Photosynthesis-dependent biosynthesis of medium chain-length fatty acids and alcohols

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ABSTRACT

Cyanobacteria can directly channel atmospheric \( \text{CO}_2 \) into a wide range of versatile carbon products such as fatty acids and fatty alcohols with applications including fuel, cosmetics, and health products. Works on alcohol production in cyanobacteria have so far focused on either long (C12-C18) or short (C2-C4) chain-length products. In the present work, we report the first synthetic pathway for 1-octanol (C8) biosynthesis in Synechocystis sp. PCC 6803, employing a carboxylic acid reductase and C8-prefering fatty acyl-ACP thioesterase. The first engineered strain produced 1-octanol but exhibited poor productivity and cellular health issues. We therefore proceeded to systematically optimize the strain and cultivation conditions in order to understand what the limiting factors were. The identification of optimal promoters and ribosomal binding sites, in combination with isopropyl myristate solvent overlay, resulted in a combined (C8–OH and C10–OH) titer of more than 100 mg/L (a 25-fold improvement relative to the first engineered strain) and a restoration of cellular health. Additionally, more than 905 mg/L 1-octanol was produced when the strain expressing sp (phosphopantetheinyl transferase) and car (carboxylic acid reductase) was fed with octanoic acid. A combination of feeding experiments and protein quantification indicated that the supply of octanoic acid from the introduced thioesterase, and possibly also native fatty acid synthesis pathway, were the main bottlenecks of the pathway.

1. Introduction

Photosynthetic microorganisms can potentially contribute towards complementing and replacing our current use of fossil fuels as a source of fuels and chemicals, without compromising our ability to also produce food. In order to directly synthesize such ready-to-use products, genetically engineered microorganisms need to be employed. Although great progress has recently been made in laboratory-based development of proof-of-principle systems (Lan et al., 2013; Nozzi et al., 2017), we know of no examples where such engineered photosynthetic microorganisms are used today for commercial production of either chemicals or fuels. Several challenges need to be overcome before this can be realized. One of many such challenges is the creation of efficient biocatalysts, here defined as cellular systems that both synthesize and excrete carbon-containing products.

Fatty acids and their derivatives are highly versatile products in the chemicals, food additives and fuel markets. Further reduction of fatty acids yields corresponding alcohols that directly can act as a replacement of fossil fuel products without further chemical modification (Fig. 1). Production of fatty alcohols from fatty acids has been demonstrated in several heterotrophic model microorganisms, such as Escherichia coli (Akhtar et al., 2013) and Saccharomyces cerevisiae (Zhou et al., 2016). Cyanobacteria have also been engineered to produce either long (C18) or short (e.g. C4) chain-length alcohols by direct reduction of acyl-ACP (Kaczmarzyk et al., 2018; Kaiser et al., 2013) or introduction of acyl-CoA pathways (Anfelt et al., 2015; Lan and Liao, 2012; Lan et al., 2013). Whilst longer chain-length alcohols suffer from challenges with product separation, the shorter alcohols (e.g. ethanol and butanol) do not satisfy all consumer needs, especially as a fuel (Kremer et al., 2015).

In the present work, we have therefore focused on developing biocatalytic strains for the production of intermediate chain-length products, i.e. the C8 fatty acid (caprylic acid) and corresponding alcohol (1-octanol). Effective C8-specific thioesterases have previously been identified (Jing et al., 2011) and used to construct synthetic pathways for 1-octanol biosynthesis in Escherichia coli (Akhtar et al., 2015). Whilst caprylic acid is commercially attractive as a food and feed ingredient (Rosenblatt et al., 2017), 1-octanol has been identified as a direct diesel replacement without the need to alter the existing vehicle and distribution infrastructure (Kremer et al., 2015). The implementation of these pathways in Synechocystis sp. PCC 6803 resulted in the biosynthesis of C8 and C10 acids and alcohols that were both excreted in to the extracellular media. Optimization of ribosomal binding sites and promoters led to further enhancement of productivity. Through quantification of...
Tes3 and in vitro feeding experiments we could conclude that the overall productivity is limited by flux through the native fatty acid synthesis pathway and the thioesterase in the introduced synthetic pathway.

2. Materials and methods

2.1. Culture medium and growth condition for Escherichia coli

E. coli DH5α (Thermo Fisher Scientific) was used to propagate all the plasmids used in the study. The strain was cultivated in lysogeny broth (LB) medium (LB Broth, Sigma Aldrich), 37 °C, 180 rpm, and supplemented with appropriate antibiotics (final concentration: carbenicillin 100 μg/ml, chloramphenicol 37 μg/ml, kanamycin 50 μg/ml, and erythromycin 200 μg/ml). The broad-host-range RSF1010 plasmid was used to transform E. coli HB101 (obtained from Prof. Peter Wolk, Michigan State University), a cargo strain carrying the pRL623 plasmid. Cultures were plated on LB agar containing chloramphenicol and erythromycin to maintain and screen for colonies carrying pRL623 and RSF1010 plasmids, respectively. The conjugate strain E. coli ED8654 (obtained from Prof. Peter Wolk, Michigan State University) (Elhai and Wolk, 1988), used for triparental conjugation, was grown in LB medium containing carbenicillin.

2.2. Culture medium and growth condition for Synechocystis sp. PCC 6803

The Synechocystis sp. PCC 6803 (hereafter Synechocystis) wild-type strain was obtained from Prof. Klaas Hellingwerf (University of Amsterdam, Netherlands). All strains were cultivated in BG11 medium (Stanier et al., 1971) without cobalt (hereafter BG11-Co) unless stated otherwise. The preculture was grown in 5 ml BG11-Co containing appropriate antibiotic(s) (final concentration: kanamycin 50 μg/ml and erythromycin 20 μg/ml) in a 6-well plate at 30 °C, 180 rpm, with continuous illumination (cool-white LED) at 60 μmol photons m⁻² s⁻¹ and 1% (v/v) CO₂ in an Algaetron AG 230 (Photon Systems Instruments) (PSI). For production experiments, when the OD₇₃₀ reached 3–4, the culture was transferred to a 100-ml Erlenmeyer flask and the OD₇₃₀ was adjusted to 0.2 by adding BG11-Co medium to a final volume of 25 ml containing appropriate antibiotic(s). The culture was grown for 10 days in an Algaetron AG 230 (PSI) at 30 °C, 180 rpm, with continuous illumination (cool-white LED) at 60 μmol photons m⁻² s⁻¹ and 1% (v/v) CO₂. Recombinant protein synthesis was induced at 48 h with 5 μM cobalt and the OD₇₃₀ was measured every 48 h thereafter. For the 1-octanol toxicity experiment, the Synechocystis sp. PCC 6803 strain lacking acyl-ACP synthetase gene (Δaas strain) was used and spiked with 1-octanol (≥98%, Alfa Aesar) to achieve a different final concentration of 1-octanol in the liquid medium. Isopropyl myristate solvent overlay (10% v/v) (Sigma Aldrich) was added immediately and the culture was grown for 72 h.

2.3. Transformation methods for Synechocystis sp. PCC 6803

For knocking out the acyl-ACP synthetase gene, a Synechocystis wild-type strain freshly prepared on BG11-Co agar plates (1.5% w/v) was used to inoculate 25 ml of BG11-Co with a starting OD₇₃₀ of 0.02. Cells were harvested when the OD₇₃₀ reached 0.4–0.7, washed in 10 ml BG11-Co twice, and resuspended in 500 μL of BG11-Co. One hundred microliters of concentrated liquid culture were mixed with four to seven micrograms of plasmid pPB43 and incubated at 30 °C with continuous illumination at 60 μmol photons m⁻² s⁻¹ and 1% (v/v) CO₂ for 12–16 h prior to plating on BG11-Co agar containing 10% strength of antibiotic. To promote segregation, individual colonies were restreaked on BG11-Co agar with higher antibiotic concentration. For diagnostic colony PCR, biomass was resuspended in sterile ddH₂O and exposed to two times of freeze and thaw cycles at 95 °C and 80 °C. Cultures were centrifuged and 3 μL of the supernatant was used as a template for polymerase chain reaction (PCR) with primers IY113 and IY114. A fully segregated mutant was used for subsequent experiments.

For triparental conjugation, one hundred microliters of the cargo strain, conjugate strain, and Synechocystis (OD₇₃₀ ~1) were mixed and incubated for 2 h (30 °C, 60 μmol photons m⁻² s⁻¹). Prior to mixing, all the E. coli and cyanobacteria strains were washed with fresh LB medium and BG11-Co medium, respectively, to remove the antibiotics. After 2 h of incubation, the culture preparations were transferred onto BG11 agar plates without antibiotics and incubated for two days (30 °C, 60 μmol photons m⁻² s⁻¹). After 2 days of incubation, cells were scrapped from the agar plate, resuspended in 500 μL of BG11-Co medium, and transferred onto a new agar plate containing 20 μg/ml erythromycin. Cells were allowed to grow for one week until colonies appeared. Individual colonies were restreaked onto a new plate containing 20 μg/ml erythromycin and used for subsequent experiments. All plasmids and strains used in this study are listed in Supplementary Tables 1 and 2, respectively.

2.4. Plasmid construction

All plasmids used for triparental conjugation in this study were assembled using the BASIC Assembly method (Storch et al., 2015). Linkers were designed using R2ODNA software: http://www.r2odna.com/ and ordered from Integrated DNA Technologies (IDT). A schematic diagram describing the plasmid construction procedure is shown in Fig. 2A. The details of prefix and suffix neutral linkers, ribosome

![Fig. 1. Graphic illustration of the engineered synthetic pathway in cyanobacteria. The associated enzymes are shown in green: (1) Acyl-ACP is synthesised via the native fatty acid biosynthesis (FAS) pathway, followed by (2) the production of free fatty acid by a thioesterase (tes3; Anabaenococcus tetras) and yielded via either (3) Acyl-ACP synthetase (aua) is deleted to block the recycling pathway of free fatty acid to acyl-ACP. With the aid of a maturase (3) phosphopantetheiny1 transferase (sfp; Bacillus subtilis), free fatty acids are converted into their corresponding medium-chain-length fatty aldehydes by (4) carboxylic acid reductase (car; Mycobacterium marinum). Finally, the fatty aldehyde intermediate is converted to medium-chain-length fatty alcohols by (5) native aldehyde reductase(s) (ahr; Synechocystis sp. PCC 6803).](image-url)
Fig. 2. Graphic overview of engineered parts and results from transformation. (A) Schematic diagram describing plasmid construction according to Storch et al., 2014. DNA parts were stored in a pJET1.2 blunt vector (Thermo Fisher Scientific), digested with BsaI and ligated with their respective prefix and suffix linkers. Ligated DNA parts and linkers were purified (Nimagen) prior to plasmid assembly. Next, the GFP dropout gene was replaced with the gene of interest (GOI) with an RBS linker preceding the GOI. (B) Plasmid organisation for Tes3 expression. (C) Transformation results of Tes3 expressing plasmids in both *Escherichia coli* (*E. coli*) and *Synechocystis* sp. PCC 6803 (Δaas) strains. (D) Plasmid organisation for Tes3, Sfp, and CAR expression. (E) Transformation results with the Tes3, Sfp, and CAR expressing plasmids in both *Escherichia coli* (*E. coli*) and *Synechocystis* sp. PCC 6803 (Syn-Δaas) strains.
binding site linkers, methylated linkers, and genetic parts used to construct the plasmids are provided in Supplementary Table 1. The genes comprising: tes3 (a thioesterase from *Anaerococcus tetrasaw* with accession No. EE182564), sfp (a phosphopantetheinyl transferase from *Bacillus subtilis* with UniProt accession No. P39135), and car (a carboxylic acid reductase from *Mycobacterium marinum* with UniProt accession No. B2HN69) were amplified from plasmid pET-TPC3 (*Akhbar et al., 2015*) using primers listed in Supplementary Table 1. The primer sequences are listed in Supplementary Table 3.

2.5. Free fatty acids and fatty alcohols extraction and analysis

For extraction of free fatty acids from the whole liquid culture, liquid cultures in flasks were mixed well by shaking prior to extraction. Two milliliters of liquid culture were transferred into a PYREX round bottom threaded culture tube (Corning, Manufacturer Part Number: 99449-13). Two hundred microliters of 1 M H₃PO₄ were added to acidify the culture. Each culture was spiked with 100 µg pentadecanoic acid (Sigma Aldrich) as internal standard and the free fatty acids were extracted with four milliliters of n-hexane (VWR Chemicals). The mixture was vortexed vigorously prior to centrifugation (3500g, 3 min) and the upper hexane layer was then transferred to a fresh PYREX round bottom threaded culture tube and evaporated completely under a stream of N₂. Five hundred microliters of 1.25 M HCl in methanolic solution were added to methyl esterify the free fatty acid at 85 °C for 2 h. Samples were cooled down to room temperature prior to extraction of the fatty acid methyl ester (FAE). FAMEs from the methanolic HCl solution were extracted by adding 500 µL of n-hexane. The solvent was briefly vortexed and 100 µL of the upper n-hexane layer was transferred to an insert in a gas chromatography vial. For fatty alcohol extraction, 2 ml of the *Synechocystis* culture was transferred to a PYREX round bottom threaded culture tube and spun down (3500g, 3 min). The supernatant was transferred to a fresh PYREX round bottom threaded culture tube for extracellular fatty alcohol extraction. The remaining cell pellet was washed twice with BG11-Co and resuspended in two milliliters of BG11-Co medium for intracellular fatty alcohol extraction. The 2 mL solution was spiked with 50 µg 1-nonalanol and 100 µg 1-pentadecanol (as internal standards) and mixed with four milliliters of chloroform:methanol (2:1 v/v) solution. After vortexing the solution vigorously and briefly the preparation was centrifuged (3500g, 3 min). The lower organic phase was then transferred into a new glass tube and extraction was repeated one more time. The lower organic phase was combined and dried under a stream of N₂. For fatty alcohol derivatization, the dried extract was resuspended in 100 µL chloroform and mixed with 100 µL of N, O-Bistrifluoroacetamide (BSTFA) (TCI Chemicals). The mixture was transferred to an insert in a GC vial, and incubated at 60 °C for 1 h prior to GC analysis. When overlay was used, 10 µL of the overlay was transferred to an insert in a GC vial, mixed with 90 µL of BSTFA, and incubated at 60 °C for 1 h prior to GC analysis. For all metabolites, one microliter of sample was analyzed using GC-MS Agilent Technologies (Santa Clara, CA, USA) 7890B Series Gas Chromatograph (GC) equipped with HP-5MS column, pulsed split ratio 10:1 and split flow 10 ml/min. The oven temperature was initially held at 70 °C for 30 s, followed by a first ramp at 30 °C/min to 250 °C and a second ramp at 40 °C/min to 300 °C with a final hold for 2 min. The free fatty acid and alcohol concentrations were normalized relative to the internal standards methyl pentadecanoate (fatty acids), 1-nonalanol (for C8-OH to C12-OH) and 1-pentadecanol (for C14 above). When overlay was used, a serial dilution of derivatized 1-octanol and 1-decanol standards was used to determine the concentration of 1-octanol and 1-decanol in the sample.

2.6. Protein quantification

The relative quantity of the heterologously expressed Tes3 protein was quantified as described previously (*Picotti et al., 2009*; Schumacher et al., 2014; Vuorijoki et al., 2016). Briefly, total soluble proteins were purified from wild-type (control) and Δaas-P_car-ΔTes3 (treatment, where n denotes RBS number 1–5) strains cultured for 8 days following induction. Following a tryptic digest (terminated by acidification) the centrifuged preparations were subjected to multiple reaction monitoring-mass-spectrometry (MRM-MS) analysis using an Agilent 1100 LC connected to an ABSciex 6500 Qtrap MS. The native protein encoded by slr1329 (ATTP SYN3, Uniprot P26527; peptide VDLITTPYR) was used as an internal standard. The peptide ETVDIYDK was selected using Skyline analysis and preliminary MRM-MS trials indicating it was uniquely present in strains engineered to express Tes3. The abundance of three proteotypic fragments originating from the ETVDIYDK peptide was averaged and normalized relative to the internal standard and the optical density (OD₅₆₀) of the culture.

3. Results and discussion

3.1. Expression of Tes3, Sfp, and Car resulted in extracellular excretion of medium-long chain fatty alcohols

It has been demonstrated previously that *E. coli* over-expressing the *Synechocystis* sp. PCC 6803 acyl-ACP synthetase (aas, encoded by slr1609) is able to extend 8-phenyloctanoic acid to 10-phenyldodecanic acid (*Beld et al., 2014*). Similarly, feeding 8-bromooctanoic acid to the *Synechocystis* sp. PCC 6803 wild-type strain (hereafter WT) led to the consumption of the acid (*Beld et al., 2014*). In this work, a *Synechocystis* sp. PCC 6803 acyl-ACP synthetase knockout strain (hereafter Δaas) was therefore generated as described previously (*von Berlepsch et al., 2012*) in order to minimize the recycling of free fatty acids back into corresponding acyl-ACPs. Genes encoding the key-constituents of the 1-octanol pathway previously used in *E. coli* (*Akhbar et al., 2015*), tes3, sfp, and car, were expressed in the Δaas mutant strain using a broad-host range RSP1010 plasmid (*Guerrero et al., 2012*) harboring the synthetic TPC3 operon (tes3, sfp, car) under the control of an IPTG-inducible *P_plac143* promoter (*Markley et al., 2015*). After 10 days of cultivation, the Δaas-*P_plac143*-3-TPC3 strain produced a wide range of fatty alcohols (Fig. 3A) with more than 95% and 50% (w/v) of 1-octanol and 1-decanol, respectively, secreted extracellularly into the medium (Fig. 3B). This was the first time that the CAR enzyme had been used to modify fatty acids in cyanobacteria.

The fatty alcohol titer was poor relative to what has previously been achieved with fatty acid dependent pathways in cyanobacteria (*Liu et al., 2011; Ruffing, 2014*) and with the TPC3 pathway in *E. coli* (*Akhbar et al., 2015*), although not too far off the highest reported fatty alcohol titer achieved previously (10.3 mg/g DCW) using an acyl-ACP reductase pathway in *Synechocystis* (*Kaczmarzyk et al., 2018*). In addition, the strain exhibited very poor growth compared to controls (Fig. 3C) and turned pale green after 10 days of cultivation (Supplementary Fig. 1). Thus, we were dealing with two possibly inter-related problems: (1) poor cellular health and (2) poor productivity. We speculated that the toxicity of the CAR-derived aldehydes and/or corresponding alcohols and sub-optimal control and strength of heterologous protein expression was to blame and proceeded to find a remedy for these potentially limiting factors.

3.2. The use of isopropyl myristate overlay alleviated the toxicity of 1-octanol

Solvent overlay has previously been used to capture hydrophobic compounds in microbial cultures, also with cyanobacteria and eukaryotic algae (*Davies et al., 2014*; *Kato et al., 2017; Lauersen et al., 2016*). The use of overlay, however, has not previously been demonstrated to capture 1-octanol nor to alleviate its toxicity to cyanobacteria. We therefore evaluated whether solvent overlay could alleviate the toxicity of 1-octanol. The parental strain (Δaas), not the 1-octanol producing strain, was used in the following experiments in order to rule out growth limitations caused by metabolic burden or toxicity from over-expression of *tes3*, *sfp*, and *car*. To choose the best
suitable overlay, we tested two commonly used solvents, dodecane and isopropyl myristate (hereafter IM). Both dodecane (data not shown) and IM could alleviate the toxicity of 1-octanol. In the presence of 10% (v/v) IM overlay, the Δ\text{aas} strain showed no growth defect when 500 mg/L 1-octanol was spiked to the liquid culture (Fig. 4A and Supplementary Fig. 2A). Without overlay, the growth was inhibited already with 25 mg/L 1-octanol (Fig. 4B and Supplementary Fig. 2B). The presence of 10% (v/v) IM also did not affect the growth (Supplementary Fig. 2C), suggesting good biocompatibility of IM towards cyanobacteria cells. When the overlay was sampled for analysis, however, dodecane eluted at the same time as 1-octanol using our standard GC-MS HP-5MS column (Supplementary Fig. 3A and 3B), whilst IM separated well from 1-octanol (Supplementary Fig. 3C). As expected, 1-octanol was located in the IM fraction (Supplementary Fig. 3D), thereby facilitating product:water separation prior to analysis. Together, this indicated that IM was a suitable choice solvent overlay for subsequent experiments as it could be used to both alleviate the toxicity and capture 1-octanol from the liquid medium.

The results demonstrated that the CAR enzyme was functional in cyanobacteria even though the 1-octanol titer was very low (Fig. 3A). Previous studies have found a close relationship between enzyme concentration and pathway flux (Angermayr et al., 2014). This comes with a risk, however, as when taken to the extreme this also increases the risk for genetic instability (Du et al., 2017). Given the toxicity of the product, and despite the positive impact of the IM solvent overlay, we were concerned whether improved productivity from further optimization might result in increased problems associated with product toxicity (e.g. cellular health, genetic instability). As the 1-octanol pathway is dependent on three rather than just one enzyme, we were also uncertain which enzyme was mainly limiting pathway flux.

We therefore evaluated (i) different promoters and RBS elements in order to more tightly regulate heterologous protein expression, (ii) quantified the intracellular accumulation of the thioesterase and (iii) carried out feeding studies.

### 3.3. The use of a cobalt inducible promoter improved free fatty acid and fatty alcohol production

Firstly, we tested a selection of known inducible (P\text{AlacO-1}, P\text{clac143})
P_{tes3}, P_{osa}, P_{psbA2S}) and constitutive (P_{lac}, lacking a lacI repressor) promoters, both with Tes3 alone (Fig. 2B) and with the whole TPC3 operon (Fig. 2D). We included, on purpose, promoters that were known to be strong and constitutive, alongside promoters that are known to be weaker and more tightly repressed (Guerrero et al., 2012). Transformation of these plasmids to the Δαas strain only yielded colonies with the first four constructs, whilst constructs with the two stronger promoters (P_{psbA2S} and P_{lac}) did not yield any transformant colonies in Synechocystis (Fig. 2C), and in the case of the latter promoter, it was not even possible to transform *E. coli* when it was combined with TPC3 (Fig. 2E). The challenge in obtaining transformants with some of the promoters highlighted the sensitivity of *Synechocystis* to the introduced metabolic pathways (both acids and alcohols). Although it would be difficult to quantify the accumulation of the heterologous proteins or metabolites in the very early stages of transformation with the differing promoters, we suspected that product toxicity caused by the expression of one or more of the pathway enzymes from a leaky or strong promoter was the reason why constructs with some promoters failed to yield any transformants.

The successfully transformed *Synechocystis* strains were cultivated in BG11-Co media and the free fatty acid and fatty alcohol content, as appropriate, was analyzed in whole culture samples. In contrast to earlier studies with ethylene biosynthesis (Guerrero et al., 2012), the P_{coa} promoter was found to result in greater productivity than any of the Lac promoters (Fig. 5A). Expression of *tes3*, *spa*, and *car* under the cobalt-inducible promoter P_{coa} increased the production of extracellular fatty alcohols by nearly 4-fold (compare Figs. 3A and 5B) and improved growth (compare Figs. 3C and 5C) compared to the fatty alcohol producing strain driven by the P_{clai43} promoter. There were no C8-C10 free fatty acids present in the medium (Supplementary Fig. 4), suggesting efficient conversion of medium chain-length free fatty acids to their corresponding fatty alcohols in *Synechocystis* by the CAR-dependent pathway and endogenous aldehyde reductase(s). The Δαas-P_{osa}-3-TPC3 strain was also cultured in the presence of 10% (v/v) IM, resulting in no improvement in growth (Fig. 5D) but more than a two-fold increase of the 1-octanol and 1-decanol titer relative to strains cultured in the absence of solvent overlay (Fig. 5E). One possibility is that the IM overlay captured some of the 1-octanol and 1-decanol that otherwise would have been lost to the environment by volatilization during 10 days of cultivation without any overlay. Informal olfactory evaluation supported this idea. Attempts to further improve the titer by increasing the cobalt inducer concentration to 15 µM resulted in lower titers of 1-octanol and 1-decanol (Supplementary Fig. 5A), hence 5µM cobalt was found to be optimal. This is likely because higher concentrations of cobalt are toxic to the cells, therefore preventing growth and metabolism (Supplementary Fig. 5B). Additionally, the use of 20% (v/v) IM also failed to improve growth and titer further, suggesting that the capacity of the IM overlay was not limiting (Figs. 5D, 5E). Overall, P_{coa} exhibited both the greatest inducibility and productivity relative to the other tested promoters (Fig. 5F).

3.4. RBS optimization increased the free fatty acid pool and fatty alcohol titer

Neither the inducer concentration nor the capacity of the overlay was limiting the productivity of the strains, and none of the medium chain-length fatty acid substrates remained at the end of each culture. We therefore suspected that the octanoic acid supply was limiting 1-octanol production and decided to evaluate the effect of Tes3 expression strength on 1-octanol production by varying the ribosome binding site (RBS) in *tes3* and quantifying the accumulation of Tes3. RBS optimization has previously been successfully employed in cyanobacteria (Thiel et al., 2018). We selected three RBS sequences (RBS No. 1) (Heidorn et al., 2011) (Supplementary Table 4). All of these RBS elements have been characterized in previous studies with cyanobacteria (Heidorn et al., 2011, Englund et al., 2016, Thiel et al., 2018), although most such studies (with the exception of Thiel et al., 2018) only employed fluorescent protein to determine the relative strengths of the RBSs. Five different constructs were created in which different RBS elements were placed in front of the *tes3* open reading frame, all employing the P_{coa} promoter. Three RBS elements (No. 1, 4, and 5) resulted in improved free fatty acid titers relative to the original RBS element (No. 3) used in the preceding experiments (Fig. 6A). We also used targeted multiple reaction monitoring mass spectrometry (MRM-MS) to measure the relative quantity of Tes3 in each culture. A near-linear relationship (R² = 0.96) between the relative quantity of Tes3 and the accumulation of fatty acids was observed (Fig. 6B). This suggested that the quantity of Tes3 was potentially limiting the overall pathway.

Subsequently, a construct was prepared in which RBS No.4 was used to drive the expression of Tes3, Spa, and CAR. This new strain displayed a combined (C8/C10) alcohol titer of ~100 mg/L (Fig. 6C), equivalent to a yield of ~80 mg/g DW of 1-octanol and 1-decanol (Fig. 6D). No remaining C8 and C10 free fatty acids or fatty aldehydes could be detected in the IM overlay or the liquid culture. Both the titer and yield of Δαas-P_{osa}-4-TPC3 surpassed that previously obtained with an *E. coli* strain expressing TPC3 (Akhbar et al., 2015), albeit over a different time span (8 days vs. 14 h). The speed of glucose production (for the heterotrophic *E. coli* cultures) by plants was not considered however.

In order to confirm the hypothesis that the availability of substrate for the fatty acid biosynthesis pathway was limiting the 1-octanol pathway, we also carried out feeding studies with octanoic acid and an Δαas strain expressing only Spa and CAR (not Tes3) with a 30% (v/v) isopropyl myristate solvent overlay. Surprisingly, the strain continued to grow and metabolize all of the supplied octanoate reaching 1-octanol titers > 10 times that produced from glucose in *E. coli* TPC3 strains. No octanoate or octanal could be found in the IM overlay and a maximum of 905 ± 35 mg/L 1-octanol was recovered. The cultures were smelling of 1-octanol suggesting that some of the product was lost through volatilization. Both the final OD₆₅₀ and the percent recovery of 1-octanol, relative to the molar amount of added octanoate, decreased successively with increasing octanoate concentrations added (Fig. 6E). This suggests that the capacity of the IM overlay was limited and that a more elaborate system for product capture could possibly improve the productivity further. Altogether, both the Tes3 quantification (Fig. 6B) and octanoate feeding experiments (Fig. 6E) indicated that the availability of free fatty acid substrate is the main limiting factor in the CAR-dependent fatty alcohol production pathway in cyanobacteria. Clearly, at least part of the flux limitation lies with the Tes3 reaction. Although at best < 10% dry weight matter was diverted towards fatty alcohols, we might also expect an impact on growth in cultures expressing the synthetic pathway, if there is a strong metabolic "pull" from the introduced synthetic 1-octanol pathway. Indeed, a growth effect was observed (Figs. 3C, 3D). However, such a growth effect may also have been due to the effects of cobalt, heterologous protein expression and product toxicity (as also indicated by Supplementary Figures 2 and 5). Hence, it is difficult to conclude exactly what factor has caused what outcome but a number of potential leads for further optimization have been identified. Although a number of strategies (e.g. acetyl-CoA carboxylase over-expression) have demonstrated enhanced FAS flux in *E. coli* (Davis et al., 2000), similar efforts have so far not yielded any improvement in cyanobacteria (Liu et al., 2011; Ruffing, 2013).

3.5. The 1-octanol producing strains displayed reproducible genetic instability

Despite the improved health of the later 1-octanol strains, especially when grown in the presence of the isopropyl myristate solvent overlay, there were still signs of growth limitations in several experiments (e.g. Figs. 5C, 5D, 6E). We noted also that continuous sub-culturing of the
Fig. 5. Biosynthesis of medium chain-length free fatty acids and fatty alcohols with differing promoters and levels of induction. (A) Free fatty acid production under different inducible promoters. (B) Extracellular fatty alcohol profiles extracted from Δaas-Pcoa-3-TPC3 and controls (Δaas and Δaas-Pcoa-3-Tes3) cultured in the absence of an isopropyl myristate (IM) solvent overlay. (C) Growth curves of Δaas-Pcoa-3-TPC3 strains shown in (B), during 10 days of cultivation when induced with 0, 2, and 5 μM cobalt without solvent overlay. (D) Growth curves of Δaas-Pcoa-3-TPC3 strains shown in (E), during 10 days of cultivation in the presence of IM overlay with different inducer and solvent overlay concentrations. (E) Production of 1-octanol and 1-decanol from Δaas-Pcoa-3-TPC3 with varying induction when cultured in the presence of an IM overlay. (F) Summary of promoter evaluation including the total fatty alcohol titer after 8 days of induction in the presence of IM solvent overlay and the difference (fold) between strains that were induced (0.5 mM IPTG for PAlacO1 and Pclac143, 5 μM nickel for PnrsB, and 5 μM cobalt for Pcoa) and those that were not induced. All data are average from three biological replicates and the error bars display the standard deviation.
created strains sometimes resulted in strains that produced less 1-octanol compared to earlier generation strains. Consequently, all of the above described results were generated with strains that had not been sub-cultured once they had been confirmed to have the correct genotype. Nevertheless, in order to understand more about what was going on, we carried out an experiment with the specific aim of capturing this phenomenon. Fig. 7A shows the schematic diagram describing the serial dilution of Δaas-Pcoa-4-TPC3. The strain (in three biological replicates) was cultured without any induction and subcultured every four days into two new flasks with fresh media at a starting OD730 of 0.2. One flask was induced with 5 µM cobalt on day 2 for fatty alcohol production in the presence of 10% (v/v) IM. The other flask was incubated for another four days without induction and used for inoculation of the subsequent generation. By the 2nd generation, the 1-octanol and 1-decanol titers dropped by more than 50% compared to that of the 1st generation. By the 4th generation, the strain had completely lost its ability to produce fatty alcohols (Fig. 7B) while still maintaining its resistance to erythromycin (Fig. 7C). To find out whether the genetic parts Pcoa, Tes3, Sfp, and CAR were still intact, four different set of primers were used to amplify the four main parts of the engineered construct: Pcoa, Tes3, Sfp, and CAR (Supplementary Figure 6A) from samples taken at time points a, b, and c (Fig. 7A). The PCR fragments were gel purified (Supplementary Figure 6B) and sequenced.

Our intention with the DNA sequencing was only to obtain preliminary insight, rather than to exhaustively define what changes had occurred, and to confirm whether there were any differences or not in any part of the construct. Whilst there was no anomaly in the sequences of Pcoa, Sfp, and CAR in samples taken at any time points, we found that the Tes3 sequencing results gave poor reading from samples at time points b and c (Supplementary Figure 6C and 6D). For example, at time point b, one of the Tes3 sequences showed a high sequencing quality and alignment to the original sequence from nucleotide No. 30–176 (3′–5′) and thereafter showed almost no homology at all (Supplementary Figure 6C, compare panels i and ii). At time point c, the trace from one
of the Tes3 amplicons (Supplementary Figure 6D, panel iii) appeared excellent from nucleotide No. 30–120 (3′–5′) but thereafter displayed only smaller peaks with partial homology to the original sequence (Supplementary Figure 6D, panel iii). Similarly, when the Tes3 amplicon was sequenced from the other (N-terminal) end at time point c, the sequence aligned perfectly with the original sequence from nucleotide No. 20–130 (5′–3′) and thereafter completely lacked any recognizable homology (Supplementary Figure 6D, compare panels iv and v). The appearance of partial homology in the sequencing results might have resulted from heterogeneity in the template, as the bulk culture was used as a PCR template. Nevertheless, the sequencing results suggested that genetic rearrangements had occurred in the Tes3 encoding part after time point b in the serial dilution experiment. Similar instances of genetic instability have been reported also in other studies where cyanobacteria have been subjected to metabolic engineering (Angermayr et al., 2014; Du et al., 2017, 2018; Jones, 2014) and is clearly a topic that deserves to be further studied in order to speed up or even enable any translational activities with genetically engineered Synechocystis.

4. Conclusions

The introduction of an appropriately selected thioesterase and carboxylic acid reductase into Synechocystis enabled the biosynthesis of C8 caprylic acid and 1-octanol, alongside of 1-decanol for the first time. The first engineered strain was unhealthy and exhibited poor productivity. The addition of an isopropyl myristate solvent overlay and optimization of promoters and RBS elements resulted in strains with > 25-fold enhancement in productivity and better cellular health. Quantification of the thioesterase indicated a close relationship between protein accumulation and pathway yield. This together with precursor feeding experiments, reaching a titer of > 905 mg/L over 8 days, indicated that the supply of octanoate was limiting the synthetic 1-octanol pathway. The strains were sufficiently stable to allow for proper characterization but exhibited genetic instability when serially cultured over several successive generations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2018.07.015.

References


