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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Biotechnology of cyanobacterial isoprene production

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Abstract

Heterologous cyanobacterial production of isoprene (C_5H_8) presents an opportunity to develop renewable resources for fuel and industrial chemicals. Isoprene can be generated photosynthetically in these microorganisms from dimethylallyl-diphosphate (DMAPP) by the recombinant enzyme isoprene synthase (ISPS), as a transgenic product of the isoprenoid biosynthetic pathway. The present work sought to combine recent enhancements in the cellular level of reactant (DMAPP) and enzyme (ISPS), as a means in the further development of this technology. This objective was approached upon the heterologous overexpression of *fni*, an isopentenyl isomerase from *Streptococcus pneumoniae*, which increased the amount of the DMAPP reactant at the expense of its isomer, isopentenyl-diphosphate (IPP), in the cells. In addition, the cellular concentration of ISPS was substantially enhanced upon expression of the *ISPS* gene, as a fusion construct with the highly expressed in cyanobacteria *cpcB* gene, encoding the abundant β-subunit of phycocyanin. Synergy between these two modifications, i.e., enhancement in DMAPP substrate availability and enhancement in the concentration of the ISPS enzyme, improved the isoprene-to-biomass production ratio in cyanobacteria from 0.2:1 mg g^{-1} (w:w), attained with the *ISPS* transgene alone, up to 12.3:1 mg g^{-1} (w:w), measured when the combined two modifications were applied to the same cell. This is the highest verifiable yield of heterologous photosynthetic isoprene production reported so far. Findings in this work constitute a step forward in the development of the cyanobacterial biotechnology for isoprene production.

Keywords Bioenergy · Isoprene · Isopentenyl diphosphate isomerase · Metabolic engineering · *Synechocystis* PCC 6803 · Synthetic biology

Introduction

Cyanobacteria have gained attention for their potential to be engineered as photocatalysts for the production of a variety of fuels and useful chemicals (Ducat et al. 2011; Lindblad et al. 2012; Ungerer et al. 2012; Bentley et al. 2013; Oliver and Atsumi 2014; Davies et al. 2015; Savakis and Hellingwerf 2015; Branco dos Santos et al. 2016; Ruffing et al. 2016; Formighieri and Melis 2017; Knoot et al. 2018). Terpenoid products comprise an attractive group of chemicals, as they are diverse, ranging in the tens of thousands of different molecules across all living organisms. This diversity lends itself to the ability to use the terpenoid biosynthetic pathway for the generation of a multitude of products including pharmaceuticals, plastics, flavors and fragrances, nutrition supplements, personal health care compounds, fuels, and general feedstock for the synthetic chemistry industry. Cyanobacteria naturally produce a number of terpenoids through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler 2007; Bentley et al. 2013; Kudoh et al. 2017), mostly in the form of pigments and prenyl molecules for photosynthesis. Of interest in this respect is the ability to divert carbon flux from the MEP pathway and to heterologously produce the hemiterpene isoprene (C_5H_8). The recent literature corroborates the platform of heterologous photosynthetically generated isoprene in cyanobacteria, derived from sunlight, CO_2, and H_2O (Lindberg et al. 2010; Bentley and Melis 2012; Pade et al. 2016; Gao et al. 2016; Chaves et al. 2016, 2017; Kudoh et al. 2017; Xiong et al. 2017).

Julie E. Chaves and Anastasios Melis have read and approved the manuscript.

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In all biological systems, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) are the universal precursors of all terpenoids. The MEP intermediate precursor enzyme 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) reductase generates both DMAPP and IPP from the HMBPP substrate. However, this reductase preferentially catalyzes the synthesis of IPP and thus generates a DMAPP:IPP ratio of ~ 1:6 (Tritsch et al. 2010). In photosynthetic organisms, this ratio is then adjusted through the reversible isomerization of IPP to DMAPP by the isopentenyl diphosphate isomerase (Idi). The chemical mechanism of this isomerization was elegantly described by Street et al. (1990). Several methods have been used to quantify the amount of DMAPP or the combined amount of DMAPP plus IPP but the prevailing DMAPP/IPP ratio is subject to experimental conditions, the Idi enzyme used, and the organism in which this was measured. Working with *Escherichia coli*, (Zhou et al. 2013) reported an in vitro equilibrium ratio of DMAPP/IPP of 2.1:1. The same *E. coli* strain transformed with the isoprene synthase (*ISPS*) gene had a lower DMAPP level and a DMAPP/IPP ratio of 1.05:1 (Zhou et al. 2013). This observation showed that DMAPP is depleted in the presence of the ISPS and is consistent with the notion that DMAPP is the sole substrate of the ISPS enzyme. In *Synechocystis* strain PCC 6803 (*Synechocystis*), IPP to DMAPP isomerization leads to a steady-state DMAPP:IPP ratio of 1:3 (Barkley et al. 2004). This ratio is optimal for longer chain terpenoids of the photosynthetic apparatus that require 3–4 IPP molecules per DMAPP, such as the phytol tail of chlorophyll, carotenoids, and quinone prenyl tails, among other (McGarvey and Croteau 1995; Lichtenthaler 2007). However, as DMAPP is the sole reactant for the synthesis of isoprene (no IPP is involved), the low DMAPP:IPP ratio limits the in-vivo rate and yield of isoprene production. In this respect, function of the native cyanobacterial Idi was deemed to be insufficient for optimal isoprene generation (Gao et al. 2016; Chaves et al. 2016), as it tends to maintain a relatively low (< 1) DMAPP-to-IPP ratio in the cells. Independent work showed that heterologous expression of the *fni* gene, an alternative IPP isomerase from the non-photosynthetic bacterium *Streptococcus pneumoniae*, shifted the DMAPP-IPP isomerization toward DMAPP, thereby helping to enhance rate and yield of isoprene production (Chaves et al. 2016).

A separate line of work addressed a different major factor limiting the rate and yield of cyanobacterial isoprene production. This pertained to the poor expression and low-specific activity of the isoprene synthase (ISPS). Recently, work from this lab successfully addressed this issue through a fusion of the *ISPS* gene to the highly expressed *cpcB*-encoded β-subunit of phycocyanin (Chaves et al. 2017). This fusion-protein approach resulted in up to 270-fold increase in transgenic ISPS protein accumulation and up to a 27-fold increase in the yield of isoprene production.

Objective of this work was to stack together the abovementioned two promising cyanobacterial modifications, which were designed and tested separately, in one and the same host cell. Such undertaking is not trivial but, rather, the required systematic way of conducting research, particularly toward the development of a fully optimized biosynthetic pathway for chemicals production. The task was addressed through the heterologous overexpression of two enzymes, the isoprene synthase fused to the highly expressed cpcB protein (the β-subunit of phycocyanin, Glazer et al. 1983), coupled with the concomitant overexpression of the isopentenyl diphosphate isomerase Fni protein. The synergy of these modifications resulted in a significant isoprene production enhancement and constitutes a step forward in the development of this cyanobacterial engineering platform.

Materials and methods

Strains and culturing conditions

Synechocystis sp. PCC 6803 (*Synechocystis*) was employed as the experimental strain and is referred to as the wild type (WT). Transgenic isoprene-producing recipient strains contain a codon optimized *ISPS* gene from *Pueraria montana* (kudzu) (Sharkey et al. 2005; Lindberg et al. 2010) fused to the *cpcB* gene with a spacer nucleotide sequence encoding either a 7-amino acid linker PMPWRVI (*cpcB*L7*ISPS*) or a 16-amino acid linker EAAAKEAAAKEAAAKA (*cpcB*L16*ISPS*), followed by a chloramphenicol resistance cassette. Fusion constructs were inserted between the *cpcB* upstream region and the *cpcA* gene in the phycocyanin-encoding *cpc* operon of *Synechocystis*.

All strains employed in this work were maintained on 1% agar-BG11 media supplemented with 10 mM TES-NaOH pH 8.2, and 0.3% Na-thiosulfate. Agar plates were supplemented with chloramphenicol (30 μg/mL) and/or streptomycin (50 μg/mL) to maintain the transformants. Liquid cultures were grown in BG11 media buffered with 25 mM NaH_2PO_4 (pH 7.5) at 28 °C, under continuous aeration and gradually increasing illumination. Cultures inoculated from a plate started in the lowest illumination at 30 μmol photons m^{-2} s^{-2} until an $OD_{730\ nm} = 0.3$ was reached. Illumination was then increased to 50 μmol photons m^{-2} s^{-2} until an $OD_{730\ nm} = 0.65–0.75$ was reached. Illumination was further increased to 100 μmol photons m^{-2} s^{-2} until the culture reached a density sufficient to support dilution in a total volume of 700 mL growth medium so as to attain an $OD_{730\ nm} = 0.65$. Equal PAR intensity of fluorescent and incandescent light sources was used for these experiments.

fni-containing constructs

Transformations were performed as previously described according to established procedures (Williams 1988; Eaton-Rye 2004; Nagarajan et al. 2011; Kirst et al. 2014). DNA constructs were designed to insert the isopentenyl diphosphate isomerase (*fni*) gene from *Streptococcus pneumoniae* (Zurbriggen et al. 2012) into *glgA1* (the locus of a gene encoding a glycogen synthase) of the *cpcB*L7*ISPS* and *cpcB*L16*ISPS* recipient strains. The *fni* gene was expressed under control of the Ptrc promoter, followed by a streptomycin resistance cassette. Nucleotide sequences of all constructs employed in this work are given in the Supplementary Materials.

Protein analysis

Liquid cultures were grown in 300-mL volumes to an OD_{730} of 2.5 and re-suspended in 5–10 mL of 50 mM Tris-HCl (pH 8) after pelleting by room temperature centrifugation at 4000 *g* for 15 min. Cells were then treated with protease inhibitor (1 mM PMSF; Sigma-Aldrich) and lysed by French press (2 × 1500 psi). Cell lysates were centrifuged at 2250 *g* for 3 min to pellet glycogen grains and unbroken cells. A small sample from the supernatant was used for chlorophyll *a* concentration determination, as previously described (Chaves et al. 2016). The lysate was supplemented with an equal volume of solubilization solution, comprising 250 mM Tris-HCl, pH 6.8, 7% *w*/*v* SDS, 20% w/v glycerol, 2 M urea, and a few grains of bromophenol blue (about 0.1% *w*:*v*). Samples were then incubated for 2 h at room temperature, followed by supplementation with β-mercaptoethanol to make a 5% final concentration. The solubilized total cellular proteins were resolved by SDS-PAGE and Western blot analysis. Proteins separated by SDS-PAGE were either stained with Coomassie Brilliant Blue for protein visualization or transferred to a nitrocellulose membrane for immunodetection using rabbit immune serum containing specific polyclonal antibodies against the ISPS (Lindberg et al. 2010) or the Fni protein (Zurbriggen et al. 2012).

Isoprene and biomass accumulation

Biomass accumulation and isoprene production were measured in sealed liquid cultures grown photoautotrophically in the absence of antibiotics. Gaseous-aqueous two-phase photobioreactors were designed in this lab specifically for quantitative biomass and isoprene production measurements (Bentley and Melis 2012). The 1 L bioreactors were filled with ~ 700 mL liquid BG11 growth medium containing 25 mM NaH_2PO_4 (pH 7.5), and then seeded with *Synechocystis* starter cultures to an $OD_{730\ nm} = 0.65$. The bioreactors were then slowly bubbled with 500 mL of 100% CO_2 gas through the bottom of the liquid culture to fill the reactor headspace. Bioreactors were then sealed for up to 48 h and stirred slowly and continuously at 28 °C under continuous illumination at 100 μmol photons m^{-2} s^{-2}.

Isoprene production was quantified by sampling 1 mL of the gaseous phase from the reactor headspace via gas chromatography (Shimadzu 8A GC-FID). Isoprene quantification was based on a calibration curve obtained with an isoprene standard (Acros Organics, Fair Lawn, NJ, USA), as described (Chaves et al. 2015). Biomass accumulation in the liquid phase of the reactor was determined upon collection of 50-mL aliquots, followed by centrifugation, rinsing with deionized water, and cell re-suspension in 2 mL of deionized water. Re-suspended samples were transferred and dried on aluminum trays for 6 h at 90 °C and weighed to determine the dry cell weight (dcw). Cell growth was also determined spectrophotometrically by measuring the optical density of live cell cultures at 730 nm with a Shimadzu UV-1800 UV-visible spectrophotometer.

Accession numbers

The detailed nucleotide sequences of the constructs used in this work are shown in the Supplementary Materials. In addition, the following GenBank accession numbers were issued for the constructs used in this work:

Title: Nucleotide sequence of the *cpcB*L7*IspS* fusion construct for expression in *Synechocystis* sp. PCC 6803. GenBank Accession number, MG855740.
Title: Nucleotide sequence of the cpcB*L16*IspS fusion construct genes for expression in *Synechocystis* sp. PCC 6803. GenBank Accession number, MG855741.
Title: Nucleotide sequence of *Synechocystis* codon optimized *Streptococcus pneumoniae* IPP isomerase *fni* gene. GenBank Accession number, MG855742.

Results

Transformations

Recipient *Synechocystis* strains encoding the isoprene synthase fusion proteins cpcB*L7*ISPS and cpcB*L16*ISPS were generated upon introduction of the *cpcB*L7*ISPS* and *cpcB*L16*ISPS* fusion constructs with the attendant chloramphenicol resistance cassette (*CmR*) into the *cpc* operon locus (Fig. 1a) (Chaves et al. 2017). The *cpc* operon encodes components of the phycocyanin peripheral rods in the phycobilisome light-harvesting antenna of cyanobacteria. Each of the two constructs used in this work replaced the native *cpcB* gene, while maintaining the rest

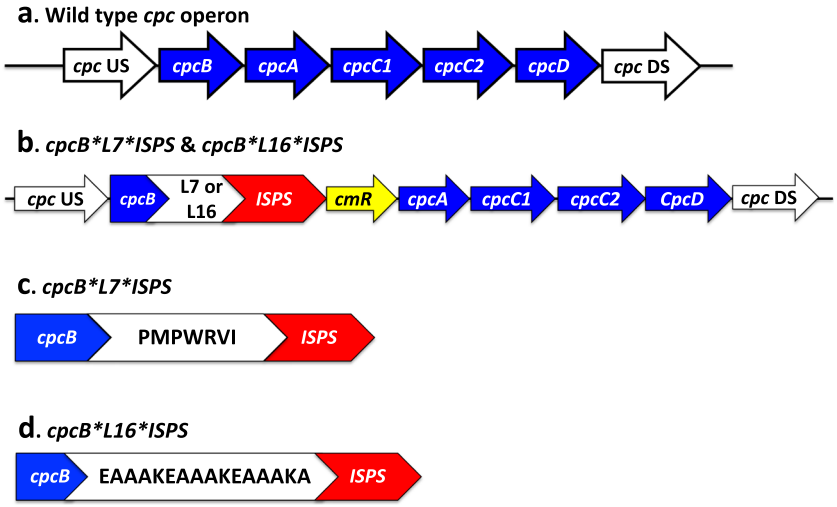

Fig. 1 Configurations of the ***cpc*** operon, as used in this work. **a** Gene sequence in the native (WT) *Synechocystis* ***cpc*** operon. **b** *cpcB*L7*ISPS* and *cpcB*L16*ISPS* fusion constructs and the attendant chloramphenicol resistance cassette designed to replace the *cpcB* gene upon a double homologous recombination. The amino acid sequences of the linker / spacers introduced between the cpcB and ISPS are given for the fusion proteins in **c** for the cpcB*L7*ISPS and **d** for the cpcB*L16*ISPS construct. [Adapted with permission from Chaves et al. (2017)]

of the native *cpc* operon (*cpcA*, *cpcC1*, *cpcC2*, *cpcD* genes) in place (Fig. 1b). The above fusion constructs contained a defined oligopeptide linker/spacer between the cpcB and ISPS proteins. Detailed descriptions of the linker L7 and L16 amino acid sequences are given in Fig. 1c, d, respectively. These linkers were shown to enhance the specific activity of the isoprene synthase in the cpcB fusion construct (Chaves et al. 2017).

The *fni* construct with the attendant streptomycin resistance cassette (SmR) was inserted into the *glgA1* locus (sll0945, encoding the glycogen synthase 1, which is a protein of the glycogen biosynthetic pathway, Fig. 2a). In this case, the *cpcB*L7*ISPS* and *cpcB*L16*ISPS* transformants acted as the recipient strains. The *fni* transformation replaced the native *glgA1* gene (Fig. 2b). Genomic DNA PCR analysis was employed to test for the state of homoplasmy achieved in these transformants. We previously confirmed that the recipient's strains *cpcB*L7*ISPS* and *cpcB*L16*ISPS* have reached a state of homoplasmy (Chaves et al. 2017). State of homoplasmy for the subsequent *fni* transformants was tested and verified, as described in detail in the Supplementary Materials (Fig. S1).

Absorbance spectra and protein analyses

Wild type and transformant cells were lysed upon passing the respective suspensions through a French press apparatus and absorbance spectra of the cell lysates were recorded. Results showed absence of the phycocyanin pigments from the transformants (Fig. S2), consistent with recent findings (Chaves et al. 2017).

Protein profiles of each strain were examined by SDS-PAGE and Western blot analysis. In the SDS-PAGE Coomassie-stain analysis, the wild type showed dominant bands at 55, 20, and 15 kD attributed to the large subunit of Rubisco (RbcL), the *cpcB*-encoded β-subunit of phycocyanin, and the *cpcA*-encoded α-subunit of phycocyanin, respectively (Fig. 3a; WT). The recipient strains showed dominant bands at 84 and 55 kD, attributed to the cpcB*L7*ISPS or

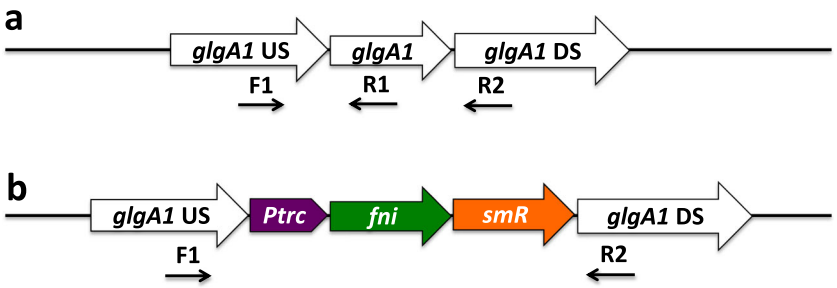

Fig. 2 Configurations of the *glgA1* locus, as used in this work. **a** Wild-type schematic of the *glgA1* gene and location of the F1, R1, and R2 primers for genomic DNA PCR analyses. **b** Schematic of the *glgA1* locus in which the *glgA1* gene was replaced by a construct comprising the *Ptrc* promoter, the *fni* transgene, and the streptomycin resistance cassette. Location of the F1 and R2 primers for genomic DNA PCR analyses is also shown

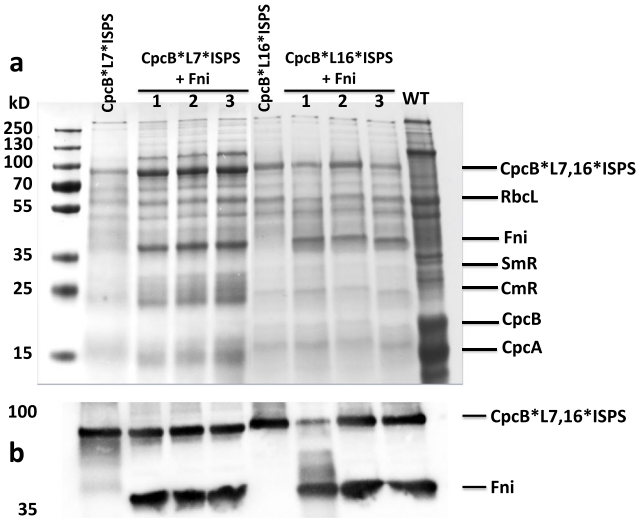

Fig. 3 SDS-PAGE and Western blot analysis of *Synechocystis* wild type (WT) and transgenic cell proteins. **a** Total protein extracts were resolved by SDS-PAGE and Coomassie stain, loading about 1 μg Chl per lane. The wild type showed a dominant protein band at about 58 kD attributed to the large subunit of Rubisco, and abundant protein bands at about 20 kD from the cpcB phycocyanin β-subunit, as well as at about 15 kD from the cpcA phycocyanin α-subunit. The cpcB*L7*ISPS, cpcB*L7*ISPS+Fni, cpcB*L16*ISPS, and the cpcB*L16*ISPS+Fni transformants failed to accumulate the 20- and 15-kD phycocyanin subunits. The transformants showed substantial accumulation of the fusion proteins cpcB*L7*ISPS, cpcB*L16*ISPS, and Fni proteins, migrating to about 80–85 kD and 37 kD, respectively. A faint band at ~24 kD in the SDS-PAGE analysis was attributed to expression of the chloramphenicol resistance protein. **b** Western blot analysis was conducted using polyclonal antibodies specific to the ISPS and Fni. Strong cross-reactions at the 84-kD region were identified as the cpcB*(L7 or L16)*ISPS fusions, and at the 37-kD region as the Fni

cpcB*L16*ISPS fusion proteins, and the RbcL, respectively (Fig. 3a; lanes 1 and 5). The CpcB and CpcA proteins were absent from the recipient strains, resulting in a Δcpc (phycocyanin-less) phenotype (Kirst et al. 2014). Three independent lines of the cpcB*L7*ISPS+Fni double transformant (Fig. 3a; lanes 2–4) and three independent lines of the cpcB*L16*ISPS+Fni double transformant (Fig. 3a; lanes 6–8) showed presence of the same dominant ~84 kD protein bands as the recipient strains. In addition, substantial amounts of the heterologous Fni recombinant protein were noted at ~37 kD. Antibiotic resistance proteins showed faint bands at 23 kD (CmR) and 27 kD (SmR).

Western blot analysis (Fig. 3b) showed strong cross-reactions of the ISPS polyclonal antibodies with a protein band at 84 kD in all transformant strains. Likewise, a strong cross reaction of the Fni polyclonal antibodies was observed with a protein band at 37 kD in the cpcB*L7*ISPS+Fni (Fig. 3b; lanes 2–4) and cpcB*L16*ISPS+Fni (Fig. 3b; lanes 6–8) transformants. These observations confirmed that transformations performed in this work truly resulted in overexpression of the heterologous ISPS fusion and Fni recombinant proteins in *Synechocystis*.

Cell biomass and isoprene accumulation

Liquid cultures of all strains were grown for biomass and isoprene accumulation measurements using the gaseous-aqueous two-phase bioreactors designed by Bentley and Melis (2012). Biomass accumulation was determined spectrophotometrically by OD at 730 nm (Fig. 4a), and dry cell weight (Fig. 4b). The rates of biomass accumulation were determined from the slope of the average accumulation in nine separate cultures from the 48–96-h growth period. Strains cpcB*L7*ISPS and cpcB*L7*ISPS+Fni accumulated biomass at a slightly slower rate (about 4 and 5 mg L^{-1} h^{-1}, respectively) than the cpcB*L16*ISPS and the cpcB*L16*ISPS+Fni (6 and 8 mg L^{-1} h^{-1}, respectively) (Table 1). These small variations in the rate may be attributed to greater isoprene production in the L7 strains (see below), thus leading to slower carbon partitioning for biomass accumulation than in the L16 transformants.

Isoprene production was measured concurrently with biomass accumulation and was found to vary greatly between different transformants. As a point of reference, a simple

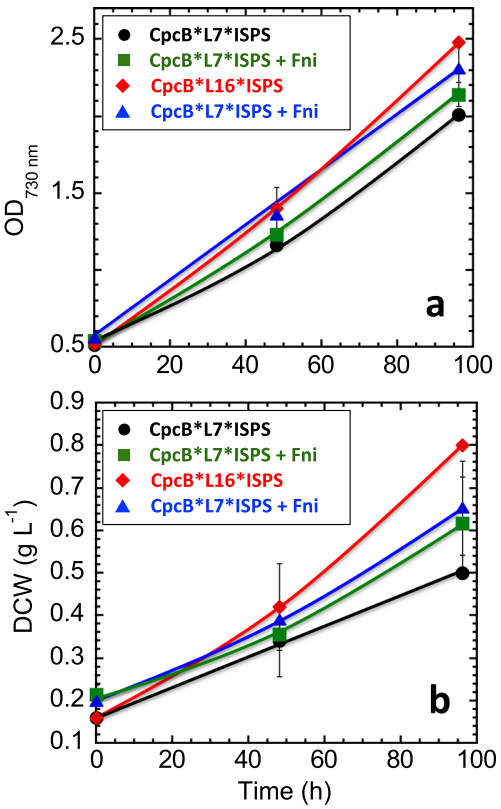

Fig. 4 Liquid cultures of all strains used in this work were grown using the gaseous-aqueous two-phase bioreactor developed in this lab (Bentley and Melis 2012), bubbled with 500 mL 100% CO_2 through the culture to fill the reactor headspace, sealed and stirred slowly by a magnetic stir bar, under continuous illumination of 100 μmol photons m^{-2} s^{-1}. Biomass accumulation was measured every 48 h, for a total of 96 h, by optical density at 730 nm (OD_{730}) and dry cell weight (dcw). Results from nine biological replicates for each measurement are shown

Table 1 Liquid cultures were grown in gaseous-aqueous two-phase photobioreactors for 96 h with provision of 500 mL of 100% CO_2 slowly bubbled through the culture to fill the reactor headspace (Bentley and Melis 2012). Continuous illumination of 100 μmol photons m^{-2} s^{-1} was also provided. Rates of biomass accumulation (mg dcw L^{-1} h^{-1}), isoprene (μg L^{-1} h^{-1}), and isoprene-to-biomass (w:w) ratios (mg g^{-1}) were calculated for each strain. (± SD of nine biological replicates for each parameter measured)

Strain	Rate of biomass accumulation (mg L^{-1} h^{-1})	Rate of isoprene accumulation (μg L^{-1} h^{-1})	Isoprene to biomass ratio (mg g^{-1})
ISPS[1]	8.0 ± 0.3	1.7 ± 0.3	0.2 ± 0.05
cpcB*L16*ISPS	6.0 ± 1.2	6.3 ± 0.8	1.5 ± 0.2
cpcB*L16*ISPS+Fni	8.0 ± 1.1	27.0 ± 5.0	5.5 ± 0.5
cpcB*L7*ISPS	4.0 ± 0.1	27.0 ± 1.0	6.0 ± 0.5
cpcB*L7*ISPS+Fni	5.0 ± 0.6	46.0 ± 8.8	12.3 ± 1.6

[1] Results from Chaves et al. (2017)

ISPS transformant generated 1.7 μg isoprene L^{-1} h^{-1} (Table 1). Among the fusion construct *plus* Fni transformants, the slowest isoprene producer was the cpcB*L16*ISPS, accumulating 6.3 μg L^{-1} h^{-1}. Addition of the *fni* transgene to this recipient strain, converting it to cpcB*L16*ISPS+Fni, boosted isoprene production nearly 4-fold to 27 μg L^{-1} h^{-1} (Fig. 5a and Table 1). The cpcB*L7*ISPS strain by itself produced isoprene at 27 μg L^{-1} h^{-1}, and the addition of the *fni* transgene, converting it to cpcB*L7*ISPS+Fni, boosted isoprene production up to 46 μg L^{-1} h^{-1} (Fig. 5a and Table 1). These results translated into different isoprene-to-biomass carbon partitioning ratios for the transformants examined in this work. The lowest yield of 0.2 mg isoprene per g biomass was measured with a simple *ISPS* transformant. Among the fusion construct *plus* Fni transformants, 1.5 mg isoprene per g biomass was measured with the cpcB*L16*ISPS construct, and the highest yield of 12.3 mg isoprene per g biomass was observed with the cpcB*L7*ISPS+Fni double transformant (Fig. 5). The latter is the highest verifiable constitutive photosynthetic isoprene production measured with homoplasmic lines, in which the heterologous isoprene biosynthesis pathway genes are encoded by the *Synechocystis* genomic DNA.

Discussion

Rerouting carbon flux from the MEP pathway to isoprene was improved in this work upon alleviation of two key bottlenecks, i.e., inadequate ISPS enzyme concentration in the cell and limited MEP pathway flux to DMAPP, which is the sole isoprene precursor. We showed that overexpression of the combination of the *cpcB*L7*ISPS* and *fni* transgenes can improve isoprene production up to 62-fold (12.3 mg g^{-1}), relative to previous results from the heterologous expression of a single isoprene synthase gene, which yielded a mere 0.2 mg isoprene per g biomass generated (Table 1, Chaves et al. 2017, 2016, 2015; Bentley and Melis 2012). This yield improvement was due to the synergy of the effects from the overexpression of Fni, shifting the DMAPP-to-IPP steady state ratio in the cells more toward DMAPP, and also due to the

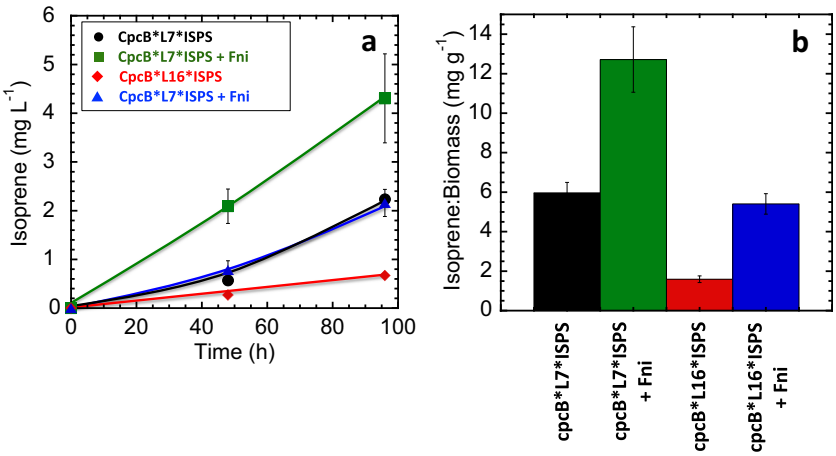

Fig. 5 **a** Isoprene accumulation measurements were conducted concurrently with biomass measurements, every 48 h for a total of 96 h. Gas chromatography was used to analyze 1-mL samples taken from the gaseous headspace of the reactor using a gas tight syringe. Isoprene quantification was determined based on a calibration curve of an isoprene standard (Acros Organics, Fair Lawn, NJ, USA), as described (Chaves et al. 2015). **b** Isoprene production was calculated relative to biomass accumulation in mg isoprene produced per g of dry cell weight. Results from nine biological replicates for each measurement are shown

overexpression of the cpcB*L7*ISPS fusion protein, aiding in the catalytic conversion of DMAPP to isoprene.

A summary of the resulting state of the art in *Synechocystis* isoprene production is presented by the MEP pathway flux schematic shown in Fig. 6. In wild type cyanobacteria, substrate flux through the MEP pathway leads to the synthesis of HMBPP, which then partitions unevenly with a preference toward accumulation of IPP and, secondarily, DMAPP. The initial DMAPP:IPP ratio was reported to be 1:6 (Tritsch et al. 2010), which increased upon the action of the *Synechocystis* Idi isomerase to 1:3 (Barkley et al. 2004). The latter is consistent with the DMAPP:IPP ratios needed for the synthesis of native photosynthetic terpenoids but not optimal for isoprene production. Overexpression of the Fni enzyme shifted this steady-state ratio toward DMAPP, thereby enhancing the pool size of this isoprene synthase reactant (Fig. 6). The additional overexpression of the isoprene synthase as a fusion with the highly expressed cpcB protein (the β-subunit of phycocyanin) effectively converted the extra DMAPP to isoprene, thereby enhancing the overall isoprene-to-biomass partitioning ratio.

In addition to the novel metabolic pathway phenotype, the work highlighted the assembly of a truncated phycobilisome antenna in the transformants, which contained only the allophycocyanin core-cylinders (Kirst et al. 2014) and were devoid of the peripheral phycocyanin rods. The transformants, therefore, possessed a *T*runcated *L*ight-harvesting *A*ntenna size phenotype, evident in their absorption spectra (Fig. S2). Such altered cellular and metabolic organization may have implications for the internal structure and thylakoid membrane development of these cyanobacteria (van de Meene et al. 2006). Further, the TLA property of the transformants bodes well for enhanced productivity under mass-culture and bright sunlight conditions (Kwon et al. 2013; Kirst et al. 2014), as these would prevail in biotechnology applications. An additional benefit emanating from the absence of phycocyanin rods in the transformants is resource economy, as these strains need not synthesize one of the cell's most abundant proteins, the cpcA α-subunit of phycocyanin, and the associated bilin tetrapyrrole pigments, translating in less per cell nitrogen investment and a lower per cell endogenous metabolic burden that can be used to generate the desirable isoprene product instead.

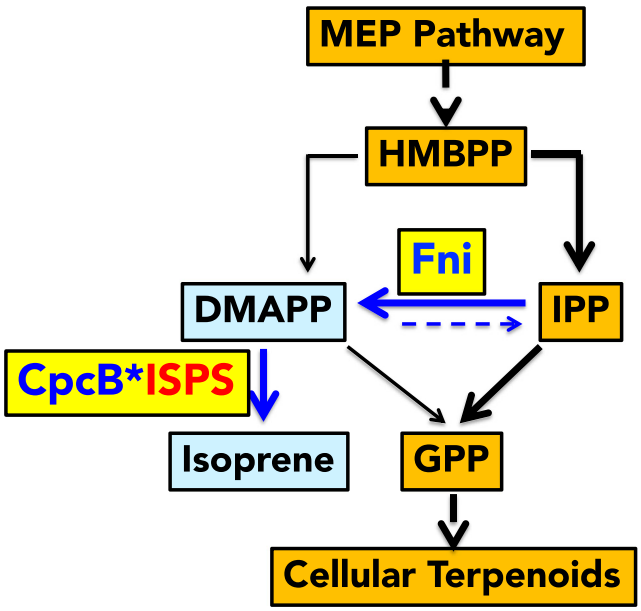

Fig. 6 Schematic of carbon flow through the MEP biosynthetic pathway leading to isoprene production. The IspH enzyme utilizes a single substrate, (E)-4-Hydroxy-3-methyl-but-2-enyl di-phosphate (HMBPP), to generate the two precursor molecules for the synthesis of all terpenoids, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). Native cyanobacterial terpenoid production requires substrate flux primarily through the IPP precursor and a steady state IPP:DMAPP ratio of 3:1 in the cell. Isoprene synthesis requires only the DMAPP reactant. Overexpression of the heterologous isopentenyl diphosphate isomerase (Fni) in *Synechocystis* shifted the IPP to DMAPP steady state ratio toward DMAPP, thereby enhancing the catalytic activity of the cpcb*ISPS fusion enzyme and leading to greater yields of isoprene production

Previous studies of the terpenoid biosynthetic pathway enzyme sequences have shown that the terpene synthases evolved by gene duplication and then diverged to form the enormous variety of enzymes leading to different terpenoid structures (Tholl 2006). Evolution of these enzymes is facilitated by the promiscuous nature of their activity (Christianson 2008), thus leading to poor catalytic activity and unwanted alternative product formation from the point of view of metabolic engineering objectives (Nobeli et al. 2009). In this respect, isoprene synthase enzymes have been shown to also bind geranyl diphosphate (GPP), which competes with the binding of DMAPP (Koksal et al. 2010), thereby slowing down isoprene production. Future work on the terpenoid pathway for isoprene production will involve optimization of the pertinent protein concentration and engineering the pathway's properties of enzyme catalysis. Combining the strategies of enhanced enzyme concentration and improved rates of catalysis (k_{cat}) has the potential to further increase terpenoid product generation (Leonard et al. 2010) above and beyond of what is achieved today. Our approach in this work has provided a key piece of the complex puzzle toward the renewable photosynthetic production of isoprene.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Barkley SJ, Desai SB, Poulter CD (2004) Type II isopentenyl diphosphate isomerase from *Synechocystis* sp. strain PCC 6803. J Bacteriol 186(23):8156–8158

Bentley FK, Melis A (2012) Diffusion-based process for carbon dioxide uptake and isoprene emission in gaseous/aqueous two-phase photobioreactors by photosynthetic microorganisms. Biotechnol Bioeng 109(1):100–109

Bentley FK, García-Cerdán JG, Chen H-C, Melis A (2013) Paradigm of monoterpene (β-phellandrene) hydrocarbons production via photosynthesis in cyanobacteria. BioEnergy Res 6:917–929

Branco Dos Santos F, Du W, Hellingwerf KJ (2016) *Synechocystis*: not just a plug-bug for CO_2, but a green *E. coli*. Front Bioeng Biotechnol 2:36

Chaves JE, Kirst H, Melis A (2015) Isoprene production in *Synechocystis* under alkaline and saline growth conditions. J Appl Phycol 27: 1089–1097

Chaves JE, Romero P, Kirst H, Melis A (2016) Role of isopentenyl-diphosphate isomerase in heterologous cyanobacterial (*Synechocystis*) isoprene production. Photosyn Res 130:517–527

Chaves JE, Rueda-Romero P, Kirst H, Melis A (2017) Engineering isoprene synthase expression and activity in cyanobacteria. ACS Synth Biol 6:2281–2292

Christianson DW (2008) Unearthing the roots of the terpenome. Curr Opin Chem Biol 12(2):141–150

Davies FK, Jinkerson RE, Posewitz MC (2015) Toward a photosynthetic microbial platform for terpenoid engineering. Photosynth Res 123: 265–284

Ducat DC, Way JC, Silver PA (2011) Engineering cyanobacteria to generate high-value products. Trends Biotechnol 29:95–103

Eaton-Rye JJ (2004) The construction of gene knockouts in the cyanobacterium *Synechocystis* sp. PCC 6803. In Methods of Molecular Biology, Vol 274: Photosynthesis Protocols (Carpentier R, Ed) pp 309–324, Humana Press, Totowa, NJ

Formighieri C, Melis A (2017) Heterologous synthesis of geranyllinalool, a diterpenol plant product, in the cyanobacterium *Synechocystis*. Applied Microbiol Biotechnol 101:2791–2800

Gao X, Gao F, Liu D, Zhang H, Nie X, Yang C (2016) Engineering the methylerythritol phosphate pathway in cyanobacteria for photosynthetic isoprene production from CO_2. Energy Env Sci 9(4):1400–1411

Glazer AN, Lundell DJ, Yamanaka G, Williams RC (1983) The structure of a "simple" phycobilisome. Ann Microbiol (Paris) 134B(1):159–180

Kirst H, Formighieri C, Melis A (2014) Maximizing photosynthetic efficiency and culture productivity in cyanobacteria upon minimizing the phycobilisome light-harvesting antenna size. Biochim Biophys Acta Bioenerg 1837(10):1653–1664

Knoot CJ, Ungerer J, Wangikar PP, Pakrasi HB (2018) Cyanobacteria: promising biocatalysts for sustainable chemical production. J Biol Chem 293:5044-5052. https://doi.org/10.1074/jbc.R117.815886

Köksal M, Zimmer I, Schnitzler JP, Christianson DW (2010) Structure of isoprene synthase illuminates the chemical mechanism of teragram atmospheric carbon emission. J Mol Biol 402(2):363–373

Kudoh K, Hotta S, Sekine M, Fujii R, Uchida A, Kubota G, Kawano Y, Ihara M (2017) Overexpression of endogenous 1-deoxy-d-xylulose 5-phosphate synthase (DXS) in cyanobacterium *Synechocystis* sp. PCC6803 accelerates protein aggregation. J Biosci Bioeng 123: 590–596

Kwon J-H, Bernát G, Wagner H, Rögner M, Rexroth S (2013) Reduced light-harvesting antenna: consequences on cyanobacterial metabolism and photosynthetic productivity. Algal Res 2:188–195

Leonard E, Ajikumar PK, Thayer K, Xiao WH, Mo JD, Tidor B, Stephanopoulos G, Prather KLJ (2010) Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. Proc Natl Acad Sci U S A 107(31):13654–13659

Lichtenthaler HK (2007) Biosynthesis, accumulation and emission of carotenoids, α-tocopherol, plastoquinone, and isoprene in leaves under high photosynthetic irradiance. Photosynth Res 92(2):163–179

Lindblad P, Lindberg P, Oliveira P, Stensjö K, Heidorn T (2012) Design, engineering, and construction of photosynthetic microbial cell factories for renewable solar fuel production. Ambio 41:163–168

Lindberg P, Park S, Melis A (2010) Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. Metabol Engin 12:70–79

McGarvey DJ, Croteau R (1995) Terpenoid metabolism. Plant Cell 7(7): 1015–1026

Nagarajan A, Winter R, Eaton-Rye J, Burnap R (2011) A synthetic DNA and fusion PCR approach to the ectopic expression of high levels of the D1 protein of photosystem II in *Synechocystis* sp PCC 6803. J Photochem Photobiol B 104:212–219

Nobeli I, Favia AD, Thornton JM (2009) Protein promiscuity and its implications for biotechnology. Nat Biotech 27(2):157–167

Oliver JWK, Atsumi S (2014) Metabolic design for cyanobacterial chemical synthesis. Photosynth Res 120:249–261

Pade N, Erdmann S, Enke H, Dethloff F, Dühring U, Georg J, Wambutt J, Kopka J, Hess WR, Zimmermann R, Kramer D, Hagemann M (2016) Insights into isoprene production using the cyanobacterium *Synechocystis* sp. PCC 6803. Biotech Biofuels 9:89

Ruffing AM, Jensen TJ, Lucas M, Strickland LM (2016) Genetic tools for advancement of *Synechococcus* sp PCC 7002 as a cyanobacterial chassis. Microb Cell Factories 15:190

Savakis P, Hellingwerf KJ (2015) Engineering cyanobacteria for direct biofuel production from CO_2. Curr Opin Biotech 33:8–14

Sharkey TD, Yeh S, Wiberley AE, Falbel TG, Gong D, Fernandez DE (2005) Evolution of the isoprene biosynthetic pathway in kudzu. Plant Physiol 137:700–712

Street IP, Christensen DJ, Poulter CD (1990) Hydrogen exchange during the enzyme-catalyzed isomerization of isopentenyl diphosphate and dimethylallyl diphosphate. J Am Chem Soc 112:8577–8578

Tholl D (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr Opin Plant Biol 9(3):297–304

Tritsch D, Hemmerlin A, Bach TJ, Rohmer M (2010) Plant isoprenoid biosynthesis via the MEP pathway: in vivo IPP/DMAPP ratio produced by (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase in tobacco BY-2 cell cultures. FEBS Lett 584(1):129–134

Ungerer J, Tao L, Davis M, Ghirardi M, Maness P-C, Yu J (2012) Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. Energy Environ Sci 5: 8998–9006

Van de Meene AML, Hohmann-Marriott MF, Vermaas WFJ, Roberson RW (2006) The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC 6803. Arch Microbiol 184:259–270

Williams JGK (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol 167:766–778

Xiong W, Cano M, Wang B, Douchi D, Yu J (2017) The plasticity of cyanobacterial carbon metabolism. Curr Opin Chem Biol 41:12–19

Zhou C, Li Z, Wiberley-Bradford AE, Weise SE, Sharkey TD (2013) Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase. Anal Biochem 440:130–136

Zurbriggen A, Kirst H, Melis A (2012) Isoprene production via the mevalonic acid pathway in *Escherichia coli* (bacteria). BioEnergy Res 5(4):814–828