2016; Doron et al., 2016; Huang and Daboussi, 2017) and for the *Nannochloropsis* genus (Poliner et al., 2018).

Vectors and Standardized Assembly

The "pOptimized" vector collection provided the first step toward standardized genetic tools in *Chlamydomonas*, including several reporter genes and a collection of targeting peptides with each gene part flanked by unique restriction sites (Lauersen et al., 2015). This toolkit is standardized but still lacks the modularity of modern cloning methodologies. More recently, we participated in the development of the first MoClo toolkit for *Chlamydomonas* (Crozet et al., 2018). It is based on standardized Golden Gate cloning (Engler et al., 2008) and allows rapid and standardized assembly of genes and multigenic constructs (Weber et al., 2011). Most of the genetic resources cited above were standardized, validated, and are openly distributed to the community (Crozet et al., 2018). The kit

comprises 119 genetic parts, including seven promoters, seven 5′UTRs, the *CrTHI4* riboswitch, eight immunological or purification tags, nine signal/targeting peptides, 12 reporter genes, five antibiotic resistance genes, the 2A peptide, two miRNA backbones and associated controls, and six 3′ UTR/terminators. This toolkit enables rapid building of engineered cells for both fundamental research and algal biotechnology. To make *Chlamydomonas* and other algae easier to engineer, we invite our readers to follow the proposed standard (Patron et al., 2015; Crozet et al., 2018) and to share their parts openly. A molecular toolbox was created for diatoms using the Gateway standard (Siaut et al., 2007). More recently, D'Adamo and colleagues used the MoClo (Weber et al., 2011) and its Plant standard (Patron et al., 2015) to build plasmids later successfully used to transform *P. tricornutum* (D'Adamo et al., 2019).

A recent breakthrough in diatoms was the design of an artificial circular chromosome delivered via bacterial conjugation to *P. tricornutum* and *Thalassiosira pseudonana* (Karas et al., 2015; Diner et al., 2017).

Genome Editing Tools

A recent breakthrough was the development of genome editing through Zn-Finger Nucleases, Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR/Cas9 for different eukaryotic algae. TALENs were successfully used for *P. tricornutum* (Weyman et al., 2015; Serif et al., 2018), whereas Zn-finger nucleases were developed for *Chlamydomonas* (Sizova et al., 2013; Greiner et al., 2017). CRISPR/Cas9 genome editing was achieved in *Chlamydomonas* (Shin et al., 2016; Greiner et al., 2017), *P. tricornutum* (Nymark et al., 2016; Serif et al., 2018; Slattery et al., 2018), and *T. pseudonana* (Hopes et al., 2016). In *Chlamydomonas*, efficient genome editing was also achieved through the Cpf1 single-stranded DNA-dependent nuclease (Ferenczi et al., 2017). In the case of *Chlamydomonas*, a library of mapped mutants generated through insertional mutagenesis is a useful resource for reverse genetics (Li et al., 2016b). A very recent study reported that DNA integration through homologous recombination was enabled through a knock-down of a LigIV homolog, thus decreasing the non-homologous end joining in profit of Homologous Recombination (Angstenberger et al., 2019).

Challenges and Future Directions

The development of all the tools described above is nourishing synthetic biology in photosynthetic eukaryotes, and is particularly useful for metabolic engineering applications. Several successes were achieved in recent years, such as the production of terpenes in *Chlamydomonas* (Lauersen et al., 2016; Wichmann et al., 2018) and *P. tricornutum* (D'Adamo et al., 2019), and other compounds such as alkanes in *Chlamydomonas* (Sorigué et al., 2016; Yunus et al., 2018) and astaxanthin in *D. salina* (Anila et al., 2016). Synthetic biology of microalgae is not exclusively restricted to metabolic engineering. For example, the possibility to harness photosynthesis to generate electrical power is currently being considered. Extraction of photocurrents from PSII using exogenous quinone as a redox mediator was investigated (Longatte et al., 2015, 2016). The redesign of the Q_A binding site of *Chlamydomonas* allowed reduction of exogenous quinones (Fu et al., 2017) and this strategy provided 10–60 $\mu A/cm^{-2}$ for 1 h on a high surface electrode (Longatte et al., 2018).

Despite all the recent advances enabling synthetic biology in eukaryotic algae, some challenges remain. Some of these challenges relate to the relatively recent development of photosynthetic microorganisms as model chassis for engineering. Relative to more established heterotrophic microbes, there are fewer genetic components and they are not as well characterized. In particular, although transcriptional characteristics in cyanobacteria that are relevant for metabolic engineering applications have recently been reviewed (Stensjö et al., 2018), much more work is needed to characterize promoters, especially the commonly used light-responsive promoters. Moreover, nonmodularity in expression (commonly observed when promoters are coupled with different gene sequences) is not well understood. These are important considerations for true standardization of components, required for predictability and reproducibility of synthetic biology approaches in photosynthetic microbes. More extensive characterization of componentry is required to improve their utility and application in photosynthetic microbes for synthetic biology projects.

Expression of transgenes is often problematic because they are randomly integrated in the genome, leading to multiple insertions, gene knockout, position effects, and long-term silencing. It should be possible in the future to solve some of these issues using, as in mammals, a landing pad for transgene insertion through site-specific recombination flanked by insulators (Raab and Kamakaka, 2010; Duportet et al., 2014). Another solution might be to use the recently published strategy demonstrated in the model diatom *P. tricornutum* in other algae, which allows a shift in the balance of DNA repair from non-homologous end joining to Homologous Recombination (Angstenberger et al., 2019). Low transgene expression, which is often problematic in *Chlamydomonas*, was recently circumvented using introns (Baier et al., 2018; Wichmann et al., 2018). It could also be improved by targeting specific loci known to be stable and highly expressed.

The recently-developed diatom artificial circular chromosome (Karas et al., 2015; Diner et al., 2017) could allow genome engineering without random integration in native chromosomes. This paves the way for the next frontier in algal engineering, which is certainly the (re) design of natural chromosomes or entire genomes as in yeast (Richardson et al., 2017).

OUTSTANDING QUESTIONS

- What genetic diversity are we missing by focusing on relatively few model photosynthetic microorganisms, and how do we most easily access it for new chassis organisms and circuits? Will expansion into new organisms come at the expense of needed development in current chassis?
- Can the problems resulting from multiple genome copies in cyanobacteria be overcome using novel technologies?
- Can new mechanisms for precise control of transgene insertion and expression be developed?
- For metabolic engineering applications, which engineered processes and products will provide the rates, yields and titers required for economic viability? Which outcomes can be delivered in a short time frame, and which will require sustained long-term investment?
- For other applications, where else will photosynthetic microorganisms shine compared to more easily-engineered model systems?

Beyond molecular tools, algal synthetic biology will require the development of single cell approaches and automation based on microfluidics (Best et al., 2016; Kim et al., 2016) and robotized platforms for DNA assembly, strain handling, and phenotyping to allow high-throughput building and testing of advanced designs, possibly improved through directed evolution.

CONCLUDING REMARKS

Photosynthetic microorganisms represent attractive chassis for synthetic biology applications, and significant recent advances demonstrate their potential. However, there are some challenges that remain to be overcome and opportunities yet to be exploited to realize this potential (see "Outstanding Questions").

Current research is focused on only a few model species; there is enormous diversity yet to be tapped in photosynthetic microorganism clades. Even in model species, metabolic processes are not fully elucidated and therefore cannot be fully exploited. Other valuable chassis organisms and component sources are waiting to be discovered. Cyanobacteria are clearly the front runner in terms of model prokaryotic photosynthetic microorganisms, but their multiple genome copies slows the production of stable transformants and creates barriers for the development and application of some valuable synthetic biology tools. For both prokaryotes and eukaryotes, random integration presents problems in control and predictability of transgene behavior; mechanisms for more precise control of insertion and expression are required.

Going forward, further development of standardized parts, high-throughput assembly systems, and other synthetic biology methods will be required. Toolboxes remain limited in eukaryotic systems, particularly for the diatom *P. tricornutum*, which has emerged even more recently as a model. An improved understanding of the biology and physiology of photosynthetic microorganisms will improve our ability to develop and apply synthetic biology tools.

For microbial cell factory/metabolic engineering applications, production rate, yield, and titer are critical, especially for high volume, low value products (Vickers et al., 2010). Photosynthesis is not an efficient process, although recent impressive advances in engineering to improve photosynthetic efficiency can deliver significant photosynthetic yield benefits (South et al., 2019). Converting this fixed carbon into improved yields, and increasing rates and titers, remain challenges for many products, including fuels and commodity biochemicals, which are particularly attractive for photosynthetic systems compared to high value/low volume products where the technoeconomic evaluation may return more positively in systems that are easier to engineer. Solving these challenges will require significant engineering and application of clever synthetic biology tools. For practical application, the potentials to use nonarable land for production and to deploy engineered organisms in extreme environments are both attractive. Photoautotrophic production may be required for applications in desert and extraplanetary (i.e. beyond Earth) environments (Llorente et al., 2018). Achieving sufficient light penetration into cultures without loss of the growth medium (especially in high-density cultures required for high titers) also represents a challenge in bulk production systems. Mixotrophic growth, although failing to fully exploit the free carbon and energy available through photosynthesis, improves growth rates and yields in cyanobacteria (Kanno and Atsumi, 2017; Matson and Atsumi, 2018) and may be required to reach maximum potential. An underdeveloped opportunity for metabolic engineering applications is the use of consortia, where a photosynthetic microbe provides the sugar or other carbon source for a heterotroph that acts as the production platform (Ducat et al., 2012; Fedeson and Ducat, 2017; Ramos-Martinez et al., 2017). This provides the benefits of both photoautotrophy and engineerability.

More broadly, expanding applications where photosynthetic microorganisms provide key advantages beyond microbial cell factories is an attractive option. Microbial fuel cells for energy storage, exploiting paired autotrophy and extremophile capabilities for bespoke

applications such as environmental remediation, and engineered symbiosis for applications such as nitrogen fixation, are likely future targets.

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