

Employing Cyanobacteria for Biofuel Synthesis and CCS

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1. Introduction

Cyanobacteria are a large group of oxygenic photoautotrophic bacteria and, like plants and algae, can capture CO₂ via the Calvin-Benson cycle and convert it to a suite of organic compounds. They are important primary producers of organic material and play significant roles in biogeochemical cycles of carbon, nitrogen, and oxygen (Jansson and Northen 2010, Sharma et al. 2010). Through their photosynthetic capacity cyanobacteria have been tremendously important in shaping the course of evolution and ecological change throughout Earth's history, and they continue to contribute to a large share of the total photosynthetic harnessing of solar energy and assimilation of CO₂ to organic compounds. For example cyanobacteria account for 30% of the annual oxygen production on Earth (Sharma et al. 2010). Our oxygenic atmosphere was originally generated by numerous cyanobacteria during the Archaean and Proterozoic Eras. Many cyanobacteria are diazotrophs and can assimilate atmospheric N₂ and convert it to organic matter. Cyanobacteria occupy a wide array of terrestrial, marine, and freshwater habitats, including extreme environments such as hot springs, deserts, bare rocks, and permafrost zones. In their natural environments, some cyanobacteria are often exposed to the highest rates of UV irradiance known on our globe (Seckbach 2007). Cyanobacteria are Gram-negative bacteria but they combine properties of both Gram-negative and Gram-positive bacteria (Stewart et al. 2006); they contain an outer membrane and lipopolysaccharides (LPS), defining characteristics of Gram-negative bacteria, and a thick, highly cross-linked peptidoglycan layer similar to Gram-positive bacteria.

Cyanobacteria and eukaryotic microalgae exhibit a carbon-concentrating mechanism (CCM), a biochemical system that allows the cells to raise the concentration of CO₂ at the site of the carboxylating enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) up to 1000-fold over that in the surrounding medium (Fig. 1) (Badger and Price 2003, Jansson and Northen 2010, Price et al. 2008). Details of the CCM differ between cyanobacteria but the salient features include a series of bicarbonate (HCO₃⁻) and CO₂ transporters and the carboxysome, a protein-enclosed micro-compartment that houses (most of) the Rubisco population and also contains the enzyme carbonic anhydrase (CA). Under low C_i (as CO₂ and HCO₃⁻) conditions the CCM is induced and activated, supporting active transport of HCO₃⁻ across the outer and plasma membranes through HCO₃⁻/Na⁺ symports or ATP-driven uniports, as well as diffusion of CO₂ into the cytosol (Price et al. 2008). Uptake of

CO₂ is facilitated by CA-harboring NADPH dehydrogenase (NDH) complexes on the thylakoid and plasma membranes that converts the incoming CO₂ to HCO₃⁻ (reaction (1)).

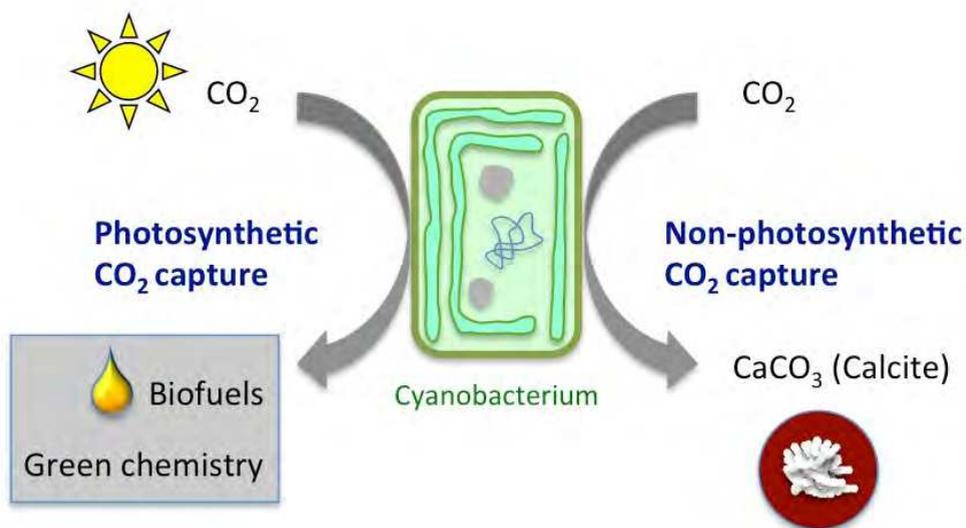
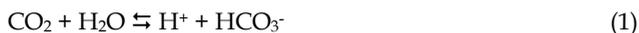


Fig. 1. The two modes of CO₂ uptake in cyanobacteria. Via photosynthesis, CO₂ is captured and converted to organic compounds, which can be exploited as biofuels or other industrial chemicals. In the calcification process, CO₂ is converted to inorganic CaCO₃, e.g. as calcite, which can be sequestered.



Under these conditions, HCO₃⁻ is the predominant C_i species taken up by the cells. The cytosolic HCO₃⁻ subsequently enters the carboxysome where CA converts it to CO₂ for the Rubisco reaction (Jansson and Northen 2010, Price et al. 2008). At non-limiting C_i concentrations the CCM recedes to a basic, constitutive level, characterized by mainly CO₂ uptake (Price et al. 2008).

In addition to photosynthetic reduction of CO₂ to organic compounds, many cyanobacteria can take up CO₂ and mineralize it to recalcitrant calcium carbonate (CaCO₃) (see Section 3 below). Thus cyanobacteria present two different modes of CO₂ uptake, via photosynthesis and the Calvin-Benson cycle, and via biomineralization (calcification) (Fig. 1).

2. Cyanobacteria as photosynthetic bioreactors for direct conversion of CO₂ to hydrocarbon fuels

Cyanobacteria are well suited for synthetic biology and metabolic engineering approaches for the phototrophic production of various desirable biomolecules, including ethanol, butanol, alkylesters, and hydrocarbon biofuels. Phototrophic biosynthesis of high-density liquid biofuels in cyanobacteria would serve as a nice complement to the microbial

production of biodiesel and hydrocarbons in heterotrophic bacteria such as *E. coli*. Two biofuels that are being considered in microbial production systems are alkanes and isoprenoids. Alkanes of defined chain lengths can be used as injection fuel similar to gasoline and jet fuel. Many cyanobacteria synthesize alkanes, albeit at minute quantities. Optimizing the expression of the alkane biosynthesis genes and enhancing the carbon flux through the fatty acid and alkane biosynthesis pathways should lead to the accumulation and/or secretion of notable amounts of alkanes. It also becomes important to understand how to control the chain lengths of the produced alkane molecules. Isoprenoids, *e.g.* the monoterpene pinene and the sesquiterpene farnesene, are considered precursors for future biodiesel or next-generation jet fuel. Cyanobacteria produce carotenoids and extending the carotenoid biosynthetic pathways by introduction of constructs for appropriate terpene synthases should allow the biosynthesis of selected mono- and sesquiterpenes.

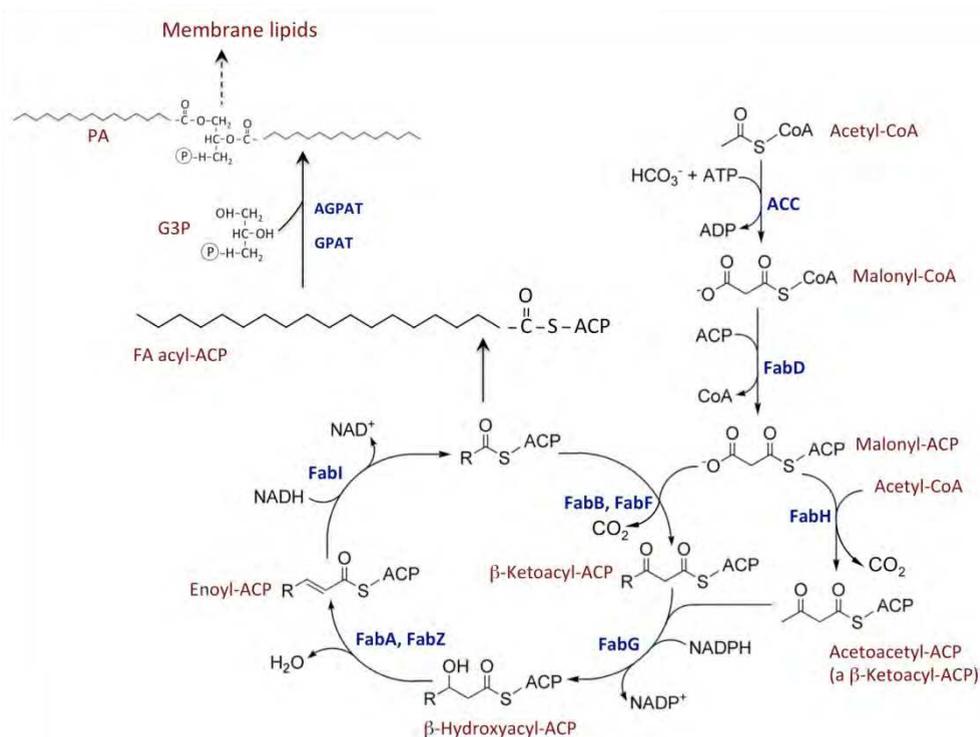


Fig. 2. Fatty acid and lipid biosynthesis in cyanobacteria. ACC, acetyl-coA carboxylase; ACP, acyl carrier protein; AGPAT, acylglycerol-3-phosphate acyltransferase; FabA/FabZ, β -Hydroxyacyl-ACP dehydratase/isomerase; FabB β -Ketoacyl-ACP synthase I; FabD, malonyl-CoA:ACP transacylase; FabF, β -Ketoacyl-ACP synthase II; FabG, β -Ketoacyl-ACP reductase; FabH, β -Ketoacyl-ACP synthase III; FabI, enoyl-ACP reductase I; G3P, glycerol-3-PGPAT, glycerol-r-P acyltransferase; PA, phosphatidic acid.

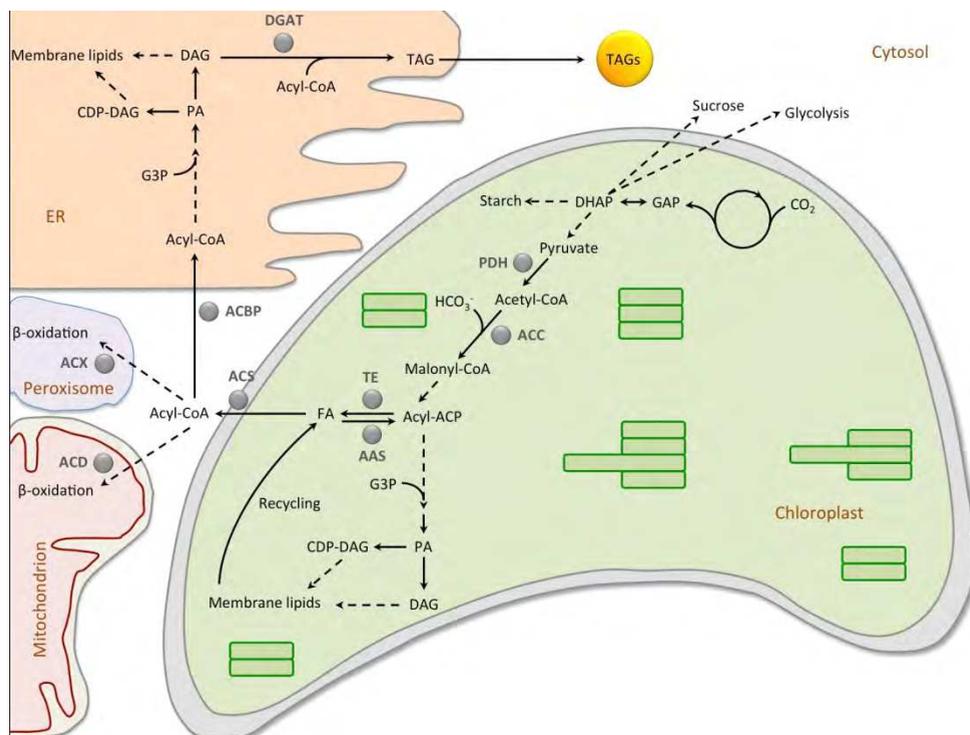


Fig. 3. Fatty acid and lipid biosynthesis in plants. AAS, acyl-ACP synthetase; ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACPB, acyl-CoA binding protein; ACS, acyl-CoA synthetase; ACD, acyl-CoA dehydrogenase; ACX, acyl-CoA oxidase; CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; G3P, glycerol-3-P; GAP, glyceraldehyde 3-P; TAG, triacylglyceride.

2.1 Biosynthesis of alkanes

The pathway for alkane synthesis in cyanobacteria is a two-step process downstream of fatty acid (FA) synthesis and seems to proceed via decarbonylation of fatty aldehydes (Schirmer et al. 2010), the major route for alkane synthesis in most organisms (Ladygina et al. 2006). FA synthesis in bacteria is accomplished by a type II FA synthase (FASII), a multienzyme system, utilizing a freely dissociable acyl carrier protein ACP. The products of FASII are released as acyl-ACPs and may be directly incorporated into membrane lipids by acyltransferases that attach a FA to the glycerol 3-phosphate backbone to form the key intermediate, phosphatidic acid. This is in contrast to FA synthesis in eukaryotes, where acyl-ACPs are either hydrolyzed by acyl-ACP thioesterases (TE; EC 3.1.2.14) to yield free FAs, or directly transferred to CoA for generation of acyl-CoA. For example, in plants and algae, where FA synthesis takes place on FASII complexes in the plastids, the release of free FAs are required for transport across the plastid envelope. Upon arrival at the outer plastid surface, the free FAs are re-activated by acyl-CoA synthetase (FadD; EC 6.2.1.3) to form acyl-

CoA. Acyl-CoA is the starting substrate for synthesis of TAGs but can also be used for β -oxidation and for synthesis of membrane lipids.

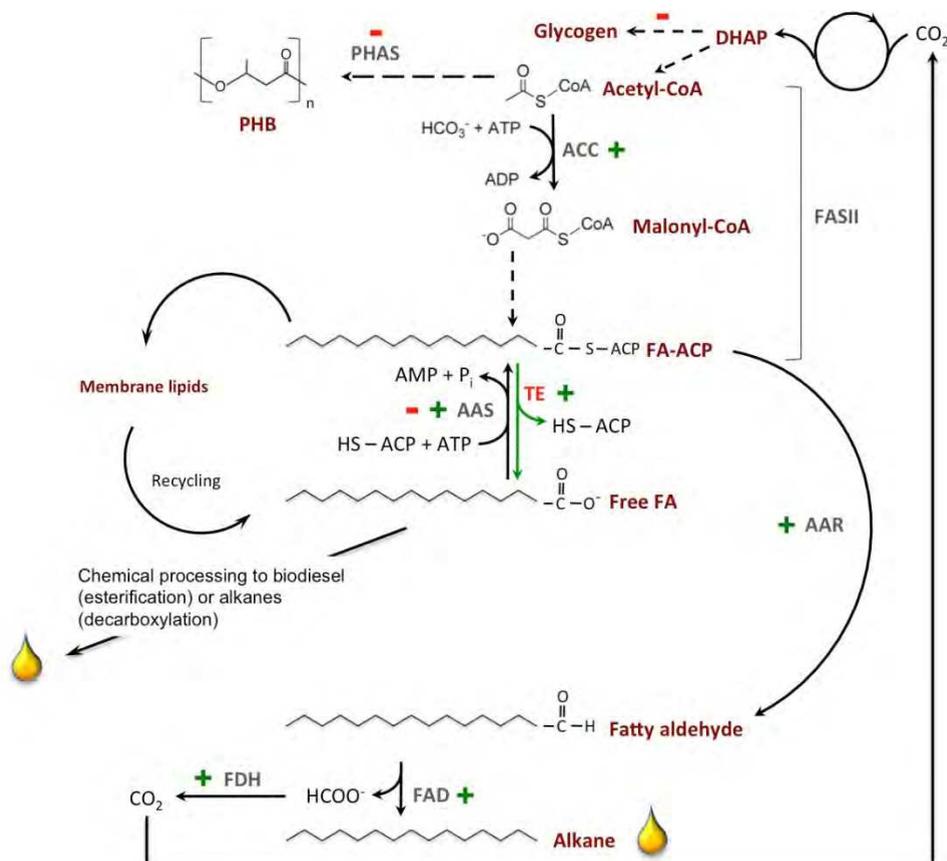


Fig. 4. Rationale for biosynthesis of alkane fuels in cyanobacteria. AAR; acyl-ACP reductase; AAS, acyl-ACP synthetase; ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; FAD, fatty acyl decarboxylase; FA, fatty acid; FASII, fatty acid synthase complex type II; FDH, formate dehydrogenase; PHAS, polyhydroxyalkanoate synthase; PHB, polyhydroxybutyrate; TE, thioesterase.

Most bacteria lack intracellular TEs that act on FA-ACPs, and the formation of free FAs mainly occurs during recycling of membrane lipids or degradation of acylated proteins. *E. coli* and other bacteria that can take up and metabolize exogenous FAs possess periplasmic TEs (e.g. *TesA* in *E. coli* (Cho and Cronan 1994)) that liberate FAs for import. Heterologous expression of TEs, primarily from plants, in bacteria has resulted in high production of free FAs (Jha et al. 2006, Jones et al. 1995, Steen et al. 2010, Voelker and Davies 1994, Yuan et al. 1995). The concomitant decrease in acyl-ACP levels also relieves the rigorous feedback inhibition of acetyl-CoA carboxylase (ACC; EC 6.4.1.2) (and other FA-biosynthesis enzymes) exerted by this end product. ACC catalyzes the rate-limiting step in FA synthesis and thus

expression of TEs in the cytosol of bacteria has the dual effect of producing free FAs and enhancing FA synthesis.

The decarbonylation pathway implies the involvement of the Fatty acyl-CoA or Fatty acyl-ACP reductase (FadR; EC 1.2.1.50), and Fatty aldehyde decarbonylase (FAD; EC 4.1.99.5) (Walsh et al. 1998; Ladygina et al. 2006) (Fig. 2). Gene sequences for *FadR* and *FAD* have recently been identified from several cyanobacteria (Schirmer et al. 2010). Interestingly, the decarbonylation step in cyanobacterial alkane biosynthesis may involve the release of formate (HCOO⁻) rather than CO (Warui et al. 2011).

To generate alkanes of desired chain lengths (e.g., C₈, C₁₀, and C₁₂ saturated species) for diesel, jet fuel or gasoline alternatives, cyanobacteria can be engineered to contain genes encoding TEs (Fig. 4) with different substrate specificities. For example, *FatB* from *Arabidopsis* (Accession NP_172327), *FatB2* from *Cuphea hookeriana* (GenBank: U39834.1), *FatB1* (pCGN3822) from *Umbellularia californica* (GenBank: M94159.1), and *FatB1* from *C. hookeriana* (GenBank: Q39513.1). Another potential TE is the mature TES enzyme from *E. coli* (Cho and Cronan 1994). In addition to inserting an appropriate TE, high-yield production of free FAs in cyanobacteria also require additional optimization by increasing the carbon flux towards FA synthesis. Such efforts can entail the insertion of extra copies of the gene for ACC, which catalyzes the rate-limiting step in FA-ACP synthesis. ACC is a heterotetramer consisting of AccA, AccB, AccC, and AccD. The genes for the different subunits are distributed in most, if not all, cyanobacterial genomes. For the sake of increasing ACC activity, an ACC operon can be constructed behind a strong promoter. Intuitively, another optimizing step would be to inactivate the AAS gene to prevent re-thioesterification of free FAs. However, since AAS rather than FadD may serve as the sole FA-activating enzyme in cyanobacteria, the yield of metabolites downstream of acyl-ACP, like alkanes, might benefit from increasing the copy number of AAS genes so as to speed up the activation of recycled FAs from the degradation of membrane lipids (Figs. 2, 4). With few exceptions, AAS exists as a single-copy gene in cyanobacteria, encoding an enzyme with broad substrate specificity (Kaczmarzyk and Fulda 2010). For the single purpose of free FA production, a simultaneous increase in AAC activity and inactivation of the gene for AAS is likely to improve the yield.

The physiological role(s) of alkanes in cyanobacteria is unknown. Not all cyanobacteria synthesize alkanes and in those that do, alkanes accumulate in very small amounts. It is possible that alkanes are required for proper membrane fluidity or function. Alternatively, they serve as carbon storage compounds under excess carbon and/or nutrient deficiency conditions. Although heptadecane (C₁₇) is the predominant *n*-alkane among cyanobacteria, many strains synthesize a wide array of linear, branched, and cyclic alkanes, some of which, e.g. branched methyl- and ethylalkanes, are only found in these microorganisms (Dembitsky et al. 2001, Jansson 2011). For example, the cyanobacterium *Microcoleus vaginatus* produces four *n*-alkanes and more than 60 different branched alkanes (Dembitsky et al. 2001). Another strain that merits emulation is *Anabaena cylindrica*, which was shown to form C₉-C₁₆ *n*-alkanes under high NaCl stress conditions (Bhadauriya et al. 2008), presumably due to an increase in short-chain FA during salt stress. It should be noted that C₁₂-C₁₆ *n*-alkanes are particularly well suited as jet fuel. Whether the difference in alkane composition observed between cyanobacterial strains and growth conditions reflect the existence of FAR and FAD enzymes with different chain length specificities, or whether alkane chain length is determined at the FA level, is not yet clear. In the latter case, FAR and FAD would be expected to exhibit broad substrate specificities.

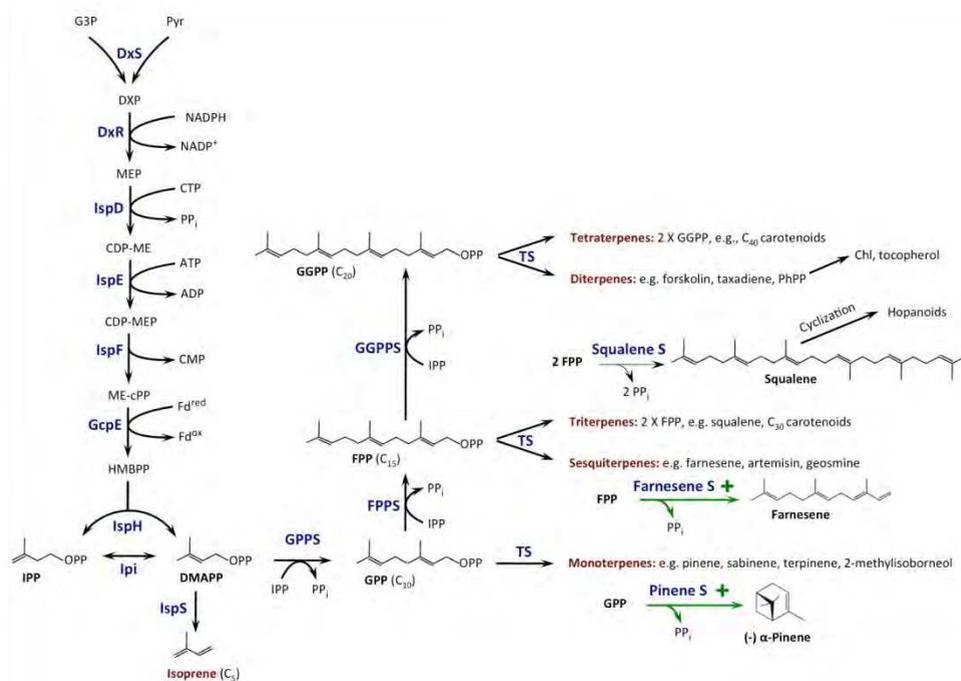


Fig. 5. Rationale for biosynthesis of isoprenoid fuels in cyanobacteria. CDP-ME diphosphocitidyl methylerythritol; CDP-MEP, diphosphocitidyl methylerythritol 2-P; Chl, chlorophyll; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylose 5-P; DxS, DXP synthase; DxR, DXP reductoisomerase; FPP, farnesyl diphosphate; G3P, Glyceraldehyde 3-P; GcpE (IspG), HMBPP synthase; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GPPS, GPP synthase; GGPS, GGPP synthase; HMBPP, hydroxymethylbutenul; IspD, CDP-ME synthase; IspE, CDP-ME kinase; IspF, Me-cPP synthase; IspH, HMBPP reductase; IspS, isoprene synthase; Ipi, IPP isomerase; IPP, isopentenyl diphosphate; ME-cPP, methylerythritol 2,4-cyclodiphosphate; MEP, methylerythritol 4-P; Pyr, pyruvate; TS, terpene synthase.

2.2 Biosynthesis of isoprenoids

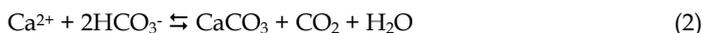
Branched hydrocarbons, which have higher octane rating than *n*-alkanes, can be produced by engineering the carotenoid pathway in cyanobacteria. While it is possible to use carotenoids themselves to make gasoline, e.g. via hydrocracking (Hillen et al. 1982), many carotenoids are solid at room temperature, complicating refining approaches. Cyanobacteria contain genes for carotenoid synthesis and thus synthesize geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), which are precursors for monoterpenes, sesqui- and triterpenes, and di- and tetraterpenes, respectively (Fig. 3). Most, if not all cyanobacteria produce sesquiterpenes such as geosmine, and monoterpenes such as 2-methylisoborneol (Agger et al. 2008) but synthesis of isoprene in naturally occurring cyanobacteria has not been reported. By introduction of an isoprene synthase (IspS) gene based on the mature enzyme from the Kudzu plant (*Pueraria montana*;

GenBank: AY316691), Lindberg *et al.*, (2010) demonstrated the production of volatile isoprene hydrocarbons in the cyanobacterium *S. 6803* (Lindberg *et al.* 2010). The rationale for engineering cyanobacteria for isoprene, monoterpene, or sesquiterpene synthesis is straightforward as it involves the addition of a single gene, *IspS* or different terpene synthases (TS). A desirable objective will be to extend the carotenoid pathway for synthesis of pinene (a monoterpene), and farnesene (a sesquiterpene). Pinene is being considered for next-generation jet fuel, and farnesene is being developed as precursors to diesel fuels (Rude and Schirmer 2009). For example, synthetic gene constructs could be based on the mature proteins of (-)- α -pinene synthase from *Pinus taeda* (GenBank: AF543527.1), and α -farnesene synthase from *Pyrus communis* (GenBank: AY566286.1).

3. Cyanobacteria as catalysts for biomineralization of CO₂ to calcium carbonate

Biomineralization offers the potential to utilize photosynthetic microorganisms like cyanobacteria as solar-powered catalysts for the conversion of CO₂ to recalcitrant carbonates, primarily calcium carbonate (CaCO₃). If implemented at scale such calcifying systems could conceivably be deployed for biological carbon capture and storage (CCS) by sequestering point-source CO₂ (Jansson and Northen 2010). Microbial calcification, *i.e.* formation and precipitation of CaCO₃, is widespread in nature and among microorganisms, and of vast ecological and geological importance. Spectacular manifestations of cyanobacterial calcification are presented by stromatolites and whitening events (Jansson and Northen 2010). Another magnificent illustration of microbial calcification is the White Cliffs of Dover, which are mainly eukaryotic microalgal in origin.

Precipitation of CaCO₃ can proceed by either or both the following reactions:

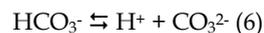


Bicarbonate (HCO₃⁻) is ubiquitous in water and is formed via dissolution of gaseous CO₂ at pH values above about 6.0 at 25 °C:



The concentration of carbonic acid (H₂CO₃) is small in circumneutral pH waters, so the dissolved CO₂ from reactions 3 and 4 occurs predominantly as HCO₃⁻.

A fraction of HCO₃⁻ dissociates to form carbonate (CO₃²⁻):



Spontaneous calcification is often impeded by thermodynamic barriers, also in systems supersaturated with Ca²⁺ and CO₃²⁻ such as the oceans, (Berry *et al.* 2002). Cyanobacteria catalyze the calcification reaction(s) on their cell surface, the exopolysaccharide substances (EPS) layer, or the proteinaceous surface layer (S-layer), by one or both of two mechanisms (Jansson and Northen 2010). The photosynthetic electron transport and the CA activity in

the carboxysome (reaction (6)) both consume cytosolic H^+ , resulting in a net increase of OH^- in the cytosol. Neutralization of this imbalance, e.g. by the activity of a Ca^{2+}/H^+ antiport, generates an alkaline microenvironment on the outer cell surface. The alkaline pH shifts the equilibrium of the bicarbonate buffer system (reactions (4) and (5)) to the right and promotes localized regions of increased CO_3^{2-} concentrations at the cell exterior (Fig. 1). A second means by which cyanobacteria can catalyze calcification is by the presence of Ca^{2+} -binding domains, e.g. glutamate and aspartate residues, or carboxylate and sulfonate groups, on the cell surface, which, together with the export of Ca^{2+} through the Ca^{2+}/H^+ translocator, raises the local Ca^{2+} concentration and serve as nucleation sites for $CaCO_3$ precipitation.

The physiological or biochemical function(s) of calcification in cyanobacteria are unclear, although some possibilities have been suggested (Jansson and Northen 2010). Since calcification will remove Ca^{2+} from chemical equilibria and may offer a means to sustain an active efflux of Ca^{2+} via the Ca^{2+}/H^+ translocator, which, in turn, generates a H^+ gradient that may enhance nutrient and HCO_3^- uptake (McConnaughey and Whelan 1997). A calcereous cell surface may also provide a protective layer against excessive light exposure.

In the context of evaluating the concept of cyanobacterial calcification for biological CCS, there are several outstanding issues that need to be addressed. First, it should be recognized that calcification as a natural phenomenon by marine or freshwater phytoplankton serves as a CO_2 source rather than a sink, i.e., calcification releases CO_2 to the atmosphere (Riebesell 2004). This can most easily be appreciated by looking at reaction (1) but, because of the HCO_3^- buffer system in oceans and lakes, it applies to reaction (2) as well (Frankignoulle 1994, Frankignoulle and Canon 1994). This global effect of calcification should not be confused with its potential use for biological CCS. In such a scenario, the comparison should be made between CO_2 in flue gas, e.g., from a coal-fired power plant, being released to the atmosphere, or being partly captured by cyanobacteria and converted to $CaCO_3$ for precipitation. Second, assuming biocalcification as a means to mitigate CO_2 emissions, the question arises as to whether such a process can operate at a level that is industrially relevant. Combining observations from whitening events in the Great Bahama Bank and microcosm experiments with the marine *Synechococcus* 8806 (*S.* 8806), Lee *et al.* (Lee *et al.* 2006) suggested that *S.* 8806 is able to produce around 2.5 MT $CaCO_3$ per year, which would translate to a removal of half of the CO_2 emitted from a 500 MW coal-fired power plant. Although these data would tend to imply that cyanobacterial calcification is a viable CCS alternative, it is not immediately obvious from the calculations at what scale (e.g. the size of the culture pond) such a system would need to run. A third question concerns the diurnal fluctuations of the calcification process. If photosynthesis is required to maintain a necessary alkaline pH at the cell surface for calcification to occur, it is not clear to what extent the formed $CaCO_3$ is stable enough to prevent its dissolution during the night.

Another issue that also relates to the pH of the cyanobacterial culture is whether or not calcification can operate at high CO_2 levels, e.g., in a pond infused with flue gas. In a high- CO_2 environment, the activity of the CCM is low and cells will preferentially take up CO_2 rather than HCO_3^- . The conversion of CO_2 during transport to the cytosol (Fig. 1) produces H^+ (reaction 6) that need to be neutralized, possibly via export to the medium (Price *et al.* 2008). This counterbalances the subsequent and opposite alkalization reaction in the carboxysome. Also, rapid infusion of gaseous CO_2 into a cyanobacterial pond will likely lower the ambient pH, impeding alkalization at the extracellular surface.

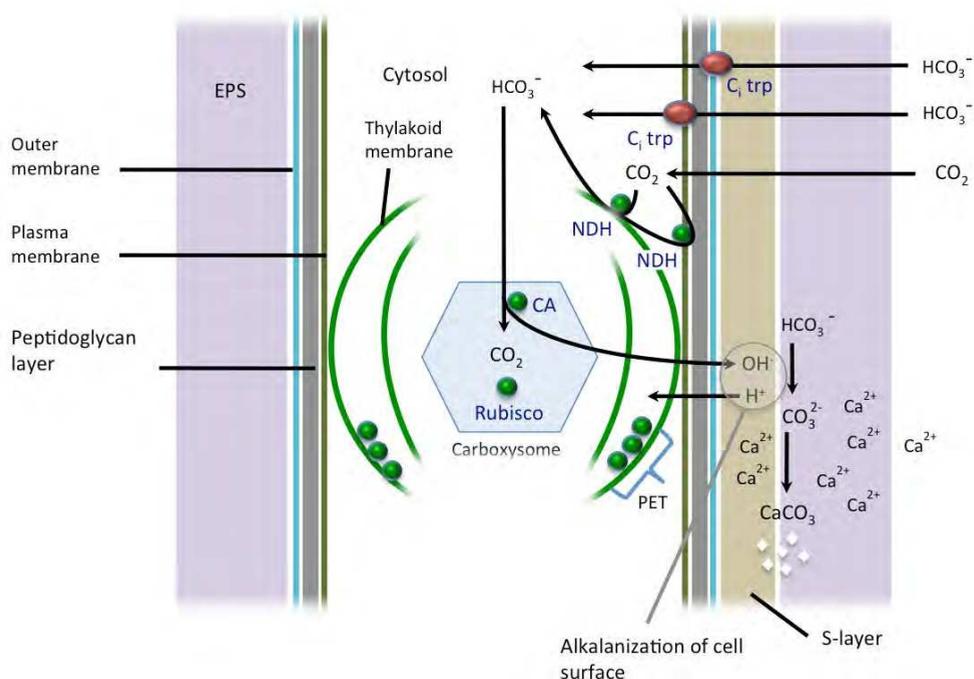


Fig. 6. Model of the carbon concentrating mechanism (CCM) and calcification in a cyanobacterial cell. CO₂ enters the cells mainly via active transport of HCO₃⁻ but also through diffusion of CO₂, which is converted to HCO₃⁻ during the uptake. Cytosolic HCO₃⁻ is subsequently imported to the carboxysome. CA, carbonic anhydrase; C₁, inorganic carbon; EPS, exopolysaccharide substances; NDH, NADPH dehydrogenase; PET photosynthetic electron transport. Modified from Jansson and Northen.

4. Conclusions

The employment of cyanobacteria as a biofuel platform offers great potential. Most of the attention in the algal biofuel space is currently devoted to eukaryotic microalgae, mainly because of their capacity to store large amounts of TAGs. However, recent demonstrations of FA ethylesters (FAEE; a biodiesel) and hydrocarbon fuels biosynthesis in *E. coli* (Kalscheuer et al. 2006; Beller et al. 2010; Schirmer et al. 2010; Steen et al. 2010) suggest that similar strategies in pathway engineering should prove achievable also in cyanobacteria, where photosynthesis, rather than organic feedstocks, will provide energy and carbon. Furthermore, cyanobacteria have previously been engineered to produce alcohol-based fuels such as ethanol and isobutanol (Deng and Coleman 1999; Atsumi et al. 2009).

The capacity of cyanobacteria to thrive in high CO₂ concentrations makes them an attractive system for beneficial recycling of CO₂ from point sources such as coal-fired power plants via biofuel synthesis, and for biological CCS via calcification. Since many cyanobacteria are halophilic, cultivation ponds can be sited away from agricultural land making use of seawater or various sources of saline wastewater.

Non-arguably, much research is needed to address challenges associated with utilization of cyanobacterial for biofuel synthesis or CCS. In addition to issues already discussed above, two more concerns are worth pointing out. Since algal cultivation requires measures for crop protection, it becomes important to learn how to construct robust consortia, or how to prevent or mitigate contamination and grazing of monocultures in open pond systems. Another hurdle in the algal biofuel industry is associated with harvesting and extraction, steps that account for 25-30% of the total biomass production cost; and strategies that facilitate, or obviate the need for, these steps need to be further developed. One solution is to use filamentous or self-flocculating strains to expedite harvesting. Another approach is to achieve release of the biofuel molecules to the medium, either through cell lysis or by secretion. An example of the former is an inducible lysis system reported for *S. 6803* (Curtiss et al. 2011, Liu and Curtiss 2009). The feasibility of secretion was illustrated by the release of free FAs from *S. 6803* and *Synechococcus elongatus* PCC 7942 cells to the medium after inactivation of the AAS gene (Kaczmarzyk and Fulda 2010).

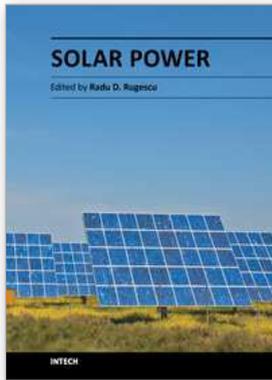
5. Acknowledgements

This work was supported in part by U. S. Department of Energy Contract DE-AC02-05CH11231 with Lawrence Berkeley National Laboratory. Financial support from LDRD projects 366190 and 366188 are acknowledged.

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Solar Power

Edited by Prof. Radu Rugescu

ISBN 978-953-51-0014-0

Hard cover, 378 pages

Publisher InTech

Published online 15, February, 2012

Published in print edition February, 2012

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How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Christer Jansson (2012). Employing Cyanobacteria for Biofuel Synthesis and CCS, Solar Power, Prof. Radu Rugescu (Ed.), ISBN: 978-953-51-0014-0, InTech, Available from: <http://www.intechopen.com/books/solar-power/employing-cyanobacteria-for-biofuel-synthesis-and-ccs>

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