1   **Synthetic metabolic pathways for photobiological conversion of CO_2 into hydrocarbon fuel**

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ABSTRACT

Liquid fuels sourced from fossil sources are the dominant energy form for mobile transport today. The consumption of fossil fuels is still increasing, resulting in a continued search for more sustainable methods to renew our supply of liquid fuel. Photosynthetic microorganisms naturally accumulate hydrocarbons that could serve as a replacement for fossil fuel, however productivities remain low. We report successful introduction of five synthetic metabolic pathways in two green cell factories, prokaryotic cyanobacteria and eukaryotic algae. Heterologous thioesterase expression enabled high-yield conversion of native fatty acyl-acyl carrier protein (ACP)ACP into free fatty acids (FFA) in *Synechocystis sp.* PCC 6803 but not in *Chlamydomonas reinhardtii* where the polar lipid fraction instead was enhanced. Despite no increase in measurable FFA in *Chlamydomonas*, genetic recoding and over-production of the native fatty acid photodecarboxylase (FAP) resulted in increased accumulation of 7-heptadecene. Implementation of a carboxylic acid reductase (CAR) and aldehyde deformylating oxygenase (ADO) dependent synthetic pathway in *Synechocystis* resulted in the accumulation of fatty alcohols and a decrease in the native saturated alkanes. In contrast, the replacement of CAR and ADO with *Pseudomonas mendocina* UndB (so named as it is responsible for 1-undecene biosynthesis in *Pseudomonas*) or *Chlorella variabilis* FAP resulted in high-yield conversion of thioesterase-liberated FFAs into corresponding alkenes and alkanes, respectively. At best, the engineering resulted in an increase in hydrocarbon accumulation of 8- (from 1 to 8.5 mg/g cdell dry weight) and 19-fold (from 4 to 77 mg/g cell dry weight) for *Chlamydomonas* and *Synechocystis*, respectively. In conclusion, reconstitution of the eukaryotic algae pathway in the prokaryotic cyanobacteria host generated the most effective system, highlighting opportunities for mix-and-match synthetic metabolism. These studies describe functioning synthetic metabolic pathways for hydrocarbon fuel synthesis in photosynthetic microorganisms for the first time, moving us closer to the commercial implementation of photobiocatalytic systems that directly convert CO_2 into infrastructure-compatible fuels.

Highlights

- Synthetic metabolic pathways for hydrocarbon fuels were engineered in algae
- Free fatty acids were effectively converted into alkenes and alkanes

44 • Transfer of algal pathway into cyanobacteria was the most effective

45 • Alkane yield was enhanced 19-fold in *Synechocystis spp.* PCC 6803

46 • Alkene yield was enhanced 8-fold in *Chlamydomonas reinhardtii*

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INTRODUCTION

Cyanobacteria (prokaryotes) and algae (eukaryotes) are photosynthetic microorganisms that have evolved to naturally accumulate C15-C19 alkanes or alkenes at very low concentrations (0.02-1.12% alkane g/g cell dry weight (CDW~~cdw~~)) (Lea-Smith et al., 2015; Schirmer et al., 2010; Sorigué et al., 2017) with the exception of naturally oleagineous species (Ajjawi et al., 2017; Metzger and Largeau, 2005; Peramuna et al., 2015). These hydrocarbons are postulated to influence the fluidity of cell membranes and are therefore essential for achieving optimal growth, indeed, the abolition of their biosynthetic capacities results in morphological defects (Lea-Smith et al., 2016). Only two enzymes, acyl-ACP reductase (AAR) and aldehyde deformylating oxygenase (ADO) are required to catalyze the bacterial conversion of acyl-ACP into alkanes (Schirmer et al., 2010). Similarly, eukaryotic microalgae also biosynthesize small quantities of alkanes and alkenes directly from fatty acids, employing the distinctly different and recently discovered fatty acid photodecarboxylase (FAP; (Sorigué et al., 2017)).

In order to engineer sustainable biotechnological systems for production of hydrocarbons for the fuel market, whether heterotrophic or light-driven, far greater yields are needed alongside other complementary non-biochemical improvements such as improved bio-process designs. Several studies have attempted to enhance alkane productivity in cyanobacteria by over-expression of the native or non-native AAR and ADO enzyme couple (Hu et al., 2013; Kageyama et al., 2015; Peramuna et al., 2015; Wang et al., 2013) which relies on acyl-ACP as the precursor. Although naturally accumulating alkane amounts have been enhanced through engineering and reported in high titres from the lipid-accumulating cyanobacteria, *Nostoc punctiforme* (up to 12.9% (g/g) CDW~~cdw~~, (Peramuna et al., 2015)), similar efforts in the non-lipid accumulating model cyanobacterium *Synechocystis sp.* PCC 6803 (hereafter *Synechocystis* 6803) have at best yielded only 1.1% (g/g) CDW~~cdw~~ (Hu et al., 2013; Wang et al., 2013). In eukaryotic algae, the native alkene/alkane path~~aw~~a~~y~~ was only recently discovered and there has been no work so far to engineer the specific pathways that synthesize such hydrocarbons. Some species of algae are also known to naturally accumulate hydrocarbons that could serve as a fuel following chemical conversion. For example, certain races of the green alga *Botrococcus braunii* naturally secrete long-chain terpene hydrocarbons as a significant portion of their biomass (Eroglu and Melis, 2010; Metzger and Largeau, 2005). However, their use as a fuel source is made impossible by the incredibly slow growth rates of this alga (Cook et al., 2017). Other oleaginous algal species can accumulate a significant portion of their biomass as triacylglycerol compounds, generally under nitrogen stress. Indeed, this phenomenon drove the push for the use of algae as third generation

82   biofuel feedstock in the first place. However, process design and downstream processing cost

83   considerations of large-scale algal cultivation have hindered the common adoption of algal

84   oils for transportation fuels (Quinn and Davis, 2015). Triacylglycerol stored by eukaryotic

85   algae can also be turned into transportation fuels via transesterification to liberate the

86   alkanes and alkenes from the glycerol backbone. An attractive alternative to the above

87   concepts is instead to directly secrete ready-to-use hydrocarbon products from algal cells as

88   this would overcome issues with biomass harvesting and chemical processing and thereby

89   greatly reduce process costs (Delrue et al., 2013).

90   In order to achieve such a one-step conversion of CO_2 into ready infrastructure-

91   compatible hydrocarbons with photosynthetic hosts, however, genetic reprogramming

92   becomes essential for introduction of synthetic metabolic pathways and optimization of the

93   entire system. Several enzymes have recently been reported to enable biosynthesis of fatty

94   aldehyde precursors (Akhtar et al., 2013), fatty alkanes (Bernard et al., 2012; Qiu et al., 2012),

95   and fatty alkenes (Rude et al., 2011; Rui et al., 2015; Rui et al., 2014). Combinatorial assembly

96   of such key enzymes into synthetic metabolic pathways consequently enabled a number of

97   novel opportunities for hydrocarbon biosynthesis, as described by many including (Akhtar et

98   al., 2013; Kallio et al., 2014; Sheppard et al., 2016; Zhu et al., 2017). Although such studies

99   have so far only been reported using heterotrophic microorganisms (*Escherichia coli* and

100   *Saccharomyces cerevisiae*) there are no reports of similar work in any phototrophic

101   microorganism.

102   In this study, we describe a first and systematic study to implement synthetic

103   metabolic pathways for the biosynthesis of hydrocarbon fuel in both prokaryotic and

104   eukaryotic photosynthetic microorganisms using the model strains *Synechocystis* 6803 and

105   *Chlamydomonas reinhardtii*. Several synthetic pathways towards saturated and unsaturated

106   hydrocarbons were functionally demonstrated in *Synechocystis* 6803, increasing the

107   hydrocarbon content up to 19-fold, and engineered *Chlamydomonas* accumulated 8-fold more

108   alkenes than the wild-type. Interestingly, the "best" system was achieved by transferring a

109   reconstructed pathway from eukaryotic algae into the prokaryotic cyanobacterium.

MATERIALS AND METHODS

2.1 Growth conditions, genetic constructs, transformation and screening of Escherichia coli and Synechocystis sp. PCC 6803

Escherichia coli DH5α was used to propagate all the plasmids used in this study. Strains were 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, gentamicin 10 μg/ml, and erythromycin 200 μg/ml).

Synechocystis sp. PCC 6803, obtained from Prof. Klaas Hellingwerf (University of Amsterdam, Netherlands), was cultivated in BG11 medium without cobalt ((hereafter BG11-Co), as the metal was used as an inducer in most cultures. All media contained appropriate antibiotic(s) (final concentration: kanamycin 50 μg/ml, gentamicin 50 μg/ml, and erythromycin 20 μg/ml). Gentamicin was only used for selection on agar plates. Precultures inoculated from colonies on agar plates were grown in 6-well plates (5 ml). When the OD_{730} reached 3-4, the culture was transferred to a 100-ml Erlenmeyer flask and the OD_{730} was adjusted to 0.2 by adding BG11-Co medium to a final volume of 25 ml containing appropriate antibiotic(s). The cultivation was carried out for 10 days at 30 °C with continuous illumination at 60 μmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2. Each main treatment culture was induced on day 2 and samples were taken for measurement of OD_{730} and metabolites day 6 and 10. All cultivations were carried out in an AlgaeTron230 (Photon Systems Instruments) (PSI) at 30 °C with continuous illumination at 60 μmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2, except where noted (100-300 μmol photons $m^{-2} s^{-1}$). A representative growth curve and all final OD_{730} values are shown in Supplementary Figure 1.

All plasmids (Supplementary Table 1A) used for transformation of cyanobacteria were assembled using the BASIC Assembly method (Storch et al., 2015). Linkers were designed using the R20DNA software: http://www.r2odna.com/ and obtained from Integrated DNA Technologies Incorporated. The details of all linkers, primers and DNA parts used to construct each plasmid are given in Supplementary Tables 1B, 1C and 1D.

For transformation by natural assimilation, each Synechocystis sp. PCC 6803 strain was inoculated from freshly prepared colonies on agar plates into 25 ml BG11-Co with a starting OD 0.02. The cells were harvested when the OD_{730} reached 0.4-0.7, washed in 10 ml BG11-Co twice, and resuspended in 500 μL BG11-Co. One hundred microliters of concentrated

liquid culture were mixed with four to seven micrograms of plasmid and incubated at 30°C

with continuous illumination at 60 µmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2 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. To

check the segregation, the biomass was resuspended in nuclease free water and exposed to

two freeze-thaw cycles (95°C, -80°C). Following centrifugation, 3 µL was used as a template

for a diagnostic polymerase chain reaction (PCR). Primers used for each PCR are listed in

Supplementary Table 1C. Only fully segregated mutants were used in further experiments. All

cyanobacteria strains used in the study are listed in Supplementary Table 2.

For transformation by triparental conjugation, one hundred microliters of the cargo

strain (*E. coli* HB101 (already carrying the pRL623 plasmid)), conjugate strain (ED8654

(Elhai and Wolk, 1988)), and *Synechocystis* sp. PCC 6803 (OD_{730} ~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 and BG11-Co medium, respectively, to

remove the antibiotics. After 2 h of incubation, the culture mix was transferred onto BG11

agar plates without antibiotic and incubated for 2 d (30 °C, 60 µmol photons $m^{-2} s^{-1}$). After 2 d

of incubation, cells were scraped 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.

2.2 Growth conditions, genetic constructs, transformation and screening of Chlamydomonas reinhardtii

C. reinhardtii strain UVM4 was used in this work (Neupert et al., 2009) graciously provided by

Prof. Dr. Ralph Bock)). The strain was routinely maintained on Tris acetate phosphate (TAP)

medium (Gorman and Levine, 1965) either with 1.5% agar plates or in liquid with 250 µmol

photons $m^{-2} s^{-2}$. Transformation was conducted with glass bead agitation as previously

described (Kindle, 1990). The amino acid sequences of *C. reinhardtii* native fatty acid

photodecarboxylase (FAP) (Uniprot: A8JHB7; (Sorigué et al., 2017)), *E. coli* thioesterase A

(TesA: P0ADA1), *Jeotgalicoccus* sp. ATCC 8456 terminal olefin-forming fatty acid

decarboxylase (OleTJE) (E9NSU2), and *Rhodococcus sp.* NCIMB 9784 P450 reductase RhFRED

(Q8KU27) were codon optimized and copies of the intron 1 of ribulose bisphosphate

carboxylase small subunit 2 (RBCS2) were added throughout the coding sequences as

previously described (Baier et al., 2018). The nucleotide sequences of optimized intron

178   containing genes have been submitted to NCBI, accession numbers can be found in

179   Supplementary Table 3. All synthetic genes were chemically synthesized (GeneArt) and

180   cloned between *Bam*HI-*Bgl*II in the pOpt2_PsaD_mVenus_Paro or pOpt2_PsaD_mRuby2_Ble

181   vectors (Wichmann et al., 2018). PsaD represents the 36 amino acid photosystem I reaction

182   center subunit II (PsaD) chloroplast targeting peptide (CTP) (Lauersen et al., 2015) between

183   *Nde*I-*Bam*HI restriction sites of the pOpt2 vectors (Wichmann et al., 2018). The native FAP

184   enzyme was designed to contain an additional glycine codon at aa position 33 to allow the

185   insertion of a *Bam*HI site at the border of the predicted CTP. The whole synthetic enzyme

186   including native targeting peptide was cloned *Nde*I-*Bgl*II and a version was created with the

187   PsaD CTP built by cloning *Bam*HI-*Bgl*II into the vectors described above. Fusions of different

188   sequences were made by digestion and complementary overhang annealing of the *Bam*HI-

189   *Bgl*II mediated restriction sites for each respective construct as needed to obtain the fusions

190   used in the present work (Supplementary Figure 2). After transformation, expression was

191   confirmed by fluorescence microscopy screening for mVenus (YFP) or mRuby2 (RFP)

192   reporters as previously described (Lauersen et al., 2016; Wichmann et al., 2018). Individual

193   mutants were subjected to Western blotting and immuno detection to determine whether

194   full-length protein products were formed (anti-GFP polyclonal HRP linked antibody, Thermo

195   Fisher Scientific). Wide-field fluorescence microscopy was used to confirm chloroplast

196   localization of YFP-linked constructs as previously described (Lauersen et al., 2016).

197

198   ### 2.3 Product analysis

199   Three different extraction and analysis protocols were used for the analysis of (1) acids, (2)

200   alcohols and (3) alkanes as well as alkenes from cyanobacteria cultures. For each analyte

201   group, liquid cultures in flasks were mixed well by shaking prior to transferring 2 mL of liquid

202   culture into a PYREX round bottom threaded culture tube (Corning, Manufacturer Part

203   Number: 99449-13).

204   For fatty acid analysis, free fatty acid extraction was performed as described

205   previously (Liu et al., 2011; Yunus and Jones, 2018). In brief, two hundred microliters of 1 M

206   H_3PO_4 were added to acidify each 2 mL culture and spiked with 100 μg pentadecanoic acid

207   (Sigma Aldrich) as an internal standard. Four millilitres of n-hexane (VWR Chemicals) was

208   added and the mixture vortexed vigorously prior to centrifugation at 3500 x g for 3 min. The

209   upper hexane layer was then transferred to a fresh PYREX round bottom threaded culture

210   tube and evaporated completely under a stream of nitrogen gas. Five hundred microliters of

211   1.25 M HCl in methanolic solution were added to methyl esterify the free fatty acid at 85 °C for

8

2 h. Samples were cooled to room temperature and 500 μL of hexane was added for extraction of the fatty acid methyl esters (FAMEs).

For fatty alcohol, alkane and alkene analysis, extraction was done as described previously (Zhou et al., 2016) with modification. Briefly, 2 mL of liquid culture were spiked with 50 μg 1-nonanol, 100 μg octadecane, and 100 μg 1-pentadecanol and mixed with 4 mL of chloroform:methanol (2:1 v/v) solution. The mixture was vortexed vigorously and centrifuged at 3500 x g for 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 nitrogen gas. For fatty alcohol derivatization, the dried extract was resuspended in 100 μL chloroform, mixed with 100 μL of N, O-bistrifluoroacetamide (BSTFA) (TCI Chemicals) and transferred to an insert in a GC vial that was incubated at 60 °C for 1 h prior to GC analysis. Note that no derivatization was needed for the analysis of hydrocarbons.

Samples (1 μL) were analyzsed using an Agilent Technologies (Santa Clara, CA, USA) 7890B Series Gas Chromatograph (GC) equipped with an HP-5MS column (pulsed split ratio 10:1 and split flow 10 ml/min), a 5988B Mass Spectrophotometer (MS) and a 7693 Autosampler. For the acids the GC oven program followed an initial hold at 40 °C for 3 min, a ramp at 10 °C.min^{-1} to 150 °C, a second ramp at 3 °C.min^{-1} to 270 °C, a third ramp at 30 °C.min^{-1} to 300 °C, and a final hold for 5 min. For alcohols and alkenes, there was an initial hold at 40 °C for 0.5 min, a ramp at 10 °C.min^{-1} to 300 °C, and a final hold for 4 min. For alkanes, the oven was initially held at 70 °C for 0.5 min, a ramp at 30 °C.min^{-1} to 250 °C, a second ramp at 40 °C.min^{-1} to 300 °C, and a final hold for 2 min. The acids, alkanes and alcohols were quantified by comparing the peak areas with that of the internal standards: pentadecanoate (for all acids), octadecane (for all alkanes), 1-nonanol (for C8 to C12 alcohols) and 1-pentadecanol (for C14 alcohols and above). The quantity of the main products (C15 and C17 alkanes, C15 alkene, and C12, C14, C16, and C18 alcohols and acids) were also corrected with their respective mass spectrometer response factors obtained using dilution series of commercial standards.

Gas chromatography mass spectroscopy (GC-MS) aimed at identification of hydrocarbon products from *C. reinhardtii* was conducted with solvent extracted samples following previously described protocols and internal standards (Lauersen et al., 2016). Quantification of 7-heptadecene was performed with serial dilutions (1 to 900 μM) of commercial 1-heptadecene standard (Acros Organics) in dodecane using extracted ion chromatograms with masses 55.00, 69.00, 91.00, 93.00, 83.00, 97.00, and 111.00.

RESULTS AND DISCUSSION

Several synthetic pathway designs were considered, all commencing with the liberation of "free" fatty acids from the native fatty acid biosynthesis pathway (Fig. 1), the presumed native precursor for many of the decarboxylating enzymes evaluated in this study.

3.1 Over-production of free fatty acids as precursor for hydrocarbon biosynthesis - Expression of Escherichia thioesterase deregulates lipid membrane biosynthesis in Chlamydomonas

In order to liberate FFAs in cyanobacteria we over-expressed the *E. coli* C16-C18 specific thioesterase TesA (Cho and Cronan, 1995) lacking its native signal sequence peptide ('TesA) and deleted the gene encoding the native fatty acyl ACP synthase (*aas*) (Kaczmarzyk et al., 2010; Liu et al., 2011)(Fig. 1). The native signal sequence peptide directs TesA to the periplasm in *E. coli* (Cho et al 1993) and its removal is assumed to maximize the liberation of "free" fatty acids also in cyanobacteria by retaining the enzyme in the cytosol. Such 'TesA/Δ*aas* engineering has previously been reported several times before in cyanobacteria (Liu et al., 2011, Ruffing et al., 2014; Work et al., 2015; Kato et al., 2017), with 13% (g/g cell dry weight (CDW)) as the highest reported fatty acid yield in *Synechocystis* 6803 (Liu et al., 2011). Further potentially stackable modifications to the strain or process have also been reported. For example, by employing a solvent overlay, Kato et al., 2017 reported up to 36% (g/g) ~~cdw~~ CDW of fatty acids excreted into the media using 'TesA/Δ*aas Synechococcus elongatus sp.* PCC 7942. In the present study, the chromosomal integration of '*tesA* into the *psbA2* site (slr1311) of *Synechocystis* 6803 Δ*aas* (Δ*aas*-'TesA), under the control of the light-inducible promoter PpsbA2S, resulted in the excretion of ~~of~~ C14:0 (3.5 mg/g CDW), C16:0 (23.2 mg/g CDW) and C18:0 (5.7 mg/g CDW) fatty acids with a chain-length distribution that is in agreement with previously reported findings (Liu et al., 2011) (Fig. 2A; Supplementary Fig. 3).

Overproduction of the same thioesterase ('TesA) and targeting of the enzyme product to the chloroplast was possible in *C. reinhardtii*. The synthetic algal optimized *E. coli* '*tesA* gene was fused with an N-terminal PsaD-based chloroplast targeting peptide and a C-terminal yellow fluorescent protein (YFP) encoding gene. Both the coding genes were interspersed by synthetic introns (Fig. 2B) as previously described to enhance transgene expression from the nuclear genome (Baier et al., 2018). Fluorescence microscopy indicated correct localization of

the 'TesA fluorescent protein fusion to the algal chloroplast (Fig. 2C). Although no FFA could be detected in the culture medium, a difference was observed in the lipid profile of the green algal cells, suggesting a de-regulation of fatty acid synthesis that specifically affected the polar lipid fraction of the alga. This was indicated by an over-accumulation of C18:1n9c chain lengths in the polar lipid membranes, with subtle changes observed in other acyl-ACP species such as C14:0 (Fig. 2D; Supplementary Fig. 4). Thus, 'TesA_YFP clearly had an impact on lipid metabolism in the eukaryotic algal host, but, the capture of liberated FFAs by acyl-ACP or -CoA synthases are likely too effective, thereby limiting the application of the same engineering principles carried out for cyanobacteria. An annotated gene product in *Chlamydomonas* Cre06.g299800 (Phytozome v5.5) has some sequence similarity to *Synechocystis aas* and therefore represents an interesting target for future strategies to block native re-uptake of FFA in the green algal cell.

Having achieved strains with enhanced accumulation of FFA in *Synechocystis*, or at least a perturbation to the lipid biosynthetic system in *Chlamydomonas*, we proceeded to investigate enzymes that further convert FFAs into hydrocarbon end-products.

3.2 Effective conversion of free fatty acids into alkenes using UndB

Three different enzymes that catalyze the conversion of fatty acids into alkenes have been recently reported, OleT (Rude et al., 2011), UndA (Rui et al., 2014), and UndB (Rui et al., 2015) (Fig. 1). So far, the best reported productivity in both *E. coli* (Rui et al., 2015) and *S. cerevisiae* (Zhou et al., 2018) has been with UndB.

In *Synechocystis* 6803, we transformed the Δ*aas*-'TesA strain with an RSF1010-based plasmid harboring a codon-optimized *undB* under the control of the Pclac143 promoter (Markeley et al., 2014), thereby generating the strain Δ*aas*-'TesA-1010-UndB (Fig. 3A). After 10 days of cultivation, both the free fatty acids and alkanes were extracted and analyzed as described in the Materials and Methods section. The accumulation of free fatty acids was markedly reduced in the Δ*aas*-'TesA-1010-UndB strain (Fig. 3B, 3C). In its place, both 1-pentadecene and 1-heptadecene accumulated with a molar yield suggesting approximately 55% conversion of 'TesA-liberated FFAs (compare Fig. 3C with Fig. 3D). More than >84% of the FFAs disappeared relative to the Δ*aas*-'TesA strain suggesting that UndB was catalytically efficient *in vivo* and that the electrons required in the UndB reaction were fortunately supplied by an unknown source. The Δ*aas*-'TesA-1010-UndB strain displayed a lower biomass accumulation than the controls (Δ*aas*-empty and Δ*aas*–'TesA strains) (Supplementary Fig. 1), presumably due to product toxicity imparted by the alkenes. A direct comparison with the

314 conversion efficiency in *E. coli* is not possible since the FFA conversion efficiency was not

315 reported in the original work (Rui et al., 2015). Despite the disappearance of C14:0 fatty acids

316 in the Δ*aas*-'TesA-1010-UndB strain, no measurable 1-tridecene (the expected corresponding

317 alkene) was observed in the whole culture extracts (Fig. 3C). None of the observed alkene

318 products were secreted extracellularly (Fig. 3E).

319   In *Chlamydomonas*, we attempted to over-produce the *Jeotgalicoccus* sp. terminal

320 olefin-forming fatty acid decarboxylase (OleT~~JE~~) and the *Rhodococcus sp.* P450 reductase

321 (RhFRED). OleT~~JE~~ was chosen as it could theoretically produce C17:1 and C15:0 hydrocarbons

322 from the major lipid species of the green algal cell, C18:1 and C16:0, respectively (Fig. 1).

323 Fusion to RhFRED has been reported to enable hydrogen peroxide-independent

324 decarboxylase activity (Liu et al., 2014). The protein products of this decarboxylase and its

325 fusion in either orientation to RhFRED could be detected by Western blotting and located to

326 the algal chloroplast in fluorescence microscopy (Supplementary Figure 5). However, no

327 differences in GC-MS profiles between the parental and expression strains could be found in

328 either dodecane solvent overlays or cell-pellet solvent extracts.

329

330 *3.3 Transfer of the CAR/ADO based pathway from E. coli to Synechocystis 6803 resulted in*

331 *the accumulation of fatty alcohols and a reduction in alkane accumulation*

332 Carboxylic acid reductases (CAR) have been previously used to construct a number of

333 synthetic pathways for alkane biosynthesis in heterotrophic microorganisms (Akhtar et al.,

334 2013; Kallio et al., 2014; Sheppard et al., 2016; Zhu et al., 2016). Although CAR appears to

335 have a high capability for converting fatty acids into corresponding fatty aldehydes (Akhtar et

336 al., 2013) (Fig. 1), a bottleneck in previous heterotrophic pathways is the subsequent

337 conversion into alkanes by kinetically slow ADO enzymes and competition with native

338 aldehyde reductases that more effectively convert aldehydes into alcohols (Kallio et al., 2014;

339 Sheppard et al., 2016).

340   Since *Synechocystis* 6803 natively harbors an aldehyde deformylating oxygenase

341 (ADO) with the appropriate substrate specificity (Khara et al., 2013) (Fig. 1), we first

342 combined TesA with CAR and evaluated its ability to supply the native ADO. A synthetic

343 operon expressing all required parts (including the CAR maturation protein Sfp) was

344 introduced to the RSF1010 plasmid backbone (Fig. 4A) and used to transform *Synechocystis*

345 6803 Δ*aas*, thus creating the strain Δ*aas*-1010-TPC2. This strain accumulated both fatty acids

346 (Fig. 4B and 4D) and fatty alcohols (Fig. 4C and 4E). The quantity of heptadecane was reduced

347 in Δ*aas*-1010-TPC2 relative to Δ*aas*-1010-'TesA (Fig. 4F). This suggests that the introduced

348   CAR-based pathway had not managed to increase the supply of fatty aldehydes to the native

349   ADO. CAR and native aldehyde reductase(s) had instead very effectively converted >90% of

350   the FFA pool (Fig. 4D) into corresponding alcohols (Fig. 4E). The most likely reason for the

351   increase in FFA in latter experiments is due to increased expression of 'TesA using the

352   RSF1010 plasmid in Δ*aas*-1010-'TesA (Fig. 4D), relative to the amount of 'TesA when

353   expressed from the chromosomal location in Δ*aas*-'TesA (Fig. 3C). Similar observations have

354   also been previously reported by Angermayr et al. (Angermayr et al., 2014). The different

355   promoters used in the two strains are also likely to have influenced the outcome, however, we

356   are not aware of any studies that directly compare the two promoters head-to-head.

357   Substantial quantities of fatty alcohols did accumulate in the Δ*aas*-1010-TPC2 strain,

358   suggesting that the supply of fatty aldehydes is not the limiting factor. One possibility is that

359   the native aldehyde reductases are simply much more active than the native ADO (Eser et al.,

360   2011; Lin et al., 2013). Another possibility is that native ADO and AAR form a close metabolon

361   *in vivo* (Warui et al., 2015) that locks out access to ADO from external supplies of fatty

362   aldehydes. In order to test this possibility, we attempted to create a variant of Δ*aas*-1010-

363   TPC2 that also included chromosomal ADO over-expression cassette under the PpsbA2S

364   promoter. Despite numerous transformation and segregation attempts, however, we were

365   unable to isolate any stable segregants. Another complementary strategy that could be

366   considered in future work would be to eliminate native aldehyde reductases, as previously

367   carried out in earlier *E. coli* studies (Kallio et al., 2014; Sheppard et al., 2016), although the full

368   complement of fatty aldehyde reductase encoding genes in cyanobacteria remains unknown.

369   Given the lack of success in producing alkanes with the CAR/ADO route in cyanobacteria we

370   then considered alternative options for both cyanobacteria and algae.

371

372   *3.4 Engineering of the native eukaryotic algae pathway and transfer to cyanobacteria results*

373   *in enhanced conversion of CO$_2$ into alkanes*

374   A fatty acid photodecarboxylase (FAP) that directly converts saturated and unsaturated FFAs

375   into alkanes and alkenes, respectively, was recently discovered in eukaryotic algae (Sorigué et

376   al., 2017). In *Chlamydomonas*, the source of free fatty acids for the native alkene pathway

377   remains unknown, although the degradation of membrane lipids may release some FFA

378   (illustrated in Fig. 1). However, we would expect increased accumulation of alkanes in algae if

379   we were able to increase the cellular quantity of the native FAP and/or introduce synthetic

380   routes to the FFA precursors.

381   Accordingly, we overproduced native FAP from *C. reinhardtii* (CrFAP) on its own or in

382   combination with co-production of *E. coli* 'TesA. The over-expression of CrFAP was carried

383   out either with its native chloroplast targeting peptide (CTP) or the robust PsaD CTP which

384   has been previously used to mediate chloroplast localization of numerous reporters

385   (Lauersen et al., 2015; Lauersen et al., 2018; Rasala et al., 2013). In order to minimize any

386   native regulation of the genomic sequence, the gene was subjected to a strategy of gene design

387   which has recently been shown to enable robust transgene expression from the nuclear

388   genome of this alga (Baier et al., 2018). Briefly, the sequence was codon optimized based on

389   its amino acid sequence and multiple copies of the first intron of the *C. reinhardtii* ribulose-

390   1,5-bisphosphate carboxylase/oxygenase (RuBisCo) small subunit 2 (rbcS2i1,

391   NCBI: X04472.1) were spread throughout the coding sequence *in silico*. This nucleotide

392   sequence was chemically synthesized and used for expression from the algal nuclear genome.

393   This strategy has previously enabled heterologous overproduction of non-native

394   sesquiterpene synthases (Lauersen et al., 2016; Lauersen et al., 2018; Wichmann et al., 2018),

395   and in the present study also the 'TesA, OleTJE, and RhFRED proteins. However, complete

396   codon optimization and synthetic intron spreading of a native gene has not yet been

397   demonstrated in eukaryotic algae. Both constructs mediated full-length target protein

398   production which was detectible in Western blots (Supplementary Fig. 5B). Replacing the

399   native CTP with the PsaD CTP enabled more reliable and robust accumulation, which was

400   detectible as YFP signal in the algal chloroplast (Supplementary Fig. 6) and strong bands in

401   transformants expressing this construct in Western blots (Supplementary Fig. 5B). The

402   parental UVM4 strain was found to contain ~0.5 mg/g 7-heptadecene as a natural product

403   (Supplementary Fig. 7). Transformants generated with the CrFAP construct (Cr8) were found

404   to contain up to 8x more of this alkene compared to the empty vector (Cr2) control strain (up

405   to 8.5 ± 1.5 mg/g, Fig. 5) which was found almost exclusively within the biomass

406   (Supplementary Fig. 7). The product was not detected in dodecane solvent overlays. CrFAP

407   accepts a very specific substrate (*cis*-vaccenic acid, C18:1*cis*Δ11) *in vivo* (Sorigué et al., 2017),

408   which corresponds to the accumulation of only 7-heptadecene as the only detected increased

409   product. This substrate is an unusual FA, and is likely not naturally abundant in the algal cell.

410   Notably, any attempts to increase the avaialability of free fatty acids using *E. coli* 'TesA did not

411   result in any increase in the quantity or diversity of accumulated alkanes. Future enzyme

412   engineering will likely be able to overcome this substrate specificity and increase overall

413   yields of liberated hydrocarbons. However, a strategy which would allow secretion of these

414   molecules, similar to the capture of heterologous terpenoids in dodecane solvent overlay

(Lauersen et al., 2016; Lauersen et al., 2018; Wichmann et al., 2018), would be an attractive next target in order to enable photo-biocatalysis of hydrocarbons from the algal biomass.

Given the success with the FAP pathway in *Chlamydomonas* (present study) and earlier work in *E. coli* (Sorigué et al., 2017), as well as finding that 'TesA expression can substantially enhance the FFA pool in cyanobacteria, a synthetic FAP pathway was an obvious choice to consider also for the prokaryotic host. We therefore proceeded to implement a reconstituted variant of the eukaryotic algae pathway in cyanobacteria by combining TesA with FAP. Given the genetic instability challenges with the CAR/ADO system (see Section 3.3) we shifted our constructs to the more tightly repressed Pcoa promoter (Peca et al., 2008) for controlling the expression of *E. coli* TesA and the *Chlorella variabilis* FAP from the RSF1010 plasmid (Fig. 6A). We noted that the yield of FFA was substantially increased when driving the expression of TesA with the Pcoa promoter (Fig. 6C) compared to Pclac143 (Fig. 4D).

Despite the dominance of C16:0 fatty acids released by 'TesA in *Synechocystis* 6803, alongside minor fractions of C14:0 and C18:0, the C17:0 alkanes dominated the hydrocarbon fraction at the lower light intensity (100 μmol photons m^{-2} s^{-1}μE) (Fig. 6B and 6D). This alkane profile in *Synechocystis* 6803 is very different to that observed in *E. coli* without over-expression of 'TesA (see Fig. S4 in (Sorigué et al., 2017)). We also observed substantial peaks of 8-heptadecene and 6,9-heptadecadiene, as suggested by comparison with a NIST mass spectrometry library, although a lack of standards prohibited confirmation (Supplementary Figure 8). Curiously, these alkenes were only detected at day 6 and were not present in samples harvested on day 10. As the fatty chain-length profiles differ when the same thioesterase is expressed in different *E. coli* strains (Akhtar et al., 2015; Jing et al., 2011), this suggests that the *in vivo* product profile of any thioesterase-dependent pathway also is dependent on what the fatty acid synthesis pathway provides, not just the substrate specificity of the thioesterase used.

Removal of the predicted chloroplast targeting sequence of FAP ('FAP) resulted in a doubling of the alkane yield, this time accompanied also by C15 pentadecane. As the FAP reaction is light-dependent, we also did a simple evaluation of this environmental factor. When the light intensity was tripled, the total alkane production with the Δ*aas*-1010-'TesA-'FAP strain increased to a yield of 77.1 mg/g CDW (19-fold enhancement relative to Δ*aas*) and a titer of 111.2 mg/L. The product profile also shifted (Fig. 6D) despite the lack of a similar shift in the remaining FFA fraction (Fig. 6B), suggesting that the substrate specificity of FAP is flexible and interestingly might change in response to a change in its cellular environment.

448   At 100 μmol photons m^{-2} s^{-1}μE the introduction of 'FAP resulted in a drop in FFA

449   accumulation of up to 90% (for C18:0), whilst for C16:0 there was only a 60% reduction (Fig.

450   6C). Despite repeated trials, the recovery in the measurable fatty acid to alkane conversion

451   remained poor for C16:0 in comparison to C18:0 and the other pathways tested in

452   *Synechocystis* 6803. This may be explained by an impact on 'TesA accumulation in the

453   constructs also carrying the gene coding for 'FAP. Nevertheless, the reconstituted eukaryotic

454   algae alkane pathway was more responsive to introduced modifications in the prokaryotic

455   cyanobacterium than in its native host, though this most likely is explained by challenges

456   associated with the release of FFA in the latter.

457   Although a substantial amount of both alkanes and alkenes were produced by the

458   engineered strains, their performance likely needs to be improved before any application can

459   be considered. Given that no genetically engineered phototrophic microalgae is currently used

460   for commercial purposes (as far as we are aware), and LCA-studies with non-catalytic systems

461   indicate a low predicted energy return on investment (EROI) (Carneiro et al., 2017), also

462   other challenges with commercial algal biotechnology (e.g. contamination, bioreactor cost,

463   energy consumption, etc) will need to be addressed.

464

CONCLUSIONS

The different biosynthetic systems presented in this study varied in terms of cellular context, compartmentation, promoters, operon structures and expression platforms, thus precluding a any direct comparison within and between the two species studied. However, the relative conversion efficiencies and absolute functionalities provide for a valid comparison. As such, it could be seen that the conversion of free fatty acids into alkenes by UndB and alkanes by FAP were effective (>50% conversion, for individual fatty acids up to >90% conversion), and that the native FAP pathway in *Chlamydomonas* was amenable to manipulation but that the inability to increase the FFA pool hindered further progress. Consequently, for alkanes, the reconstruction of the eukaryotic algae pathway in the prokaryotic cyanobacteria host provided a more productive system than the partially synthetic pathways in either of the prokaryotic (CAR-ADO) or eukaryotic hosts (TesA-FAP).

This work describes several approaches to employ synthetic metabolism and substantially exceed native capabilities for hydrocarbon biosynthesis in well-established model cyanobacteria and algae. Although even greater yields have been reported in oleaginous algae and cyanobacteria that are natively endowed to accumulate lipids, the ability to introduce synthetic metabolic pathways in model strains opens up possibilities for tailored choice of both products and hosts. Importantly, the present work is based on first generation strains and further improvement is likely with systematic optimization of both strains and cultivation conditions, including the use of superior engineered or natural enzyme variants.

17

FIGURE LEGENDS

496

497 **Figure 1. Native and synthetic metabolic pathways evaluated in the present study with**
498 **incomplete stoichiometry.** The graphic illustration shows the introduced TesA (thioesterase
499 (Cho and Cronan, 1995)), CAR (carboxylic acid reductase (Akhtar et al., 2013)), UndA
500 (responsible for 1-undecene biosynthesis in *Pseudomonas* (Rui et al., 2014)), UndB (also
501 responsible for 1-undecene biosynthesis in *Pseudomonas* (Rui et al., 2015)), OleT
502 (responsible for olefin biosynthesis in *Jeotgalicoccus* (Rude et al., 2011)) and FAP (fatty acid
503 photodecarboxylase (Sorigué et al., 2017)) enzymes alongside the native AAR/ADO (acyl-ACP
504 reductase and aldehyde deformylating oxygenase (Schirmer et al., 2010)), AHR (aldehyde
505 reductase, unknown) and FAP enzymes. Blue reactions are non-native and those in grey are
506 native. The red cross indicates deletion of the *aas* gene.

507

508 **Figure 2. Engineering for enhanced accumulation of free fatty acids.** (A) Representative total
509 ion count chromatograms for *Synechocystis* 6803 strains Δ*aas*-'TesA (black) vs. Δ*aas* only
510 (orange) extracted on day 10 of cultivation (induced day 2). Peak identities: (3) Heptadecane,
511 (4) Tetradecanoic acid, (5) Hexadecanoic acid, (6) 9,12-octadecadienoic acid, (7) 9-
512 octadecenoic acid, (8) Octadecanoic acid. (B) Graphic representation of the constructs used to
513 transform *Chlamydomonas*. CTP = Chloroplast Transit Peptide. (C) Fluorescence microscopy
514 of representative strains indicating appropriate chloroplast localization of the CTP_'TesA_YFP
515 construct. (D) Total (TL), polar (PL), and neutral (NL) gravimetric lipid fractions of
516 *Chlamydomonas* parental strain and TesA overproducing strains under nutrient replete
517 conditions (N+) and after 96 hours of nitrogen depletion (N-). PL is significantly greater in +N
518 for TesA: ttest, p:0.047 (indicated by an asterisk).

519

520 **Figure 3. Over-expression of UndB results in effective (>50%) conversion of fatty acids into**
521 **corresponding alkenes.** (A) Graphic representation of the genetic modification of
522 *Synechocystis* sp. PCC 6803 and the plasmid used for UndB expression. (B) GC-MS
523 chromatograms with extracts from the two different strains (w/wo UndB); Δ*aas*-1010-'TesA
524 (black) and Δ*aas*-1010-'TesA-UndB (orange). (C) The free fatty acid yield (relative to biomass)
525 in the whole cultures of the two strains, subdivided into the three dominant chain-lengths. (D)
526 The yield of alkenes in the whole cultures of the two strains, subdivided into the three
527 dominant chain-lengths. (E) The localization of the alkene products in whole cultures of the
528 two strains. Peak identities: (1) 1-pentadecene, (2) 1-heptadecene, (3) heptadecane, (4)

529 tetradecanoic acid, (5) hexadecanoic acid, (6) 9,12-octadecadienoic acid, (7) 9-octadecenoic

530 acid, (8) octadecanoic acid. Data are mean ± SD from three biological replicates. All samples

531 were extracted on day 10.

532

533 **Figure 4. The CAR-dependent pathway produces mainly fatty alcohols.** (A) Graphic overview

534 (not to scale) illustrating the main constructs studied in the figure. (B) Total ion

535 chromatogram from extracts of Δaas-1010-'TesA (black) and Δ*aas*-1010-TPC2 (orange). (C)

536 Fatty alcohol profile from extracts of Δ*aas*-TPC2. The yield of fatty acids (D), alcohols (E) and

537 alkanes (F). Peak identities: (2) 1-dodecanol, (3) heptadecane, (4) 1-tetradecanol, (5) 1-

538 hexadecanol, (6) 9,12-octadecadien-1-ol, (7) 9-octadecen-1-ol, (8) 1-octadecanol, (9)

539 dodecanoic acid, (10) tetradecanoic acid, (11) hexadecanoic acid, (12) octadecanoic acid. Data

540 are mean ± SD of three biological replicates. Cultures were induced on day 2 following

541 dilution and samples were extracted on day 10.

542

543 **Figure 5. CrFAP over-expression increases 7-heptadecene yield, but heterologous thioesterase**

544 **(TesA) expression, its co-expression, and C- or N-terminal fusion with CrFAP has no benefit.**

545 Mutants expressing indicated constructs (left panel) were cultivated for seven days in TAP

546 medium with 250 μmol photons s^{-1} m^{-2} constant illumination and cell pellets were extracted

547 with cell rupture by glass beads and dodecane for yield quantification of 7-heptadecene via

548 GC-MS (bar graph, right). All constructs bear a PsaD chloroplast targeting peptide (CTP) to

549 allow protein transit to the chloroplast. Arrows and plus sign indicate co-expression in double

550 transformed mutants. Error bars represent 95% confidence intervals of single strains

551 cultivated in biological triplicates.

552

553 **Figure 6. Conversion of free fatty acids into alkanes in cyanobacteria using FAP.** (A) Graphic

554 representative of the plasmids used to transform *Synechocystis* sp. PCC 6803. (B) Total ion

555 chromatogram from Δ*aas*-Pcoa-'TesA (left) and Δ*aas*-1010-Pcoa-'TesA-'FAP (100 μmol

556 photons s^{-1} m^{-2} (μE)~~mE~~, middle; 300 μmol photons s^{-1} m^{-2}~~mE~~, right). The free fatty acid (C)

557 and alkane (D) yield in all tested strains. Data are mean ± SD of three biological replicates.

558 Samples were extracted on day 10. Peak: (1) heptadecane, (2) octadecane (internal standard),

559 (3) pentadecane, (4) undecane, (5) tridecane, (6) hexadecanoic acid.

560

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721

Highlights

- Synthetic metabolic pathways for hydrocarbon fuels were engineered in algae

- Free fatty acids were effectively converted into alkenes and alkanes

- Transfer of algal pathway into cyanobacteria was the most effective

- Alkane yield was enhanced 19-fold in *Synechocystis spp.* PCC 6803

- Alkene yield was enhanced 8-fold in *Chlamydomonas reinhardtii*

1   **Synthetic metabolic pathways for photobiological conversion of CO$_2$ into hydrocarbon fuel**

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ABSTRACT

Liquid fuels sourced from fossil sources are the dominant energy form for mobile transport today. The consumption of fossil fuels is still increasing, resulting in a continued search for more sustainable methods to renew our supply of liquid fuel. Photosynthetic microorganisms naturally accumulate hydrocarbons that could serve as a replacement for fossil fuel, however productivities remain low. We report successful introduction of five synthetic metabolic pathways in two green cell factories, prokaryotic cyanobacteria and eukaryotic algae. Heterologous thioesterase expression enabled high-yield conversion of native fatty acyl-acyl carrier protein (ACP) into free fatty acids (FFA) in *Synechocystis sp.* PCC 6803 but not in *Chlamydomonas reinhardtii* where the polar lipid fraction instead was enhanced. Despite no increase in measurable FFA in *Chlamydomonas*, genetic recoding and over-production of the native fatty acid photodecarboxylase (FAP) resulted in increased accumulation of 7-heptadecene. Implementation of a carboxylic acid reductase (CAR) and aldehyde deformylating oxygenase (ADO) dependent synthetic pathway in *Synechocystis* resulted in the accumulation of fatty alcohols and a decrease in the native saturated alkanes. In contrast, the replacement of CAR and ADO with *Pseudomonas mendocina* UndB (so named as it is responsible for 1-undecene biosynthesis in *Pseudomonas*) or *Chlorella variabilis* FAP resulted in high-yield conversion of thioesterase-liberated FFAs into corresponding alkenes and alkanes, respectively. At best, the engineering resulted in an increase in hydrocarbon accumulation of 8- (from 1 to 8.5 mg/g cell dry weight) and 19-fold (from 4 to 77 mg/g cell dry weight) for *Chlamydomonas* and *Synechocystis*, respectively. In conclusion, reconstitution of the eukaryotic algae pathway in the prokaryotic cyanobacteria host generated the most effective system, highlighting opportunities for mix-and-match synthetic metabolism. These studies describe functioning synthetic metabolic pathways for hydrocarbon fuel synthesis in photosynthetic microorganisms for the first time, moving us closer to the commercial implementation of photobiocatalytic systems that directly convert CO_2 into infrastructure-compatible fuels.

Highlights

- Synthetic metabolic pathways for hydrocarbon fuels were engineered in algae
- Free fatty acids were effectively converted into alkenes and alkanes

44 • Transfer of algal pathway into cyanobacteria was the most effective

45 • Alkane yield was enhanced 19-fold in *Synechocystis spp.* PCC 6803

46 • Alkene yield was enhanced 8-fold in *Chlamydomonas reinhardtii*

47

INTRODUCTION

Cyanobacteria (prokaryotes) and algae (eukaryotes) are photosynthetic microorganisms that have evolved to naturally accumulate C15-C19 alkanes or alkenes at very low concentrations (0.02-1.12% alkane g/g cell dry weight (CDW)) (Lea-Smith et al., 2015; Schirmer et al., 2010; Sorigué et al., 2017) with the exception of naturally oleagineous species (Ajjawi et al., 2017; Metzger and Largeau, 2005; Peramuna et al., 2015). These hydrocarbons are postulated to influence the fluidity of cell membranes and are therefore essential for achieving optimal growth, indeed, the abolition of their biosynthetic capacities results in morphological defects (Lea-Smith et al., 2016). Only two enzymes, acyl-ACP reductase (AAR) and aldehyde deformylating oxygenase (ADO) are required to catalyze the bacterial conversion of acyl-ACP into alkanes (Schirmer et al., 2010). Similarly, eukaryotic microalgae also biosynthesize small quantities of alkanes and alkenes directly from fatty acids, employing the distinctly different and recently discovered fatty acid photodecarboxylase (FAP; (Sorigué et al., 2017)).

In order to engineer sustainable biotechnological systems for production of hydrocarbons for the fuel market, whether heterotrophic or light-driven, far greater yields are needed alongside other complementary non-biochemical improvements such as improved bio-process designs. Several studies have attempted to enhance alkane productivity in cyanobacteria by over-expression of the native or non-native AAR and ADO enzyme couple (Hu et al., 2013; Kageyama et al., 2015; Peramuna et al., 2015; Wang et al., 2013) which relies on acyl-ACP as the precursor. Although naturally accumulating alkane amounts have been enhanced through engineering and reported in high titres from the lipid-accumulating cyanobacteria, *Nostoc punctiforme* (up to 12.9% (g/g) CDW, (Peramuna et al., 2015)), similar efforts in the non-lipid accumulating model cyanobacterium *Synechocystis sp.* PCC 6803 (hereafter *Synechocystis* 6803) have at best yielded only 1.1% (g/g) CDW (Hu et al., 2013; Wang et al., 2013). In eukaryotic algae, the native alkene/alkane pathway was only recently discovered and there has been no work so far to engineer the specific pathways that synthesize such hydrocarbons. Some species of algae are also known to naturally accumulate hydrocarbons that could serve as a fuel following chemical conversion. For example, certain races of the green alga *Botrococcus braunii* naturally secrete long-chain terpene hydrocarbons as a significant portion of their biomass (Eroglu and Melis, 2010; Metzger and Largeau, 2005). However, their use as a fuel source is made impossible by the incredibly slow growth rates of this alga (Cook et al., 2017). Other oleaginous algal species can accumulate a significant portion of their biomass as triacylglycerol compounds, generally under nitrogen stress. Indeed, this phenomenon drove the push for the use of algae as third generation

82 biofuel feedstock in the first place. However, process design and downstream processing cost

83 considerations of large-scale algal cultivation have hindered the common adoption of algal

84 oils for transportation fuels (Quinn and Davis, 2015). Triacylglycerol stored by eukaryotic

85 algae can also be turned into transportation fuels via transesterification to liberate the

86 alkanes and alkenes from the glycerol backbone. An attractive alternative to the above

87 concepts is instead to directly secrete ready-to-use hydrocarbon products from algal cells as

88 this would overcome issues with biomass harvesting and chemical processing and thereby

89 greatly reduce process costs (Delrue et al., 2013).

90   In order to achieve such a one-step conversion of CO_2 into ready infrastructure-

91 compatible hydrocarbons with photosynthetic hosts, however, genetic reprogramming

92 becomes essential for introduction of synthetic metabolic pathways and optimization of the

93 entire system. Several enzymes have recently been reported to enable biosynthesis of fatty

94 aldehyde precursors (Akhtar et al., 2013), fatty alkanes (Bernard et al., 2012; Qiu et al., 2012),

95 and fatty alkenes (Rude et al., 2011; Rui et al., 2015; Rui et al., 2014). Combinatorial assembly

96 of such key enzymes into synthetic metabolic pathways consequently enabled a number of

97 novel opportunities for hydrocarbon biosynthesis, as described by many including (Akhtar et

98 al., 2013; Kallio et al., 2014; Sheppard et al., 2016; Zhu et al., 2017). Although such studies

99 have so far only been reported using heterotrophic microorganisms (*Escherichia coli* and

100 *Saccharomyces cerevisiae*) there are no reports of similar work in any phototrophic

101 microorganism.

102   In this study, we describe a first and systematic study to implement synthetic

103 metabolic pathways for the biosynthesis of hydrocarbon fuel in both prokaryotic and

104 eukaryotic photosynthetic microorganisms using the model strains *Synechocystis* 6803 and

105 *Chlamydomonas reinhardtii*. Several synthetic pathways towards saturated and unsaturated

106 hydrocarbons were functionally demonstrated in *Synechocystis* 6803, increasing the

107 hydrocarbon content up to 19-fold, and engineered *Chlamydomonas* accumulated 8-fold more

108 alkenes than the wild-type. Interestingly, the "best" system was achieved by transferring a

109 reconstructed pathway from eukaryotic algae into the prokaryotic cyanobacterium.

MATERIALS AND METHODS

2.1 Growth conditions, genetic constructs, transformation and screening of Escherichia coli and Synechocystis sp. PCC 6803

Escherichia coli DH5α was used to propagate all the plasmids used in this study. Strains were 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, gentamicin 10 μg/ml, and erythromycin 200 μg/ml).

Synechocystis sp. PCC 6803, obtained from Prof. Klaas Hellingwerf (University of Amsterdam, Netherlands), was cultivated in BG11 medium without cobalt ((hereafter BG11-Co), as the metal was used as an inducer in most cultures. All media contained appropriate antibiotic(s) (final concentration: kanamycin 50 μg/ml, gentamicin 50 μg/ml, and erythromycin 20 μg/ml). Gentamicin was only used for selection on agar plates. Precultures inoculated from colonies on agar plates were grown in 6-well plates (5 ml). When the OD_{730} reached 3-4, the culture was transferred to a 100-ml Erlenmeyer flask and the OD_{730} was adjusted to 0.2 by adding BG11-Co medium to a final volume of 25 ml containing appropriate antibiotic(s). The cultivation was carried out for 10 days at 30 °C with continuous illumination at 60 μmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2. Each main treatment culture was induced on day 2 and samples were taken for measurement of OD_{730} and metabolites day 6 and 10. All cultivations were carried out in an AlgaeTron230 (Photon Systems Instruments) (PSI) at 30 °C with continuous illumination at 60 μmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2, except where noted (100-300 μmol photons $m^{-2} s^{-1}$). A representative growth curve and all final OD_{730} values are shown in Supplementary Figure 1.

All plasmids (Supplementary Table 1A) used for transformation of cyanobacteria were assembled using the BASIC Assembly method (Storch et al., 2015). Linkers were designed using the R20DNA software: http://www.r2odna.com/ and obtained from Integrated DNA Technologies Incorporated. The details of all linkers, primers and DNA parts used to construct each plasmid are given in Supplementary Tables 1B, 1C and 1D.

For transformation by natural assimilation, each *Synechocystis* sp. PCC 6803 strain was inoculated from freshly prepared colonies on agar plates into 25 ml BG11-Co with a starting OD 0.02. The cells were harvested when the OD_{730} reached 0.4-0.7, washed in 10 ml BG11-Co twice, and resuspended in 500 μL BG11-Co. One hundred microliters of concentrated

144 liquid culture were mixed with four to seven micrograms of plasmid and incubated at 30°C

145 with continuous illumination at 60 µmol photons $m^{-2} s^{-1}$ and 1% (v/v) CO_2 for 12-16 h prior

146 to plating on BG11-Co agar containing 10% strength of antibiotic. To promote segregation,

147 individual colonies were restreaked on BG11-Co agar with higher antibiotic concentration. To

148 check the segregation, the biomass was resuspended in nuclease free water and exposed to

149 two freeze-thaw cycles (95°C, -80°C). Following centrifugation, 3 µL was used as a template

150 for a diagnostic polymerase chain reaction (PCR). Primers used for each PCR are listed in

151 Supplementary Table 1C. Only fully segregated mutants were used in further experiments. All

152 cyanobacteria strains used in the study are listed in Supplementary Table 2.

153   For transformation by triparental conjugation, one hundred microliters of the cargo

154 strain (*E. coli* HB101 (already carrying the pRL623 plasmid)), conjugate strain (ED8654

155 (Elhai and Wolk, 1988)), and *Synechocystis* sp. PCC 6803 (OD_{730} ~1) were mixed and

156 incubated for 2 h (30 °C, 60 µmol photons $m^{-2} s^{-1}$). Prior to mixing, all the *E. coli* and

157 cyanobacteria strains were washed with fresh LB and BG11-Co medium, respectively, to

158 remove the antibiotics. After 2 h of incubation, the culture mix was transferred onto BG11

159 agar plates without antibiotic and incubated for 2 d (30 °C, 60 µmol photons $m^{-2} s^{-1}$). After 2 d

160 of incubation, cells were scraped from the agar plate, resuspended in 500 µL of BG11-Co

161 medium, and transferred onto a new agar plate containing 20 µg/ml erythromycin. Cells were

162 allowed to grow for one week until colonies appeared. Individual colonies were restreaked

163 onto a new plate containing 20 µg/ml erythromycin and used for subsequent experiments.

164

165 *2.2 Growth conditions, genetic constructs, transformation and screening of Chlamydomonas*

166 *reinhardtii*

167 *C. reinhardtii* strain UVM4 was used in this work (Neupert et al., 2009) graciously provided by

168 Prof. Dr. Ralph Bock)). The strain was routinely maintained on Tris acetate phosphate (TAP)

169 medium (Gorman and Levine, 1965) either with 1.5% agar plates or in liquid with 250 µmol

170 photons $m^{-2} s^{-2}$. Transformation was conducted with glass bead agitation as previously

171 described (Kindle, 1990). The amino acid sequences of *C. reinhardtii* native fatty acid

172 photodecarboxylase (FAP) (Uniprot: A8JHB7; (Sorigué et al., 2017)), *E. coli* thioesterase A

173 (TesA: P0ADA1), *Jeotgalicoccus* sp. ATCC 8456 terminal olefin-forming fatty acid

174 decarboxylase (OleT) (E9NSU2), and *Rhodococcus sp.* NCIMB 9784 P450 reductase RhFRED

175 (Q8KU27) were codon optimized and copies of the intron 1 of ribulose bisphosphate

176 carboxylase small subunit 2 (RBCS2) were added throughout the coding sequences as

177 previously described (Baier et al., 2018). The nucleotide sequences of optimized intron

178 containing genes have been submitted to NCBI, accession numbers can be found in

179 Supplementary Table 3. All synthetic genes were chemically synthesized (GeneArt) and

180 cloned between *Bam*HI-*Bg*lII in the pOpt2_PsaD_mVenus_Paro or pOpt2_PsaD_mRuby2_Ble

181 vectors (Wichmann et al., 2018). PsaD represents the 36 amino acid photosystem I reaction

182 center subunit II (PsaD) chloroplast targeting peptide (CTP) (Laursen et al., 2015) between

183 *Nde*I-*Bam*HI restriction sites of the pOpt2 vectors (Wichmann et al., 2018). The native FAP

184 enzyme was designed to contain an additional glycine codon at aa position 33 to allow the

185 insertion of a *Bam*HI site at the border of the predicted CTP. The whole synthetic enzyme

186 including native targeting peptide was cloned *Nde*I-*Bg*lI and a version was created with the

187 PsaD CTP built by cloning *Bam*HI-*Bg*lI into the vectors described above. Fusions of different

188 sequences were made by digestion and complementary overhang annealing of the *Bam*HI-

189 *Bg*lI mediated restriction sites for each respective construct as needed to obtain the fusions

190 used in the present work (Supplementary Figure 2). After transformation, expression was

191 confirmed by fluorescence microscopy screening for mVenus (YFP) or mRuby2 (RFP)

192 reporters as previously described (Laursen et al., 2016; Wichmann et al., 2018). Individual

193 mutants were subjected to Western blotting and immuno detection to determine whether

194 full-length protein products were formed (anti-GFP polyclonal HRP linked antibody, Thermo

195 Fisher Scientific). Wide-field fluorescence microscopy was used to confirm chloroplast

196 localization of YFP-linked constructs as previously described (Laursen et al., 2016).

197

198 *2.3 Product analysis*

199 Three different extraction and analysis protocols were used for the analysis of (1) acids, (2)

200 alcohols and (3) alkanes as well as alkenes from cyanobacteria cultures. For each analyte

201 group, liquid cultures in flasks were mixed well by shaking prior to transferring 2 mL of liquid

202 culture into a PYREX round bottom threaded culture tube (Corning, Manufacturer Part

203 Number: 99449-13).

204   For fatty acid analysis, free fatty acid extraction was performed as described

205 previously (Liu et al., 2011; Yunus and Jones, 2018). In brief, two hundred microliters of 1 M

206 H_3PO_4 were added to acidify each 2 mL culture and spiked with 100 μg pentadecanoic acid

207 (Sigma Aldrich) as an internal standard. Four millilitres of n-hexane (VWR Chemicals) was

208 added and the mixture vortexed vigorously prior to centrifugation at 3500 x g for 3 min. The

209 upper hexane layer was then transferred to a fresh PYREX round bottom threaded culture

210 tube and evaporated completely under a stream of nitrogen gas. Five hundred microliters of

211 1.25 M HCl in methanolic solution were added to methyl esterify the free fatty acid at 85 °C for

212 2 h. Samples were cooled to room temperature and 500 μL of hexane was added for

213 extraction of the fatty acid methyl esters (FAMEs).

214 For fatty alcohol, alkane and alkene analysis, extraction was done as described

215 previously (Zhou et al., 2016) with modification. Briefly, 2 mL of liquid culture were spiked

216 with 50 μg 1-nonanol, 100 μg octadecane, and 100 μg 1-pentadecanol and mixed with 4 mL of

217 chloroform:methanol (2:1 v/v) solution. The mixture was vortexed vigorously and

218 centrifuged at 3500 x g for 3 min. The lower organic phase was then transferred into a new

219 glass tube and extraction was repeated one more time. The lower organic phase was

220 combined and dried under a stream of nitrogen gas. For fatty alcohol derivatization, the dried

221 extract was resuspended in 100 μL chloroform, mixed with 100 μL of N, O-

222 bistrifluoroacetamide (BSTFA) (TCI Chemicals) and transferred to an insert in a GC vial that

223 was incubated at 60 ^{o}C for 1 h prior to GC analysis. Note that no derivatization was needed for

224 the analysis of hydrocarbons.

225 Samples (1 μL) were analyzed using an Agilent Technologies (Santa Clara, CA, USA)

226 7890B Series Gas Chromatograph (GC) equipped with an HP-5MS column (pulsed split ratio

227 10:1 and split flow 10 ml/min), a 5988B Mass Spectrophotometer (MS) and a 7693

228 Autosampler. For the acids the GC oven program followed an initial hold at 40 ^{o}C for 3 min, a

229 ramp at 10 ^{o}C.min^{-1} to 150 ^{o}C, a second ramp at 3 ^{o}C.min^{-1} to 270 ^{o}C, a third ramp at 30 ^{o}C.min^{-}

230 ^{1} to 300 ^{o}C, and a final hold for 5 min. For alcohols and alkenes, there was an initial hold at 40

231 ^{o}C for 0.5 min, a ramp at 10 ^{o}C.min^{-1} to 300 ^{o}C, and a final hold for 4 min. For alkanes, the oven

232 was initially held at 70 ^{o}C for 0.5 min, a ramp at 30 ^{o}C.min^{-1} to 250 ^{o}C, a second ramp at 40

233 ^{o}C.min^{-1} to 300 ^{o}C, and a final hold for 2 min. The acids, alkanes and alcohols were quantified

234 by comparing the peak areas with that of the internal standards: pentadecanoate (for all

235 acids), octadecane (for all alkanes), 1-nonanol (for C8 to C12 alcohols) and 1-pentadecanol

236 (for C14 alcohols and above). The quantity of the main products (C15 and C17 alkanes, C15

237 alkene, and C12, C14, C16, and C18 alcohols and acids) were also corrected with their

238 respective mass spectrometer response factors obtained using dilution series of commercial

239 standards.

240 Gas chromatography mass spectroscopy (GC-MS) aimed at identification of

241 hydrocarbon products from *C. reinhardtii* was conducted with solvent extracted samples

242 following previously described protocols and internal standards (Lauersen et al., 2016).

243 Quantification of 7-heptadecene was performed with serial dilutions (1 to 900 μM) of

244 commercial 1-heptadecene standard (Acros Organics) in dodecane using extracted ion

245 chromatograms with masses 55.00, 69.00, 91.00, 93.00, 83.00, 97.00, and 111.00.

RESULTS AND DISCUSSION

Several synthetic pathway designs were considered, all commencing with the liberation of "free" fatty acids from the native fatty acid biosynthesis pathway (Fig. 1), the presumed native precursor for many of the decarboxylating enzymes evaluated in this study.

3.1 Over-production of free fatty acids as precursor for hydrocarbon biosynthesis - Expression of Escherichia thioesterase deregulates lipid membrane biosynthesis in Chlamydomonas

In order to liberate FFAs in cyanobacteria we over-expressed the *E. coli* C16-C18 specific thioesterase TesA (Cho and Cronan, 1995) lacking its native signal sequence peptide ('TesA) and deleted the gene encoding the native fatty acyl ACP synthase (*aas*) (Kaczmarzyk et al., 2010; Liu et al., 2011)(Fig. 1). The native signal sequence peptide directs TesA to the periplasm in *E. coli* (Cho et al 1993) and its removal is assumed to maximize the liberation of "free" fatty acids also in cyanobacteria by retaining the enzyme in the cytosol. Such 'TesA/Δaas engineering has previously been reported several times before in cyanobacteria (Liu et al., 2011, Ruffing et al., 2014; Work et al., 2015; Kato et al., 2017), with 13% (g/g cell dry weight (CDW)) as the highest reported fatty acid yield in *Synechocystis* 6803 (Liu et al., 2011). Further potentially stackable modifications to the strain or process have also been reported. For example, by employing a solvent overlay, Kato et al., 2017 reported up to 36% (g/g) CDW of fatty acids excreted into the media using 'TesA/Δaas *Synechococcus elongatus sp.* PCC 7942. In the present study, the chromosomal integration of '*tesA* into the *psbA2* site (slr1311) of *Synechocystis* 6803 Δaas (Δaas-'TesA), under the control of the light-inducible promoter PpsbA2S, resulted in the excretion of C14:0 (3.5 mg/g CDW), C16:0 (23.2 mg/g CDW) and C18:0 (5.7 mg/g CDW) fatty acids with a chain-length distribution that is in agreement with previously reported findings (Liu et al., 2011) (Fig. 2A; Supplementary Fig. 3).

Overproduction of the same thioesterase ('TesA) and targeting of the enzyme product to the chloroplast was possible in *C. reinhardtii*. The synthetic algal optimized *E. coli* '*tesA* gene was fused with an N-terminal PsaD-based chloroplast targeting peptide and a C-terminal yellow fluorescent protein (YFP) encoding gene. Both the coding genes were interspersed by synthetic introns (Fig. 2B) as previously described to enhance transgene expression from the nuclear genome (Baier et al., 2018). Fluorescence microscopy indicated correct localization of the 'TesA fluorescent protein fusion to the algal chloroplast (Fig. 2C). Although no FFA could

280   be detected in the culture medium, a difference was observed in the lipid profile of the green

281   algal cells, suggesting a de-regulation of fatty acid synthesis that specifically affected the polar

282   lipid fraction of the alga. This was indicated by an over-accumulation of C18:1n9c chain

283   lengths in the polar lipid membranes, with subtle changes observed in other acyl-ACP species

284   such as C14:0 (Fig. 2D; Supplementary Fig. 4). Thus, 'TesA_YFP clearly had an impact on lipid

285   metabolism in the eukaryotic algal host, but, the capture of liberated FFAs by acyl-ACP or -

286   CoA synthases are likely too effective, thereby limiting the application of the same engineering

287   principles carried out for cyanobacteria. An annotated gene product in *Chlamydomonas*

288   Cre06.g299800 (Phytozome v5.5) has some sequence similarity to *Synechocystis aas* and

289   therefore represents an interesting target for future strategies to block native re-uptake of

290   FFA in the green algal cell.

291   Having achieved strains with enhanced accumulation of FFA in *Synechocystis*, or at

292   least a perturbation to the lipid biosynthetic system in *Chlamydomonas*, we proceeded to

293   investigate enzymes that further convert FFAs into hydrocarbon end-products.

294

295   *3.2 Effective conversion of free fatty acids into alkenes using UndB*

296   Three different enzymes that catalyze the conversion of fatty acids into alkenes have been

297   recently reported, OleT (Rude et al., 2011), UndA (Rui et al., 2014), and UndB (Rui et al., 2015)

298   (Fig. 1). So far, the best reported productivity in both *E. coli* (Rui et al., 2015) and *S. cerevisiae*

299   (Zhou et al., 2018) has been with UndB.

300   In *Synechocystis* 6803, we transformed the Δ*aas*-'TesA strain with an RSF1010-based

301   plasmid harboring a codon-optimized *undB* under the control of the Pclac143 promoter

302   (Markeley et al., 2014), thereby generating the strain Δ*aas*-'TesA-1010-UndB (Fig. 3A). After

303   10 days of cultivation, both the free fatty acids and alkanes were extracted and analyzed as

304   described in the Materials and Methods section. The accumulation of free fatty acids was

305   markedly reduced in the Δ*aas*-'TesA-1010-UndB strain (Fig. 3B, 3C). In its place, both 1-

306   pentadecene and 1-heptadecene accumulated with a molar yield suggesting approximately

307   55% conversion of 'TesA-liberated FFAs (compare Fig. 3C with Fig. 3D). More than >84% of

308   the FFAs disappeared relative to the Δ*aas*-'TesA strain suggesting that UndB was catalytically

309   efficient *in vivo* and that the electrons required in the UndB reaction were fortunately

310   supplied by an unknown source. The Δ*aas*-'TesA-1010-UndB strain displayed a lower biomass

311   accumulation than the controls (Δ*aas*-empty and Δ*aas*–'TesA strains) (Supplementary Fig. 1),

312   presumably due to product toxicity imparted by the alkenes. A direct comparison with the

313   conversion efficiency in *E. coli* is not possible since the FFA conversion efficiency was not

314   reported in the original work (Rui et al., 2015). Despite the disappearance of C14:0 fatty acids

315   in the Δ*aas*-'TesA-1010-UndB strain, no measurable 1-tridecene (the expected corresponding

316   alkene) was observed in the whole culture extracts (Fig. 3C). None of the observed alkene

317   products were secreted extracellularly (Fig. 3E).

318   In *Chlamydomonas*, we attempted to over-produce the *Jeotgalicoccus* sp. terminal

319   olefin-forming fatty acid decarboxylase (OleT) and the *Rhodococcus sp.* P450 reductase

320   (RhFRED). OleT was chosen as it could theoretically produce C17:1 and C15:0 hydrocarbons

321   from the major lipid species of the green algal cell, C18:1 and C16:0, respectively (Fig. 1).

322   Fusion to RhFRED has been reported to enable hydrogen peroxide-independent

323   decarboxylase activity (Liu et al., 2014). The protein products of this decarboxylase and its

324   fusion in either orientation to RhFRED could be detected by Western blotting and located to

325   the algal chloroplast in fluorescence microscopy (Supplementary Figure 5). However, no

326   differences in GC-MS profiles between the parental and expression strains could be found in

327   either dodecane solvent overlays or cell-pellet solvent extracts.

328

329   *3.3 Transfer of the CAR/ADO based pathway from E. coli to Synechocystis 6803 resulted in*

330   *the accumulation of fatty alcohols and a reduction in alkane accumulation*

331   Carboxylic acid reductases (CAR) have been previously used to construct a number of

332   synthetic pathways for alkane biosynthesis in heterotrophic microorganisms (Akhtar et al.,

333   2013; Kallio et al., 2014; Sheppard et al., 2016; Zhu et al., 2016). Although CAR appears to

334   have a high capability for converting fatty acids into corresponding fatty aldehydes (Akhtar et

335   al., 2013) (Fig. 1), a bottleneck in previous heterotrophic pathways is the subsequent

336   conversion into alkanes by kinetically slow ADO enzymes and competition with native

337   aldehyde reductases that more effectively convert aldehydes into alcohols (Kallio et al., 2014;

338   Sheppard et al., 2016).

339   Since *Synechocystis* 6803 natively harbors an aldehyde deformylating oxygenase

340   (ADO) with the appropriate substrate specificity (Khara et al., 2013) (Fig. 1), we first

341   combined TesA with CAR and evaluated its ability to supply the native ADO. A synthetic

342   operon expressing all required parts (including the CAR maturation protein Sfp) was

343   introduced to the RSF1010 plasmid backbone (Fig. 4A) and used to transform *Synechocystis*

344   6803 Δ*aas*, thus creating the strain Δ*aas*-1010-TPC2. This strain accumulated both fatty acids

345   (Fig. 4B and 4D) and fatty alcohols (Fig. 4C and 4E). The quantity of heptadecane was reduced

346   in Δ*aas*-1010-TPC2 relative to Δ*aas*-1010-'TesA (Fig. 4F). This suggests that the introduced

347   CAR-based pathway had not managed to increase the supply of fatty aldehydes to the native

348 ADO. CAR and native aldehyde reductase(s) had instead very effectively converted >90% of

349 the FFA pool (Fig. 4D) into corresponding alcohols (Fig. 4E). The most likely reason for the

350 increase in FFA in latter experiments is due to increased expression of 'TesA using the

351 RSF1010 plasmid in Δ*aas*-1010-'TesA (Fig. 4D), relative to the amount of 'TesA when

352 expressed from the chromosomal location in Δ*aas*-'TesA (Fig. 3C). Similar observations have

353 also been previously reported by Angermayr et al. (Angermayr et al., 2014). The different

354 promoters used in the two strains are also likely to have influenced the outcome, however, we

355 are not aware of any studies that directly compare the two promoters head-to-head.

356   Substantial quantities of fatty alcohols did accumulate in the Δ*aas*-1010-TPC2 strain,

357 suggesting that the supply of fatty aldehydes is not the limiting factor. One possibility is that

358 the native aldehyde reductases are simply much more active than the native ADO (Eser et al.,

359 2011; Lin et al., 2013). Another possibility is that native ADO and AAR form a close metabolon

360 *in vivo* (Warui et al., 2015) that locks out access to ADO from external supplies of fatty

361 aldehydes. In order to test this possibility, we attempted to create a variant of Δ*aas*-1010-

362 TPC2 that also included chromosomal ADO over-expression cassette under the PpsbA2S

363 promoter. Despite numerous transformation and segregation attempts, however, we were

364 unable to isolate any stable segregants. Another complementary strategy that could be

365 considered in future work would be to eliminate native aldehyde reductases, as previously

366 carried out in earlier *E. coli* studies (Kallio et al., 2014; Sheppard et al., 2016), although the full

367 complement of fatty aldehyde reductase encoding genes in cyanobacteria remains unknown.

368 Given the lack of success in producing alkanes with the CAR/ADO route in cyanobacteria we

369 then considered alternative options for both cyanobacteria and algae.

370

371 *3.4 Engineering of the native eukaryotic algae pathway and transfer to cyanobacteria results*

372 *in enhanced conversion of CO₂ into alkanes*

373 A fatty acid photodecarboxylase (FAP) that directly converts saturated and unsaturated FFAs

374 into alkanes and alkenes, respectively, was recently discovered in eukaryotic algae (Sorigué et

375 al., 2017). In *Chlamydomonas*, the source of free fatty acids for the native alkene pathway

376 remains unknown, although the degradation of membrane lipids may release some FFA

377 (illustrated in Fig. 1). However, we would expect increased accumulation of alkanes in algae if

378 we were able to increase the cellular quantity of the native FAP and/or introduce synthetic

379 routes to the FFA precursors.

380   Accordingly, we overproduced native FAP from *C. reinhardtii* (CrFAP) on its own or in

381 combination with co-production of *E. coli* 'TesA. The over-expression of CrFAP was carried

out either with its native chloroplast targeting peptide (CTP) or the robust PsaD CTP which has been previously used to mediate chloroplast localization of numerous reporters (Lauersen et al., 2015; Lauersen et al., 2018; Rasala et al., 2013). In order to minimize any native regulation of the genomic sequence, the gene was subjected to a strategy of gene design which has recently been shown to enable robust transgene expression from the nuclear genome of this alga (Baier et al., 2018). Briefly, the sequence was codon optimized based on its amino acid sequence and multiple copies of the first intron of the *C. reinhardtii* ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) small subunit 2 (rbcS2i1, NCBI: X04472.1) were spread throughout the coding sequence *in silico*. This nucleotide sequence was chemically synthesized and used for expression from the algal nuclear genome. This strategy has previously enabled heterologous overproduction of non-native sesquiterpene synthases (Lauersen et al., 2016; Lauersen et al., 2018; Wichmann et al., 2018), and in the present study also the 'TesA, OleT, and RhFRED proteins. However, complete codon optimization and synthetic intron spreading of a native gene has not yet been demonstrated in eukaryotic algae. Both constructs mediated full-length target protein production which was detectible in Western blots (Supplementary Fig. 5B). Replacing the native CTP with the PsaD CTP enabled more reliable and robust accumulation, which was detectible as YFP signal in the algal chloroplast (Supplementary Fig. 6) and strong bands in transformants expressing this construct in Western blots (Supplementary Fig. 5B). The parental UVM4 strain was found to contain ~0.5 mg/g 7-heptadecene as a natural product (Supplementary Fig. 7). Transformants generated with the CrFAP construct (Cr8) were found to contain up to 8x more of this alkene compared to the empty vector (Cr2) control strain (up to 8.5 ± 1.5 mg/g, Fig. 5) which was found almost exclusively within the biomass (Supplementary Fig. 7). The product was not detected in dodecane solvent overlays. CrFAP accepts a very specific substrate (*cis*-vaccenic acid, C18:1*cis*Δ11) *in vivo* (Sorigué et al., 2017), which corresponds to the accumulation of only 7-heptadecene as the only detected increased product. This substrate is an unusual FA and is likely not naturally abundant in the algal cell. Notably, any attempts to increase the availability of free fatty acids using *E. coli* 'TesA did not result in any increase in the quantity or diversity of accumulated alkanes. Future enzyme engineering will likely be able to overcome this substrate specificity and increase overall yields of liberated hydrocarbons. However, a strategy which would allow secretion of these molecules, similar to the capture of heterologous terpenoids in dodecane solvent overlay (Lauersen et al., 2016; Lauersen et al., 2018; Wichmann et al., 2018), would be an attractive next target in order to enable photo-biocatalysis of hydrocarbons from the algal biomass.

416   Given the success with the FAP pathway in *Chlamydomonas* (present study) and

417   earlier work in *E. coli* (Sorigué et al., 2017), as well as finding that 'TesA expression can

418   substantially enhance the FFA pool in cyanobacteria, a synthetic FAP pathway was an obvious

419   choice to consider also for the prokaryotic host. We therefore proceeded to implement a

420   reconstituted variant of the eukaryotic algae pathway in cyanobacteria by combining TesA

421   with FAP. Given the genetic instability challenges with the CAR/ADO system (see Section 3.3)

422   we shifted our constructs to the more tightly repressed Pcoa promoter (Peca et al., 2008) for

423   controlling the expression of *E. coli* TesA and the *Chlorella variabilis* FAP from the RSF1010

424   plasmid (Fig. 6A). We noted that the yield of FFA was substantially increased when driving the

425   expression of TesA with the Pcoa promoter (Fig. 6C) compared to Pclac143 (Fig. 4D).

426   Despite the dominance of C16:0 fatty acids released by 'TesA in *Synechocystis* 6803,

427   alongside minor fractions of C14:0 and C18:0, the C17:0 alkanes dominated the hydrocarbon

428   fraction at the lower light intensity (100 µmol photons $m^{-2}\,s^{-1}$) (Fig. 6B and 6D). This alkane

429   profile in *Synechocystis* 6803 is very different to that observed in *E. coli* without over-

430   expression of 'TesA (see Fig. S4 in (Sorigué et al., 2017)). We also observed substantial peaks

431   of 8-heptadecene and 6,9-heptadecadiene, as suggested by comparison with a NIST mass

432   spectrometry library, although a lack of standards prohibited confirmation (Supplementary

433   Figure 8). Curiously, these alkenes were only detected at day 6 and were not present in

434   samples harvested on day 10. As the fatty chain-length profiles differ when the same

435   thioesterase is expressed in different *E. coli* strains (Akhtar et al., 2015; Jing et al., 2011), this

436   suggests that the *in vivo* product profile of any thioesterase-dependent pathway also is

437   dependent on what the fatty acid synthesis pathway provides, not just the substrate

438   specificity of the thioesterase used.

439   Removal of the predicted chloroplast targeting sequence of FAP ('FAP) resulted in a

440   doubling of the alkane yield, this time accompanied also by C15 pentadecane. As the FAP

441   reaction is light-dependent, we also did a simple evaluation of this environmental factor.

442   When the light intensity was tripled, the total alkane production with the Δ*aas*-1010-'TesA-

443   'FAP strain increased to a yield of 77.1 mg/g CDW (19-fold enhancement relative to Δ*aas*) and

444   a titer of 111.2 mg/L. The product profile also shifted (Fig. 6D) despite the lack of a similar

445   shift in the remaining FFA fraction (Fig. 6B), suggesting that the substrate specificity of FAP is

446   flexible and interestingly might change in response to a change in its cellular environment.

447   At 100 µmol photons $m^{-2}\,s^{-1}$ the introduction of 'FAP resulted in a drop in FFA

448   accumulation of up to 90% (for C18:0), whilst for C16:0 there was only a 60% reduction (Fig.

449   6C). Despite repeated trials, the recovery in the measurable fatty acid to alkane conversion

450   remained poor for C16:0 in comparison to C18:0 and the other pathways tested in

451   *Synechocystis* 6803. This may be explained by an impact on 'TesA accumulation in the

452   constructs also carrying the gene coding for 'FAP. Nevertheless, the reconstituted eukaryotic

453   algae alkane pathway was more responsive to introduced modifications in the prokaryotic

454   cyanobacterium than in its native host, though this most likely is explained by challenges

455   associated with the release of FFA in the latter.

456   Although a substantial amount of both alkanes and alkenes were produced by the

457   engineered strains, their performance likely needs to be improved before any application can

458   be considered. Given that no genetically engineered phototrophic microalgae is currently used

459   for commercial purposes (as far as we are aware), and LCA-studies with non-catalytic systems

460   indicate a low predicted energy return on investment (EROI) (Carneiro et al., 2017), also

461   other challenges with commercial algal biotechnology (e.g. contamination, bioreactor cost,

462   energy consumption, etc) will need to be addressed.

463

CONCLUSIONS

The different biosynthetic systems presented in this study varied in terms of cellular context, compartmentation, promoters, operon structures and expression platforms, thus precluding a any direct comparison within and between the two species studied. However, the relative conversion efficiencies and absolute functionalities provide for a valid comparison. As such, it could be seen that the conversion of free fatty acids into alkenes by UndB and alkanes by FAP were effective (>50% conversion, for individual fatty acids up to >90% conversion), and that the native FAP pathway in *Chlamydomonas* was amenable to manipulation but that the inability to increase the FFA pool hindered further progress. Consequently, for alkanes, the reconstruction of the eukaryotic algae pathway in the prokaryotic cyanobacteria host provided a more productive system than the partially synthetic pathways in either of the prokaryotic (CAR-ADO) or eukaryotic hosts (TesA-FAP).

This work describes several approaches to employ synthetic metabolism and substantially exceed native capabilities for hydrocarbon biosynthesis in well-established model cyanobacteria and algae. Although even greater yields have been reported in oleaginous algae and cyanobacteria that are natively endowed to accumulate lipids, the ability to introduce synthetic metabolic pathways in model strains opens up possibilities for tailored choice of both products and hosts. Importantly, the present work is based on first generation strains and further improvement is likely with systematic optimization of both strains and cultivation conditions, including the use of superior engineered or natural enzyme variants.

17

FIGURE LEGENDS

495

496 **Figure 1. Native and synthetic metabolic pathways evaluated in the present study with**

497 **incomplete stoichiometry.** The graphic illustration shows the introduced TesA (thioesterase

498 (Cho and Cronan, 1995)), CAR (carboxylic acid reductase (Akhtar et al., 2013)), UndA

499 (responsible for 1-undecene biosynthesis in *Pseudomonas* (Rui et al., 2014)), UndB (also

500 responsible for 1-undecene biosynthesis in *Pseudomonas* (Rui et al., 2015)), OleT

501 (responsible for olefin biosynthesis in *Jeotgalicoccus* (Rude et al., 2011)) and FAP (fatty acid

502 photodecarboxylase (Sorigué et al., 2017)) enzymes alongside the native AAR/ADO (acyl-ACP

503 reductase and aldehyde deformylating oxygenase (Schirmer et al., 2010)), AHR (aldehyde

504 reductase, unknown) and FAP enzymes. Blue reactions are non-native and those in grey are

505 native. The red cross indicates deletion of the *aas* gene.

506

507 **Figure 2. Engineering for enhanced accumulation of free fatty acids.** (A) Representative total

508 ion count chromatograms for *Synechocystis* 6803 strains Δ*aas*-'TesA (black) vs. Δ*aas* only

509 (orange) extracted on day 10 of cultivation (induced day 2). Peak identities: (3) Heptadecane,

510 (4) Tetradecanoic acid, (5) Hexadecanoic acid, (6) 9,12-octadecadienoic acid, (7) 9-

511 octadecenoic acid, (8) Octadecanoic acid. (B) Graphic representation of the constructs used to

512 transform *Chlamydomonas*. CTP = Chloroplast Transit Peptide. (C) Fluorescence microscopy

513 of representative strains indicating appropriate chloroplast localization of the CTP_'TesA_YFP

514 construct. (D) Total (TL), polar (PL), and neutral (NL) gravimetric lipid fractions of

515 *Chlamydomonas* parental strain and TesA overproducing strains under nutrient replete

516 conditions (N+) and after 96 hours of nitrogen depletion (N-). PL is significantly greater in +N

517 for TesA: ttest, p:0.047 (indicated by an asterisk).

518

519 **Figure 3. Over-expression of UndB results in effective (>50%) conversion of fatty acids into**

520 **corresponding alkenes**. (A) Graphic representation of the genetic modification of

521 *Synechocystis* sp. PCC 6803 and the plasmid used for UndB expression. (B) GC-MS

522 chromatograms with extracts from the two different strains (w/wo UndB); Δ*aas*-1010-'TesA

523 (black) and Δ*aas*-1010-'TesA-UndB (orange). (C) The free fatty acid yield (relative to biomass)

524 in the whole cultures of the two strains, subdivided into the three dominant chain-lengths. (D)

525 The yield of alkenes in the whole cultures of the two strains, subdivided into the three

526 dominant chain-lengths. (E) The localization of the alkene products in whole cultures of the

527 two strains. Peak identities: (1) 1-pentadecene, (2) 1-heptadecene, (3) heptadecane, (4)

528 tetradecanoic acid, (5) hexadecanoic acid, (6) 9,12-octadecadienoic acid, (7) 9-octadecenoic

529 acid, (8) octadecanoic acid. Data are mean ± SD from three biological replicates. All samples

530 were extracted on day 10.

531

532 **Figure 4. The CAR-dependent pathway produces mainly fatty alcohols.** (A) Graphic overview

533 (not to scale) illustrating the main constructs studied in the figure. (B) Total ion

534 chromatogram from extracts of Δaas-1010-'TesA (black) and Δ*aas*-1010-TPC2 (orange). (C)

535 Fatty alcohol profile from extracts of Δ*aas*-TPC2. The yield of fatty acids (D), alcohols (E) and

536 alkanes (F). Peak identities: (2) 1-dodecanol, (3) heptadecane, (4) 1-tetradecanol, (5) 1-

537 hexadecanol, (6) 9,12-octadecadien-1-ol, (7) 9-octadecen-1-ol, (8) 1-octadecanol, (9)

538 dodecanoic acid, (10) tetradecanoic acid, (11) hexadecanoic acid, (12) octadecanoic acid. Data

539 are mean ± SD of three biological replicates. Cultures were induced on day 2 following

540 dilution and samples were extracted on day 10.

541

542 **Figure 5. CrFAP over-expression increases 7-heptadecene yield, but heterologous thioesterase**

543 **(TesA) expression, its co-expression, and C- or N-terminal fusion with CrFAP has no benefit.**

544 Mutants expressing indicated constructs (left panel) were cultivated for seven days in TAP

545 medium with 250 μmol photons s^{-1} m^{-2} constant illumination and cell pellets were extracted

546 with cell rupture by glass beads and dodecane for yield quantification of 7-heptadecene via

547 GC-MS (bar graph, right). All constructs bear a PsaD chloroplast targeting peptide (CTP) to

548 allow protein transit to the chloroplast. Arrows and plus sign indicate co-expression in double

549 transformed mutants. Error bars represent 95% confidence intervals of single strains

550 cultivated in biological triplicates.

551

552 **Figure 6. Conversion of free fatty acids into alkanes in cyanobacteria using FAP.** (A) Graphic

553 representative of the plasmids used to transform *Synechocystis* sp. PCC 6803. (B) Total ion

554 chromatogram from Δ*aas*-Pcoa-'TesA (left) and Δ*aas*-1010-Pcoa-'TesA-'FAP (100 μmol

555 photons s^{-1} m^{-2} (μE), middle; 300 μmol photons s^{-1} m^{-2}, right). The free fatty acid (C) and

556 alkane (D) yield in all tested strains. Data are mean ± SD of three biological replicates.

557 Samples were extracted on day 10. Peak: (1) heptadecane, (2) octadecane (internal standard),

558 (3) pentadecane, (4) undecane, (5) tridecane, (6) hexadecanoic acid.

559

560

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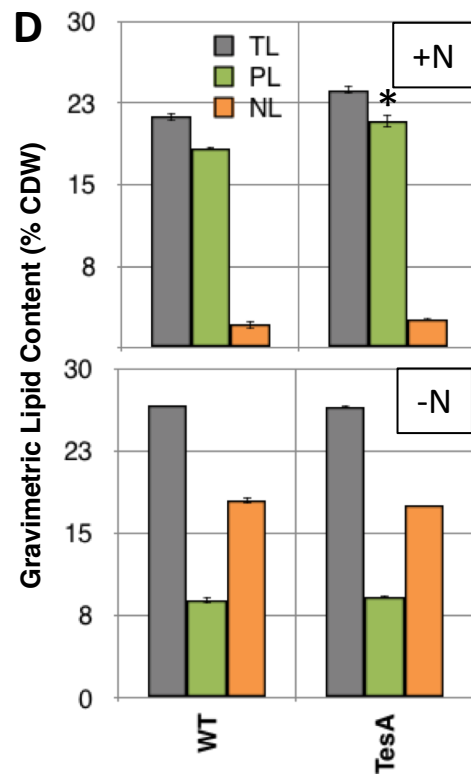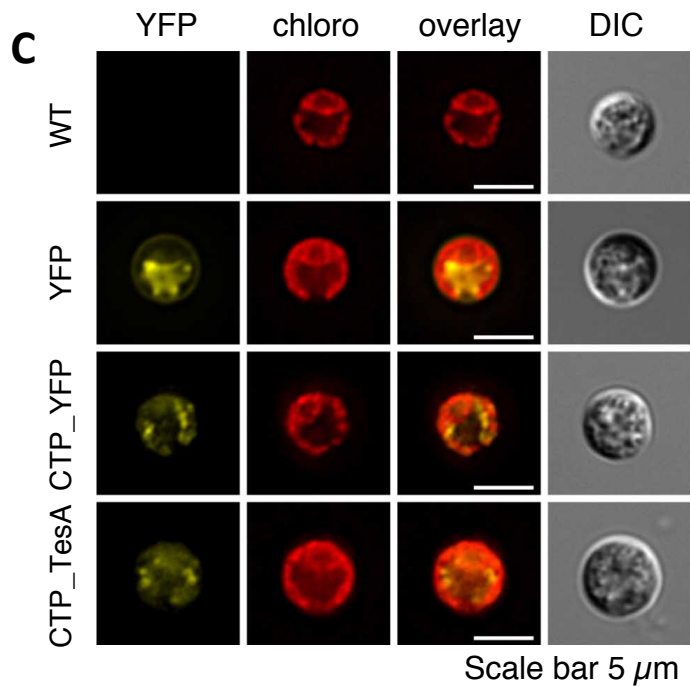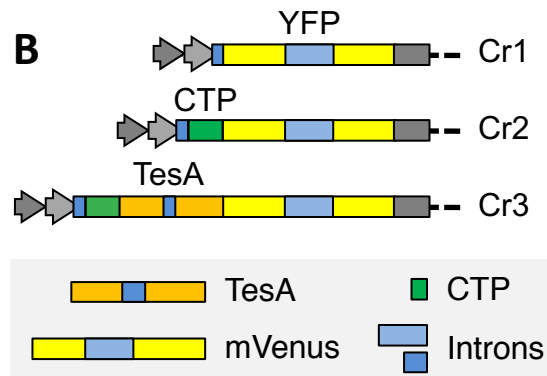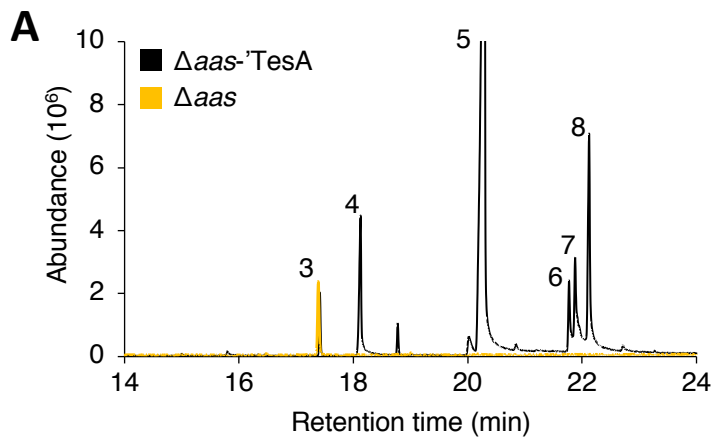713   Biotechnol. 3.

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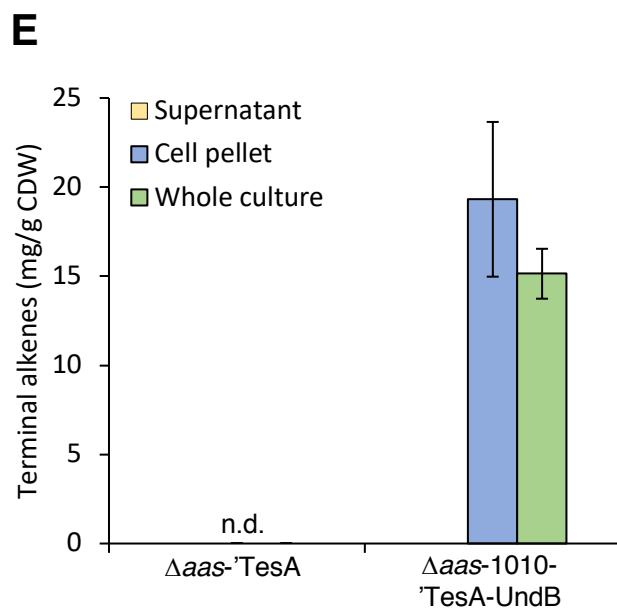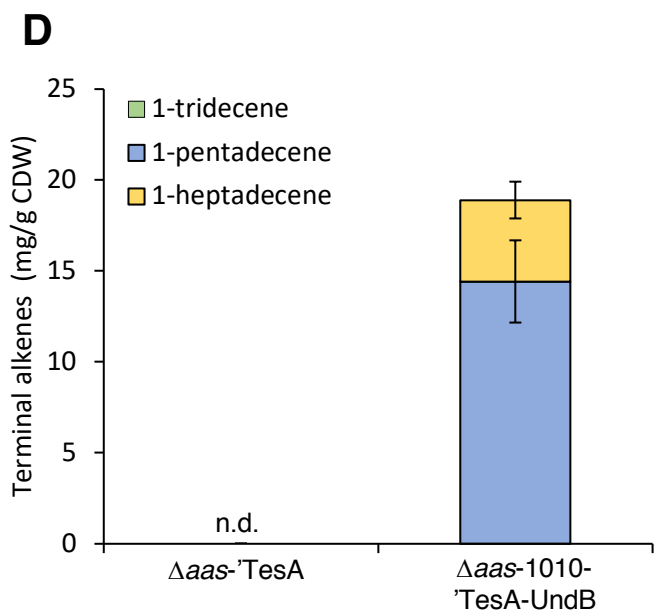715   fatty acids and alcohols. Metab Eng. 49, 59-68.
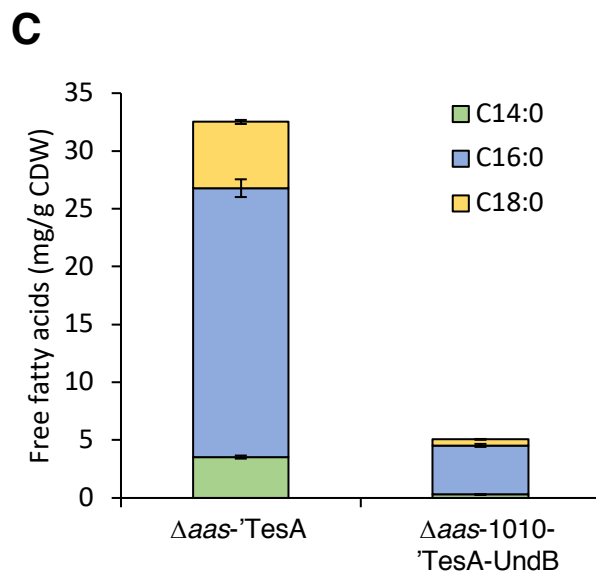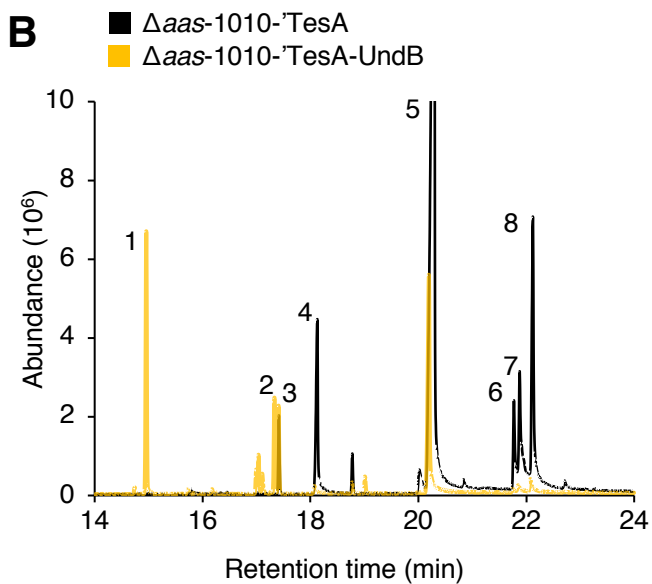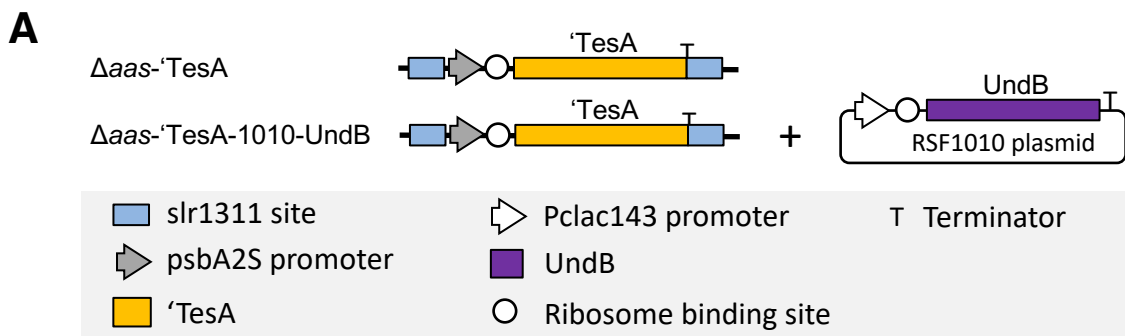
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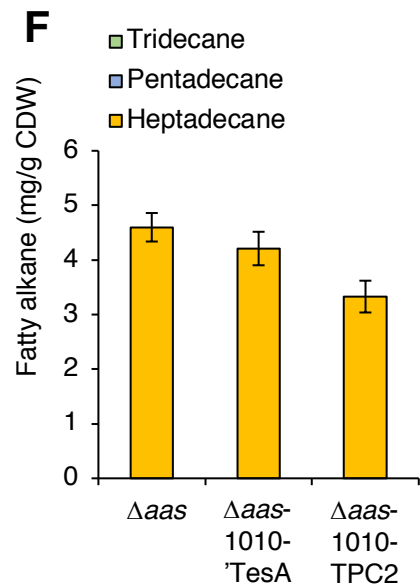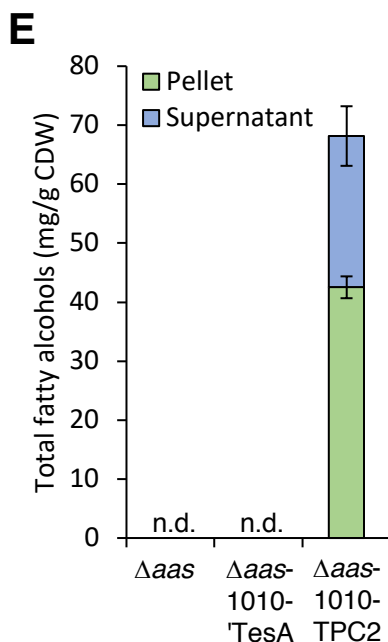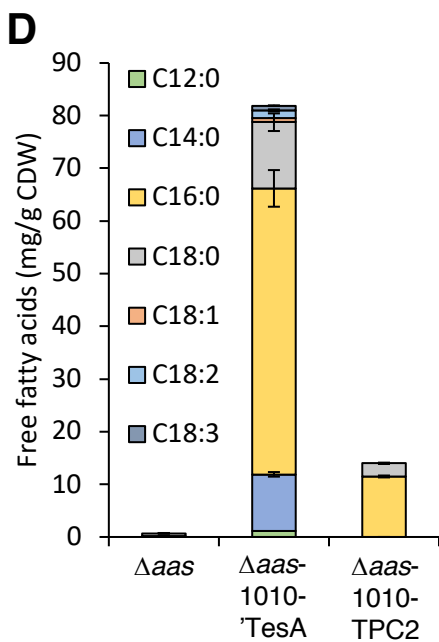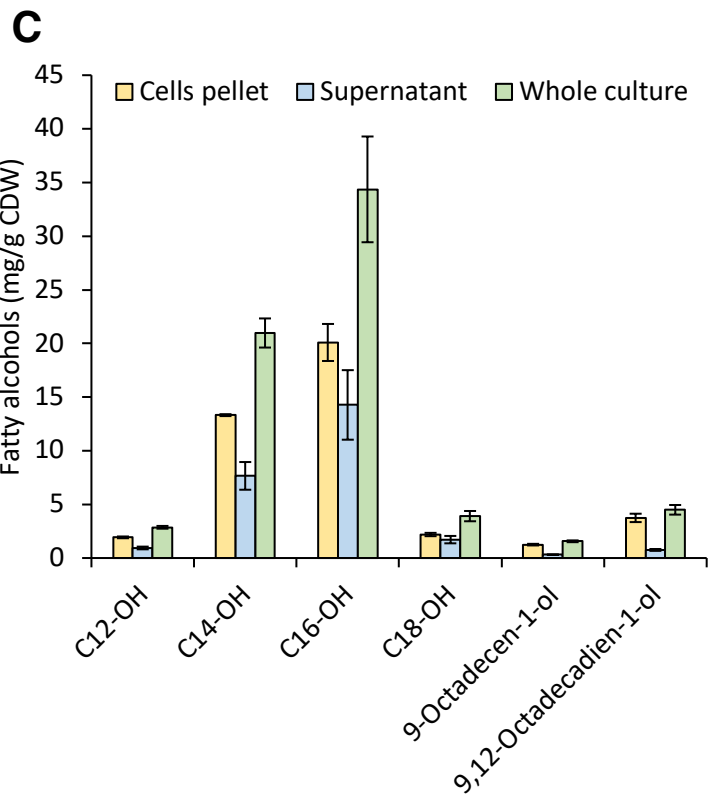
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720

Scale bar 5 μm

A

Δ*aas*-'TesA

Δ*aas*-'TesA-1010-UndB

slr1311 site	Pclac143 promoter	T Terminator
psbA2S promoter	UndB	
'TesA	Ribosome binding site	

B

- Δ*aas*-1010-'TesA
- Δ*aas*-1010-'TesA-UndB

Abundance (10^6)

Retention time (min)

C

Free fatty acids (mg/g CDW)

- C14:0
- C16:0
- C18:0

Δ*aas*-'TesA   Δ*aas*-1010-'TesA-UndB

D

Terminal alkenes (mg/g CDW)

- 1-tridecene
- 1-pentadecene
- 1-heptadecene

Δ*aas*-'TesA   Δ*aas*-1010-'TesA-UndB

n.d.

E

Terminal alkenes (mg/g CDW)

- Supernatant
- Cell pellet
- Whole culture

Δ*aas*-'TesA   Δ*aas*-1010-'TesA-UndB

n.d.

A

Δ*aas*-1010-'TesA

Δ*aas*-1010-TPC2

- ▷ Pclac143 promoter
- 🟧 'TesA
- 🟩 Phosphopantetheinyl transferase (*sfp*)
- 🟦 Carboxylic acid reductase (*car*)
- ◯ Ribosome binding site
- ⊤ Terminator

B

■ Δ*aas*-1010-'TesA
■ Δ*aas*-1010-TPC2

C

☐ Cells pellet ☐ Supernatant ☐ Whole culture

D

☐ C12:0
☐ C14:0
☐ C16:0
☐ C18:0
☐ C18:1
☐ C18:2
☐ C18:3

E

☐ Pellet
☐ Supernatant

F

☐ Tridecane
☐ Pentadecane
☐ Heptadecane

Vector number

Cr2

Cr3

Cr3 + Cr9

Cr8

Cr10

Cr11

7-heptadecene yield (mg/g)

Legend: TesA, mVenus, mRuby2, CTP, Introns, CrFAP

CTP mVenus

TesA

*Cr*FAP   mRuby2

1

A

Δ*aas*-1010-Pcoa-'TesA

Δ*aas*-1010-Pcoa-'TesA-FAP

Δ*aas*-1010-Pcoa-'TesA-'FAP

- ▷ Pcoa promoter
- 🟧 'TesA
- 🟪🟩 FAP with chloroplast transit peptide
- 🟦 FAP lacking chloroplast transit peptide
- ○ Ribosome binding site
- ⊤ Terminator

B

Δ*aas*-1010-Pcoa-'TesA (100 μE)

Δ*aas*-1010-Pcoa-'TesA-'FAP (100 μE)

Δ*aas*-1010-Pcoa-'TesA-'FAP (300 μE)

C

Free fatty acids (mg/g CDW)

C12:0, C14:0, C16:0, C18:0

300 μE

D

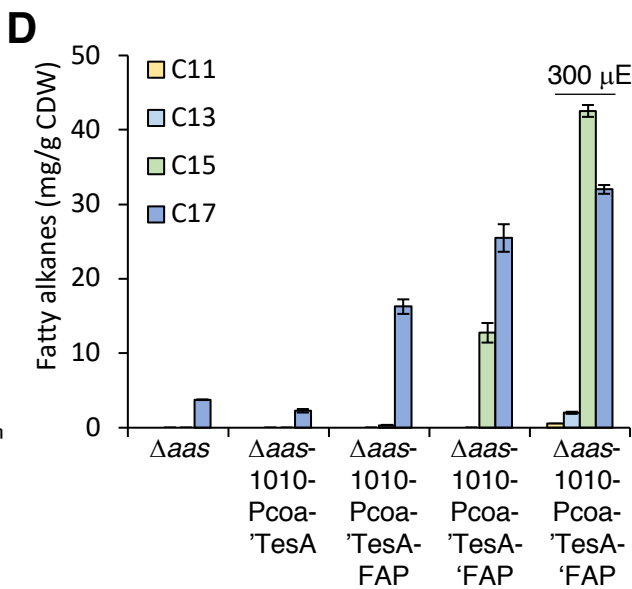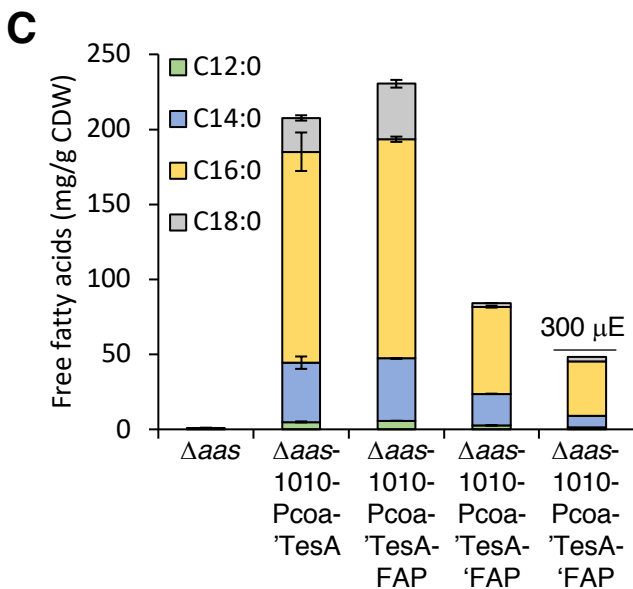Fatty alkanes (mg/g CDW)
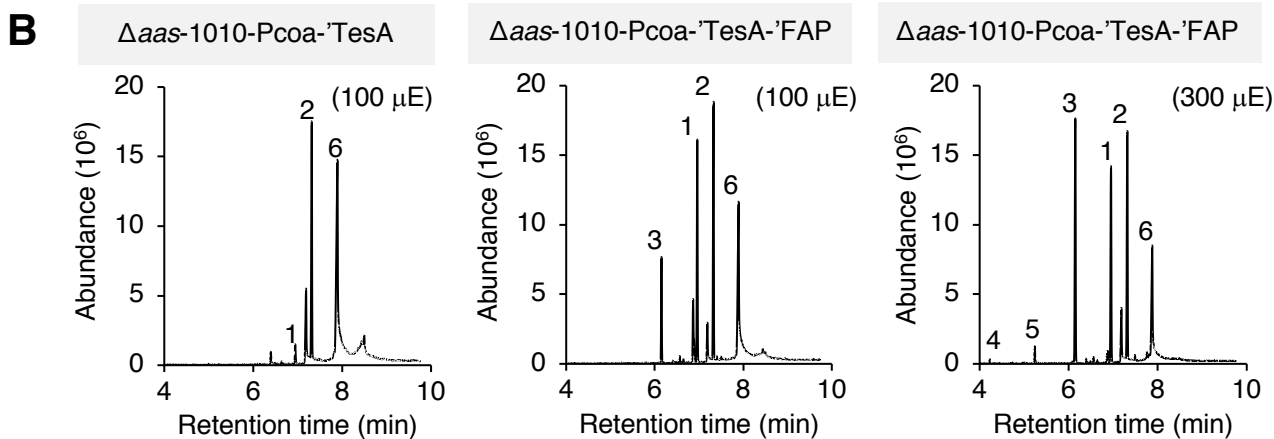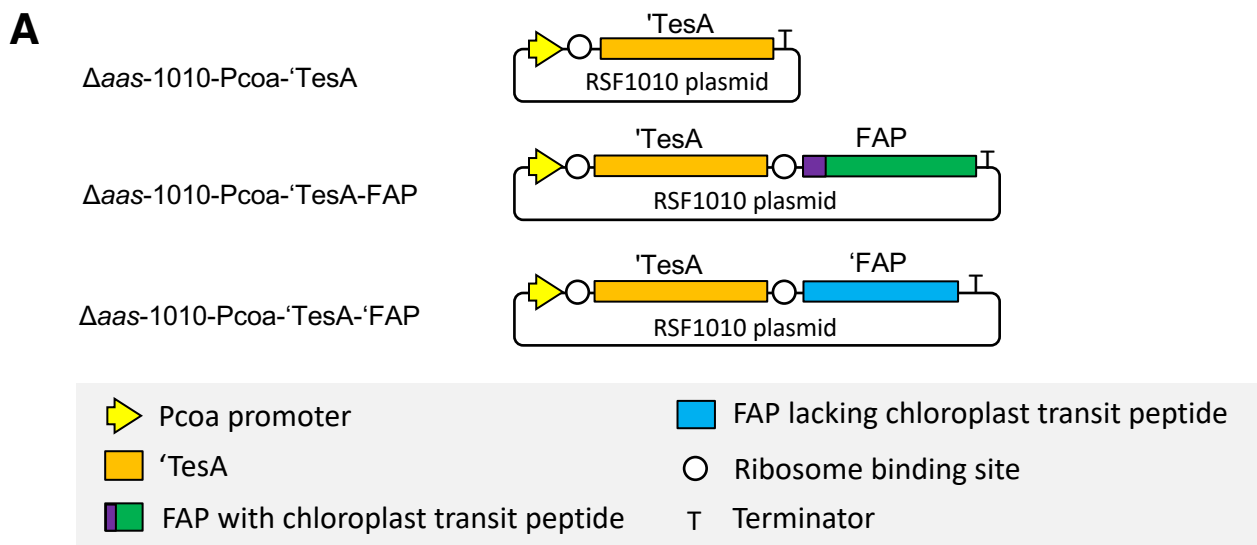
C11, C13, C15, C17

300 μE

Supplementary Information

Synthetic metabolic pathways for photobiological conversion of CO_2 into hydrocarbon fuel

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Supplementary Table 3. All DNA constructs used for engineering *Chlamydomonas* (optimized transgenes, vector backbone, reporters, selection markers, NCBI accession numbers)

Supplementary Figure 1. Growth properties of engineered cyanobacteria strains. (A) The final OD_{730} and biomass accumulation of the engineered strains on day 10 when samples were extracted. (B) Representative growth profile for the first three strains shown in (A).

Supplementary Figure 2. Graphic illustration of all (key) *Chlamydomonas* DNA constructs used in the study

Supplementary Figure 3. The inactivation of *aas* (acyl-ACP synthase).

Supplementary Figure 4. GC-MS chromatogram for 7-heptadecene in native *Chlamydomonas*.

Supplementary Figure 5. Western blot with proteins extracted from engineered *Chlamydomonas* strains. (A) OleTJE strains, (B) CrFAP strains.

Supplementary Figure 6. Fluorescence microscopy with *Chlamydomonas* OleTJE and CrFAP strains.

Supplementary Figure 7. Polar and neutral lipid profiles in TesA-overexpressing *Chlamydomonas* strains.

Supplementary Figure 8. GC-MS chromatograms of suspected alkenes in 6803-FAP strains.

Note that all of the supplementary information is presented in the order of mention in the main text.

Supplementary Table 1. The inventory of materials ((A) plasmids, (B) linkers, (C) primers, (D) DNA parts) used to prepare DNA constructs for the creation of *Synechocystis* strains.

(A) Plasmid DNA used for transformation of cyanobacteria

Assembly	Prefix Linker	Plasmid	Suffix Linker	Plasmid generated	Relevant Information
1	1MP	pIY67	1S	pIY370	pIY370 is a plasmid storage containing rrnB1 T1 terminator and T7Te terminator, gentamicin selection marker, and downstream region of slr1311 site.
	1P	pIY127	2S		
	2P	pIY353	2MS		
	2MP	pIY21	1MS		
2	1MP	pIY370	1S	pIY380	pIY380 is a plasmid used for chromosomal integration targeting the slr1311 site with PpsbA2S as a promoter. This plasmid contains a GFP dropout gene.
	1P	pIY23	2S		
	2P	pIY352	2MS		
	2MP	pIY24	1MS		
3	LRBS1-5P	pIY72	1S	pIY397	pIY397 is used to integrate 'tesA in slr1311 site under PpsbA2S promoter.
	1P	pIY380	LRBS1-5S		
4	1MP	pIY128	1S	pIY140	pIY140 is an RSF1010 overexpression plasmid with Pclac143 promoter and erythromycin as a selection marker. This plasmid contains a GFP dropout gene.
	1P	pIY51	2S		
	2P	pIY102	2MS		
	2MP	pIY24	1MS		
5	1MP	pIY128	1S	pIY417	pIY417 is an RSF1010 overexpression plasmid with Pcoa promoter and erythromycin as a selecton marker. This plasmid contains a GFP dropout gene.
	1P	pIY51	2S		
	2P	pPB345	2MS		
	2MP	pIY24	1MS		
6	LRBS1-5P	pIY72	1S	pIY397	pIY397 is used to integrate 'TesA in slr1311 site under PpsbA2S promoter.
	1P	pIY380	LRBS1-5S		
7	LRBS1-5P	pIY72	1S	pIY196	pIY196 is a 'TesA overexpression plasmid with Pclac143 promoter and erythromycin selection marker.
	1P	pIY140	LRBS1-5S		
8	LRBS1-3P	pIY395	1S	pIY402	pIY402 is an UndB overexpression plasmid with Pclac143 promoter and erythromycin selection marker.
	1P	pIY140	LRBS1-3S		
9	1P	pIY67	2S	pIY608	pIY608 is an empty RSF1010 plasmid with Pclac143 promoter and erythromycin selection marker.
	2P	pIY140	1S		
10	1P	pIY67	2S	pIY606	pIY606 is an empty RSF1010 plasmid with Pcoa promoter and erythromycin selection marker.
	2P	pIY417	1S		
11	LRBS1-4P	pIY619	LRBS2-4S	pIY625	pIY602 is used to overexpress 'TesA and FAP under Pcoa promoter.
	LRBS2-4P	pIY499	1S		
	1P	pIY417	LRBS1-4S		
12	LRBS1-4P	pIY619	LRBS2-4S	pIY630	pIY630 is used to overexpress 'TesA and truncated FAP under Pcoa promoter.
	LRBS2-4P	pIY505	1S		
	1P	pIY417	LRBS1-4S		
13	LRBS1-5P	pIY354	1S	pIY440	pIY440 is used to overexpress 'TesA, Sfp, and CAR under Pclac143 promoter.
	1P	pIY140	LRBS1-5S		
14	LRBS1-4P	pIY619	1S	pIY679	pIY679 is used to overexpress 'TesA under Pcoa promoter.
	1P	pIY417	LRBS1-4S		

(B) DNA linkers used for preparation of constructs used to engineer cyanobacteria

Adapter		Linker		Mixed Prefix Linker (P linker)
Name	Sequence (5' to 3')	Name	Sequence (5' to 3')	
1P-A	TTTATTGAACTA	1P-L	GGACTAGTTCAATAAATACCCTCTGACTGTCTCGGAG	1P
2P-A	TTCTTATTACCT	2P-L	GGACAGGTAATAAGAACTACACGACTGGATACTGACT	2P
3P-A	TGTTATTACAGA	3P-L	GGACTCTGTAATAACAATACCGATAAAGCAACGAGTG	3P
5P-A	TTGATTTATCCT	5P-L	GGACAGGATAAATCAACTCGTAAGCAATACTGTCTGT	5P
LRBS1-5P-A	ATTAGTGGAGGTTA	LRBS1-5P-L	GGACTAACCTCCACTAATTTACAACTGATACTTACCTGA	LRBS1-5P
LRBS1-4P-A	ATCACAAGGAGGTA	LRBS1-4P-L	GGACTACCTCCTTGTGATTTACAACTGATACTTACCTGA	LRBS1-4P
LRBS1-3P-A	AAAGAGGAGAAATA	LRBS1-3P-L	GGACTATTTCTCCTCTTTTTACAACTGATACTTACCTGA	LRBS1-3P
LRBS2-4P-A	ATCACAAGGAGGTA	LRBS2-4P-L	GGACTACCTCCTTGTGATTTTCTGCTACCCTTATCTCAG	LRBS2-4P
1MP-A	TCTGGTGGGT/iMe-dC/TCT	1MP-L	GGACAGAGACCCACCAGATAATAGTGTTTCCACGAAGTG	1MP
2MP-A	AACTTCGGAATC	2MP-L	GGACGATTCCGAAGTTACACCAGATTGGACTGTTATTAC	2MP

Adapter		Linker		Mixed Suffix Linker (S linker)
Name	Sequence (5' to 3')	Name	Sequence (5' to 3')	
1S-A	TGTCGTAAGTAA	1S-L	CTCGTTACTTACGACACTCCGAGACAGTCAGAGGGTA	1S
2S-A	TTTCACACCGAT	2S-L	CTCGATCGGTGTGAAAAGTCAGTATCCAGTCGTGTAG	2S
3S-A	TAGTGCCGTGAT	3S-L	CTCGATCACGGCACTACACTCGTTGCTTTATCGGTAT	3S
5S-A	GGCACTACTTCT	5S-L	CTCGAGAAGTAGTGCCACAGACAGTATTGCTTACGAG	5S
LRBS1-5S-A	GACGGTGTTCAA	LRBS1-5S-L	CTCGTTGAACACCGTCTCAGGTAAGTATCAGTTGTAA	LRBS1-5S
LRBS1-4S-A	GACGGTGTTCAA	LRBS1-4S-L	CTCGTTGAACACCGTCTCAGGTAAGTATCAGTTGTAA	LRBS1-4S
LRBS1-3S-A	GACGGTGTTCAA	LRBS1-3S-L	CTCGTTGAACACCGTCTCAGGTAAGTATCAGTTGTAA	LRBS1-3S
LRBS2-4S-A	CCAATAGTAACA	LRBS2-4S-L	CTCGTGTTACTATTGGCTGAGATAAGGGTAGCAGAAA	LRBS2-4S
1MS-A	CGAGTTCTTACC	1MS-L	CTCGGGTAAGAACTCGCACTTCGTGGAAACACTATTA	1MS
2MS-A	CGATAGGT/iMe-dC/TCC	2MS-L	nTATCGGTAATAACAGTCCAATCTGGTGT	2MS

(C) DNA primers used for preparation of constructs used to engineer cyanobacteria

Primer	Sequence (5' to 3')
IY107	TCTGGTGGGTCTCTGTCCTTTACAGCTAGCTCAGTCCTAG
IY108	CGATAGGTCTCCCGAGCCTGTGTGAAATTGTTATCCGCTCAC
IY113	GCACCAAGTACGGGAATTGGATTCGGTG
IY114	CCGAGGTAATGGAGAGGACTAAGCACCAAG
IY145	TCTGGTGGGTCTCTGTCCCCAGGCATCAAATAAAACGAAAGGCTC
IY146	CGATAGGTCTCCCGAGCCCGGCCGGCAGGAGCAGAAGAGCATAC
IY155	CGATAGGTCTCCCGAGCCGTGGCAGCAGCCTAGGGAATTCTTACAGC
IY211	TCTGGTGGGTCTCTGTCCGGCTTGATAGAACCTTACTTGGATGCC
IY212	CGATAGGTCTCCCGAGCCAACTGACTAAACTTAGTCTAAAGGATTAATGAG
IY213	TCTGGTGGGTCTCTGTCCTTCCTTGGTGTAATGCCAACTGAATAATCTG
IY214	CGATAGGTCTCCCGAGCCGGCAATCGAGACAATTATTTTCCTCTAGACG
IY238	TCTGGTGGGTCTCTGTCCATGGCGGACACGTTATTGATTCTGG
IY258	TCTGGTGGGTCTCTGTCCATGGCGTCTGCTGTAGAAGATATTCGCAAAG
IY259	CGATAGGTCTCCCGAGCCTTACG
PB208	TCTGGTGGGTCTCTGTCCCTGCACTAAAGACAAGTGAG
PB209	CGATAGGTCTCCCGAGCCGCTTTTTAACTTGGATTTTTACC
PB78	TCTGGTGGGTCTCTGTCCAAGAGGAGAAAGGTACCATGGCGGACACGTTATTGATTCTGG
PB79	CGATAGGTCTCCCGAGCCTTATGAGTCATGATTTACTAAAGGCTGCAACTGC

(D) DNA parts used for preparation of constructs used to engineer cyanobacteria. All DNA parts were amplified from DNA templates using primers listed above (C) and stored in pJET1.2 blunt (Thermo Fischer).

Template	Primer		Plasmid Generated	Plasmid Contained	Relevant information
	F	R			
pDF-lac[a]	IY145	IY146	pIY128	pJET-RSF1010	A plasmid storage containing a broad-host-range vector RSF1010 backbone
-	-	-	pIY127	pJET-Gm	A plasmid storage containing gentamicin cassette. A gift from Dr. Geoff Baldwin (Imperial College)
pPB223	IY107	IY108	pIY102	pJET-lacI-Pclac143	A plasmid storage containing a lac repressor and Pclac143 promoter
-	-	-	pIY24	pJET-GFP	A plasmid storage containing a superfold green fluorescence protein. GFP was ordered as a gBlock.
pET-TPC2[b]	PB78	PB79	pIY72	pJET-'TesA	A plasmid storage containing 'TesA
pET-TPC2[b]	PB78	IY155	pIY354	pJET-TPC2	A plasmid storage containing 'TesA, Sfp, and CAR.
-	-	-	pIY51	pJET-Ery	A plasmid storage containing erythromycin cassette. Erythromycin cassette was ordered as a gBlock.
6803-WT genomic DNA	IY211	IY212	pIY352	pJET-slr1311_up	A plasmid storage containing the upstream region of slr1311.
6803-WT genomic DNA	IY213	IY214	pIY353	pJET-slr1311_down	A plasmid storage containing the downstream region of slr1311.
6803-WT genomic DNA	PB208	PB209	pPB345	pJET-Pcoa	A plasmid storage containing CoaR and Pcoa promoter.
-	-	-	pIY395	pJET-UndB	A plasmid storage containing UndB. UndB was ordered as a gBlock from IDT DNA.
-	-	-	pIY499	pJET-FAP	A plasmid storage containing FAP. FAP was ordered as a gBlock from IDT DNA.
pIY499	IY258	IY259	pIY505	pJET-'FAP	A plasmid storage containing truncated FAP.
-	-	-	pIY21	pMB1-Kan	A plasmid backbone harbouring ColE1 ori and a kanamycin selection marker.
-	-	-	pIY23	pMB1-Amp	A plasmid backbone harbouring ColE1 ori and a ampicillin selection marker.
-	-	-	pIY67	pJET-termB15	A plasmid storage containing rrnB T1 terminator and T7Te terminator.
pIY72	IY238	PB79	pIY619	pJET-'TesA	A plasmid storage containing 'TesA.

[a]Guerrero et al., *Plos ONE*, 2012
[b]Akhtar et al., *PNAS*, 2013

Supplementary Table 2. All *Synechocystis sp*. PCC 6803 strains used in the study.

Strain	Relevant information
WT	*Synechocystis* sp. PCC 6803 wild-type strain obtained from Prof. Klaas Hellingwerf (Univ of Amsterdam)
Δ*aas*	*Synechocystis* sp. PCC 6803 acyl-ACP synthetase deletion strain.
Δ*aas*-'TesA	Δ*aas* harbouring chromosomal integration of 'tesA under PpsbA2S promoter. This strain was obtained by transforming plasmid pIY397.
Δ*aas*-'TesA-1010-UndB	Δ*aas*-'TesA carrying UndB overexpression RSF1010 plasmid pIY402.
Δ*aas*-1010-'TesA	Δ*aas* carrying 'TesA overexpression RSF1010 plasmid pIY196 using a Pclac143 promoter
Δ*aas*-1010-TPC2	Δ*aas* carrying 'TesA, Sfp, and CAR overexpression RSF1010 plasmid pIY440 using a Pclac143 promoter
Δ*aas*-1010-Pcoa-'TesA	Δ*aas* carrying 'TesA overexpression RSF1010 plasmid pIY679 using a Pcoa promoter
Δ*aas*-1010-Pcoa-'TesA-FAP	Δ*aas* carrying 'TesA and FAP overexpression RSF1010 plasmid pIY625 using a Pcoa promoter
Δ*aas*-1010-Pcoa-'TesA-'FAP	Δ*aas* carrying 'TesA and truncated FAP overexpression RSF1010 plasmid pIY630 using a Pcoa promoter

Supplementary Table 3. All constructs used for engineering *Chlamydomonas*.

Vector number in manuscript	Overexpression plasmids	Resistance in Cr	Proteins produced	StrepII tag y/n?	Gene length with introns	CDS	RBCS2 intron 1 copies in complete cassette	Protein size (aa)	Predicted molecular mass	Reference
Cr1	pOpt2_mVenus_Paro	paromomycin	mVenus (YFP)	y	1287	813	1	270	30.6	Wichmann et al. 2018
Cr2	pOpt2_PsaD_mVenus_Paro	paromomycin	PsaD_YFP	y	1395	921	1	306	34.3	Lauersen et al. 2016
Cr3	pOpt2_PsaD_TesA_mVenus_Paro	paromomycin	PsaD_TesA_YFP	y	2107	1448	2	495	55.3	This work
Cr4	pOpt2_PsaD_OleTJE_mVenus_Paro	paromomycin	PsaD_OleTJE_YFP	y	3090	2181	4	726	82.2	This work
Cr5	pOpt2_PsaD_OleTJE_RhFRED_mVenus_Paro	paromomycin	PsaD_OleTJE_RhFRED_YFP	y	4527	3183	7	1060	118.3	This work
Cr6	pOpt2_PsaD_RhFRED_OleTJE_mVenus_Paro	paromomycin	PsaD_RhFRED_OleTJE_YFP	y	4527	3183	7	1060	118.3	This work
Cr7	pOpt2_*CrFAP_mVenus_Paro	paromomycin	CrFAP_YFP	y	3694	2640	5	879	94.8	This work
Cr8	pOpt2_PsaD_CrFAP_mVenus_Paro	paromomycin	PsaD_CrFAP_YFP	y	3709	2655	5	884	95.3	This work
Cr9	pOpt2_PsaD_CrFAP_mRuby2_Ble	zeocin	PsaD_CrFAP_RFP	y	3703	2649	5	882	94.9	This work
Cr10	pOpt2_PsaD_TesA_CrFAP_mVenus_Paro	paromomycin	PsaD_TesA_CrFAP_YFP	y	4427	3228	6	1075	116.6	This work
Cr11	pOpt2_PsaD_CrFAP_TesA_mVenus_Paro	paromomycin	PsaD_CrFAP_TesA_YFP	y	4427	3228	6	1075	116.6	This work
	Genes synthesized for Cr expression in this work		**NCBI accession number for Cr optimized transgene**							
	CrcoTesA	-	TesA	MH004289	712	573	1	193	21.4	Cho and Cronan, 1995
	CrcoOleTJE	-	OleTJE	MH248837	1707	1272	3	428	48.4	Rude et al. 2011
	CrcoRhFRED	-	RhFRED	MH248838	1431	996	3	336	36.2	Liu et al. 2014
	CrcoCrFAP	-	CrFAP	MH248839	2416	1836	4	612	64.5	Soriqué et al. 2017

Notes

*Native CrFAP secretion signal

Synthesized gene sizes include any linkers designed into the genes without restriction sites

Predicted molecular weights contain targeting peptides and the StrepII tag, actual sizes of mature proteins may be variable in the algal chloroplast

The HSP70-RBCS2-i1 promoter contains an additional copy of the 145 bp rbcs2 intron 1 sequence which is added to the intron 1 copy number column for plasmids

Wichmann J., Baier T., Wentnagel E., Lauersen K. J., Kruse O. 2018. Tailored carbon partitioning for phototrophic production of (E)-α-bisabolene from the green microalga Chlamydomonas reinhardtii. Metabolic Engineering 45: 211-222.

Lauersen K. J., Baier T., Wichmann J., Wördenweber R., Hübner W., Huser T., Kruse O. 2016. Efficient phototrophic production of a high-value sesquiterpenoid from the eukaryotic microalga Chlamydomonas reinhardtii. Metabolic Engineering 38: 331-343.

Supplementary Figure 1. Growth properties of engineered cyanobacteria strains. (A) The final OD_{730} and biomass accumulation of the engineered strains on day 10 when samples were extracted. (B) Representative growth profile for the first three strains shown in (A).
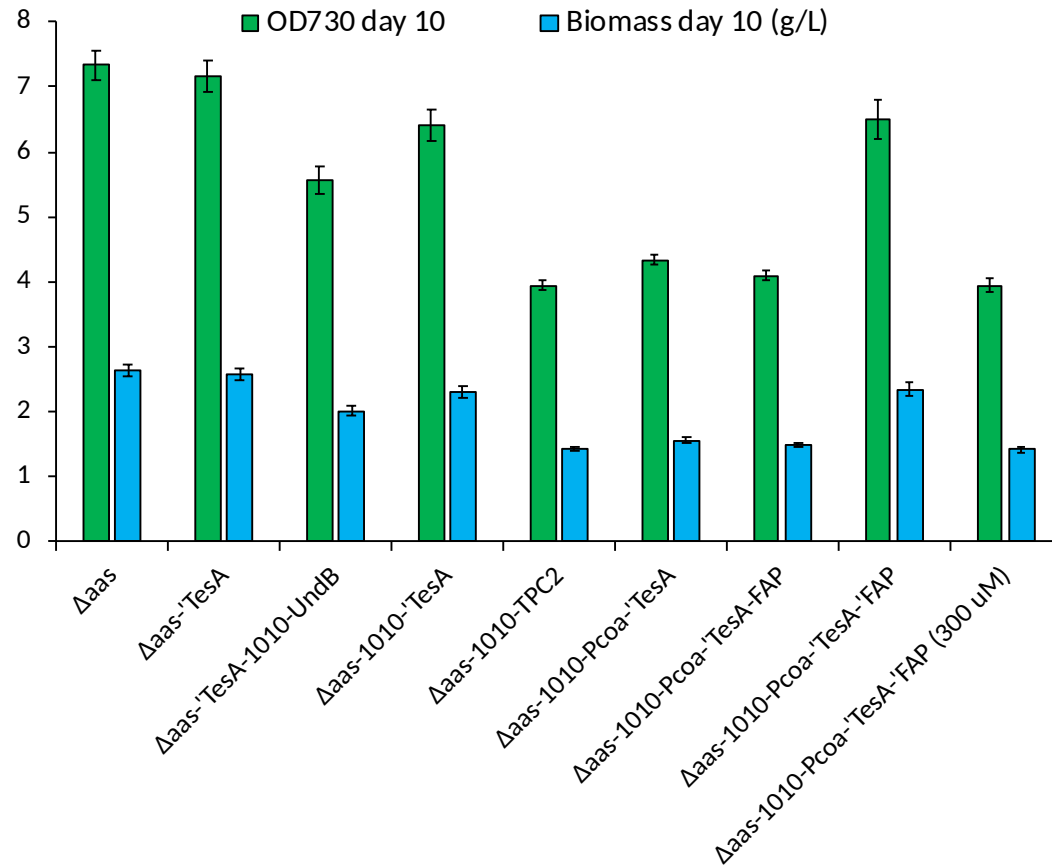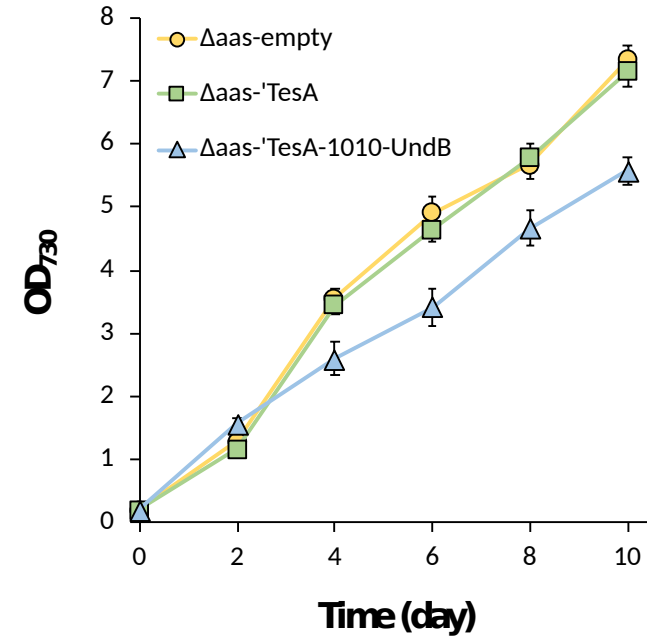
Supplementary Figure 2. Graphic illustration of all (key) *Chlamydomonas* DNA constructs used in the study. Vector constructs for nuclear transgene expression from *C. reinhardtii*. The base pOpt2_YFP_Paro vector (Wichmann et al 2018) was used to construct chloroplast targeted protein products using the photosystem I reaction center subunit II (PsaD) chloroplast targeting peptide (CTP). All other constructs were fused to create protein products with C-terminal mVenus (YFP) or mRuby2 (RFP) reporters. Nuclear expression constructs all contain the rbcs2 i1 intron in copies spread throughout their sequence (blue boxes). The fluorescent reporters contain rbcs2 i2 as previously described (Lauersen et al 2015). *E. coli* thioesterase A' (TesA), *Jeotgalicoccus* sp. terminal olefin-forming fatty acid decarboxylase (OleTJE) and its fusion to *Rhodococcus* sp. P450 reductase RhFRED. *C. reinhardtii* native fatty acid photodecarboxylase (*Cr*FAP) was synthetized with its own native CTP (*), but was also cloned with the PsaD CTP instead and expressed as YFP or RFP fusions, as well as in fusion with the TesA construct mediated by the modular nature of the pOpt2 vectors. All vectors use the common HSP70-RBCS2-i1 fusion promoter and corresponding 3' UTR and either paromomycin (Paro) or zeocin/bleomycin (Ble) resistance cassettes from the pOpt2 vectors (Wichmann et al. 2018). * indicates site of additional glycine to add *Bam*HI site and allow cloning removal of the native chloroplast targeting peptide and replacement with the PsaD CTP. Paro – all vectors confer paromomycin resistance except the single zeocin (Ble) resistance vector. The vector numbers are shown to the right.
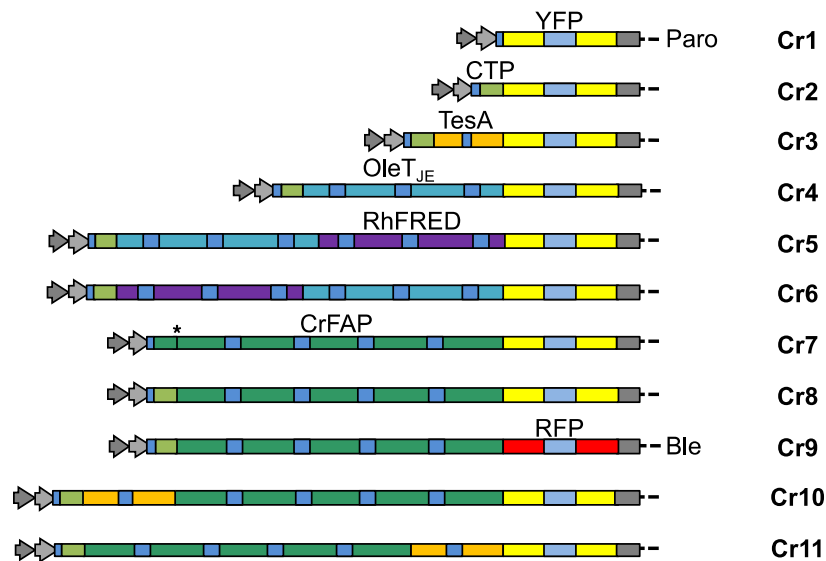
Supplementary Figure 3. Inactivation of *aas*. (A) Schematic diagram of acyl-ACP synthetase (encoded by slr1609) knockout. (B) Colony PCR analysis of Δ*aas* and the wild-type strain with positive and negative controls using primers IY113 and IY114. (C) Image of the wild-type (left) and Δ*aas* (right) strains after 10 days of cultivation. Circle highlight visible precipitates forming in the Δ*aas* culture. Representative chromatograms from extracted (D) Δ*aas* and (E) wild-type cultures. C15:0 pentadecanoic acid was used as internal standard.
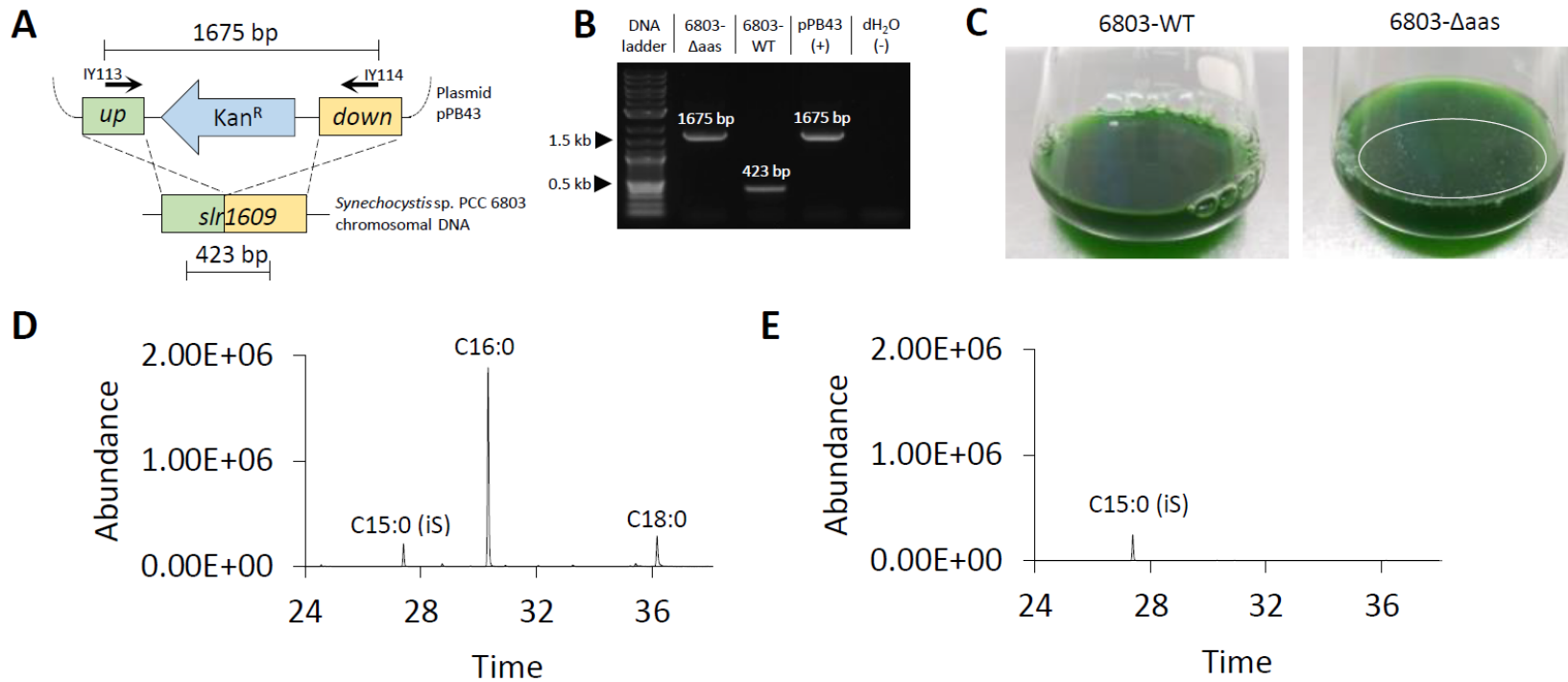
Supplementary Figure 4. Polar and neutral lipid profiles in the wild-type control (UVM4) and TesA-overexpressing *Chlamydomonas* strains under nitrogen replete (+N) and deficient (-N) conditions. Extraction of total lipids, separation into neutral and polar lipids, transesterification and GC-MS analysis were performed to determine relative lipid profiles for each strain under each condition. The left section of the graph displays quantities for C8-C12 FAMEs only, using a different axis.

**(A) Polar lipds.**
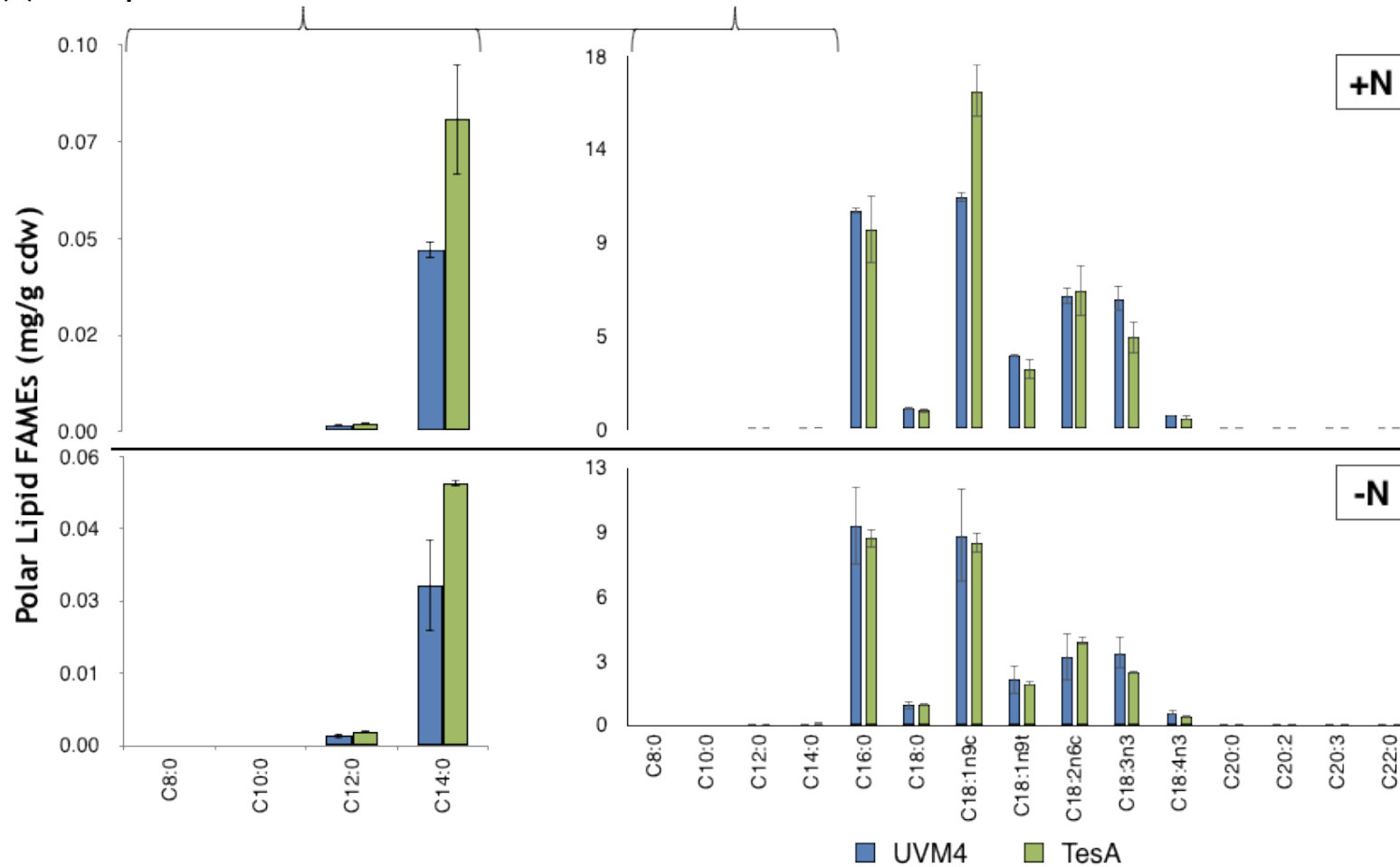
(B) Neutral lipds.
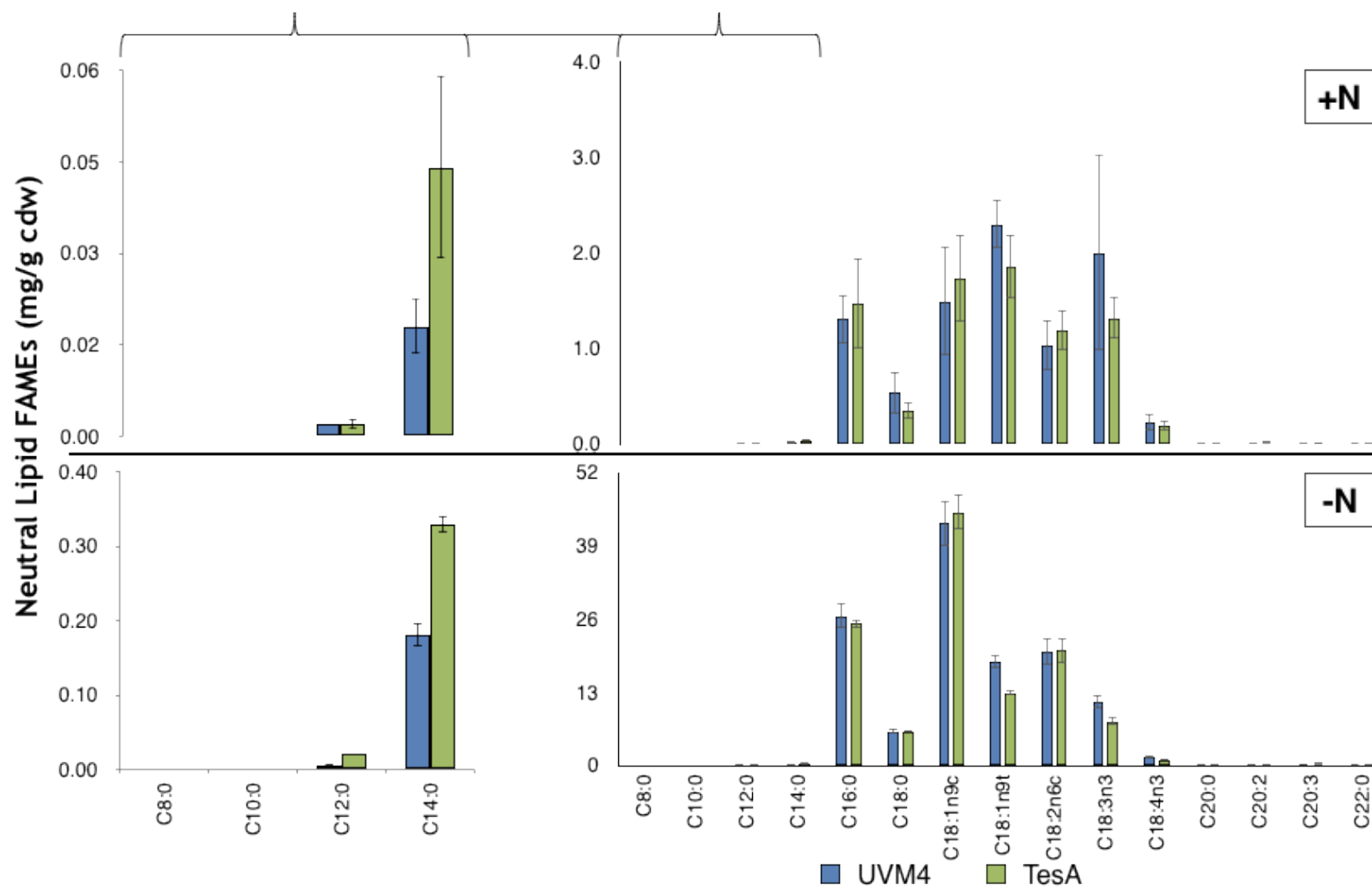
Supplementary Figure 5. Western blot to determine full-length fusion protein expression from the nuclear genome of *C. reinhardtii*.

(A) OleTJE strains. Proteins were extracted from 2 day old cultures in logarithmic phase. An anti-GFP HRP linked polyclonal antibody (Thermo Fisher Scientific) was used for detection of YFP-fused proteins on nitrocellulose membranes. Ponceau S staining of the membrane is shown as a protein loading control. M – PageRuler Prestained protein ladder (Thermo). P – UVM4 parental strain.
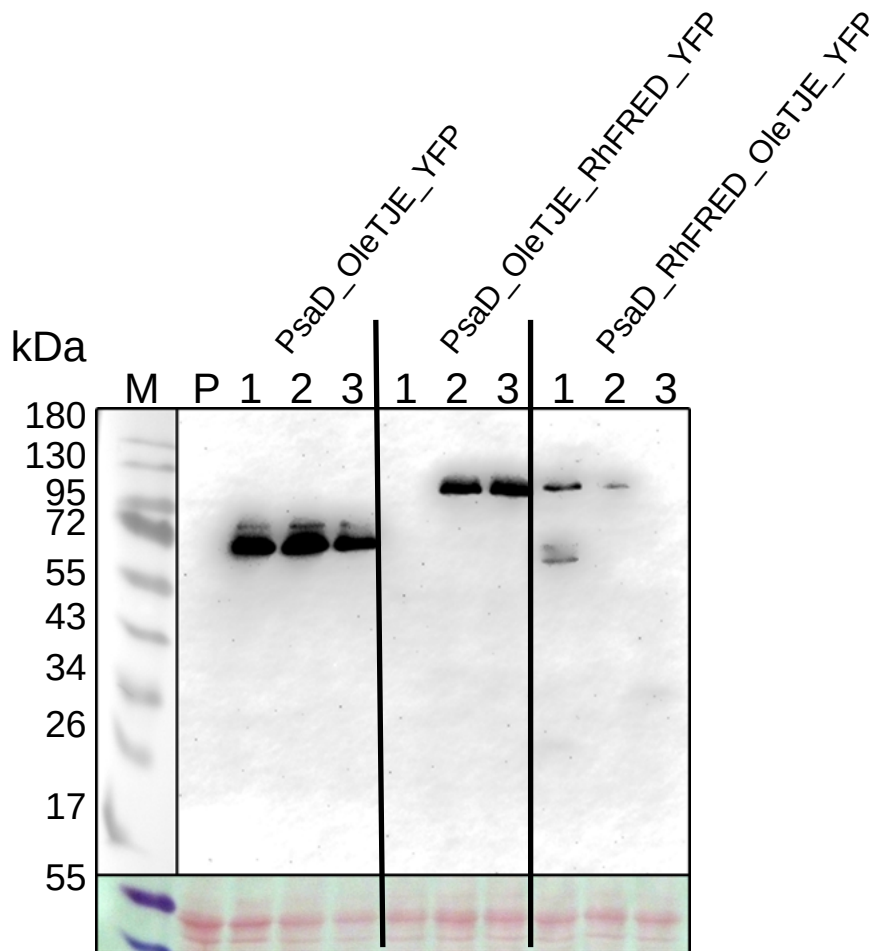

a-GFP antibody whole cell lysates
*native CrFAP CTP

(B) CrFAP and CrFAP-TesA fusion expressing strains. Same methods as above. * indicates native CrFAP chloroplast targeting peptide construct (vector Cr7).
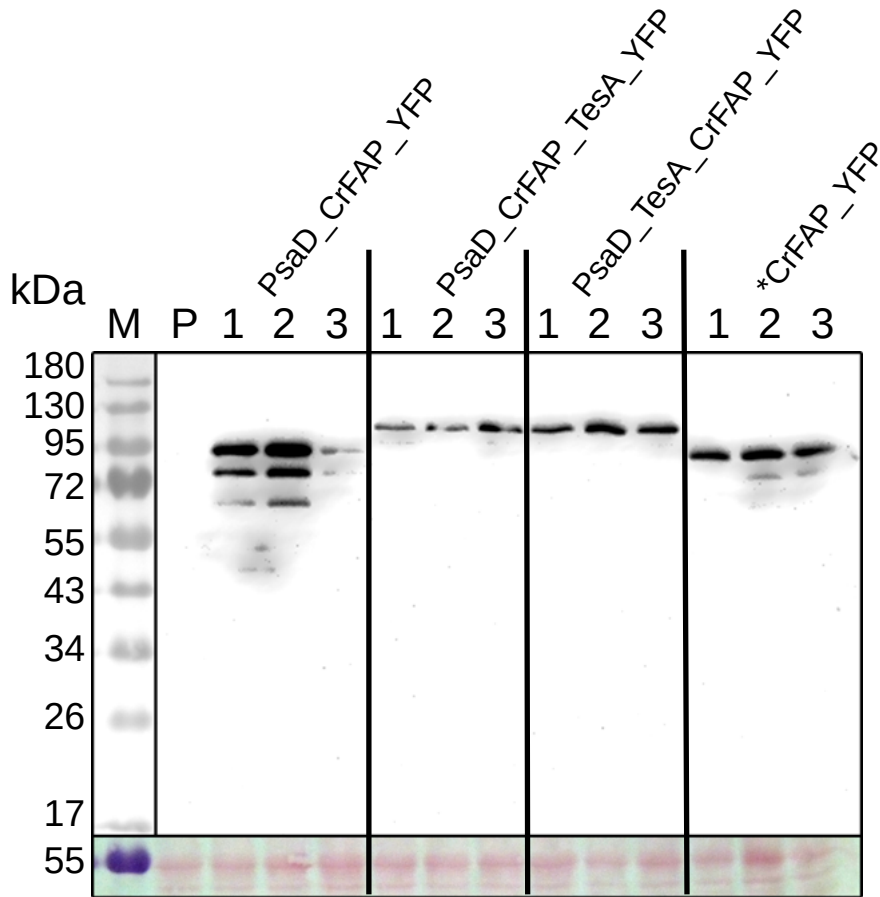
Supplementary Figure 6. Fluorescence microscopy with *Chlamydomonas* strains expressing various OleT and CrFAP constructs. Wide-field fluorescence microscopy was used to confirm chloroplast localization of YFP-linked constructs as previously described (Lauersen et al 2016). The over-expressed proteins, from vectors listed in Supp. Table 3 (above), in each strain is listed on the right, and the vector numbers are shown on the left.
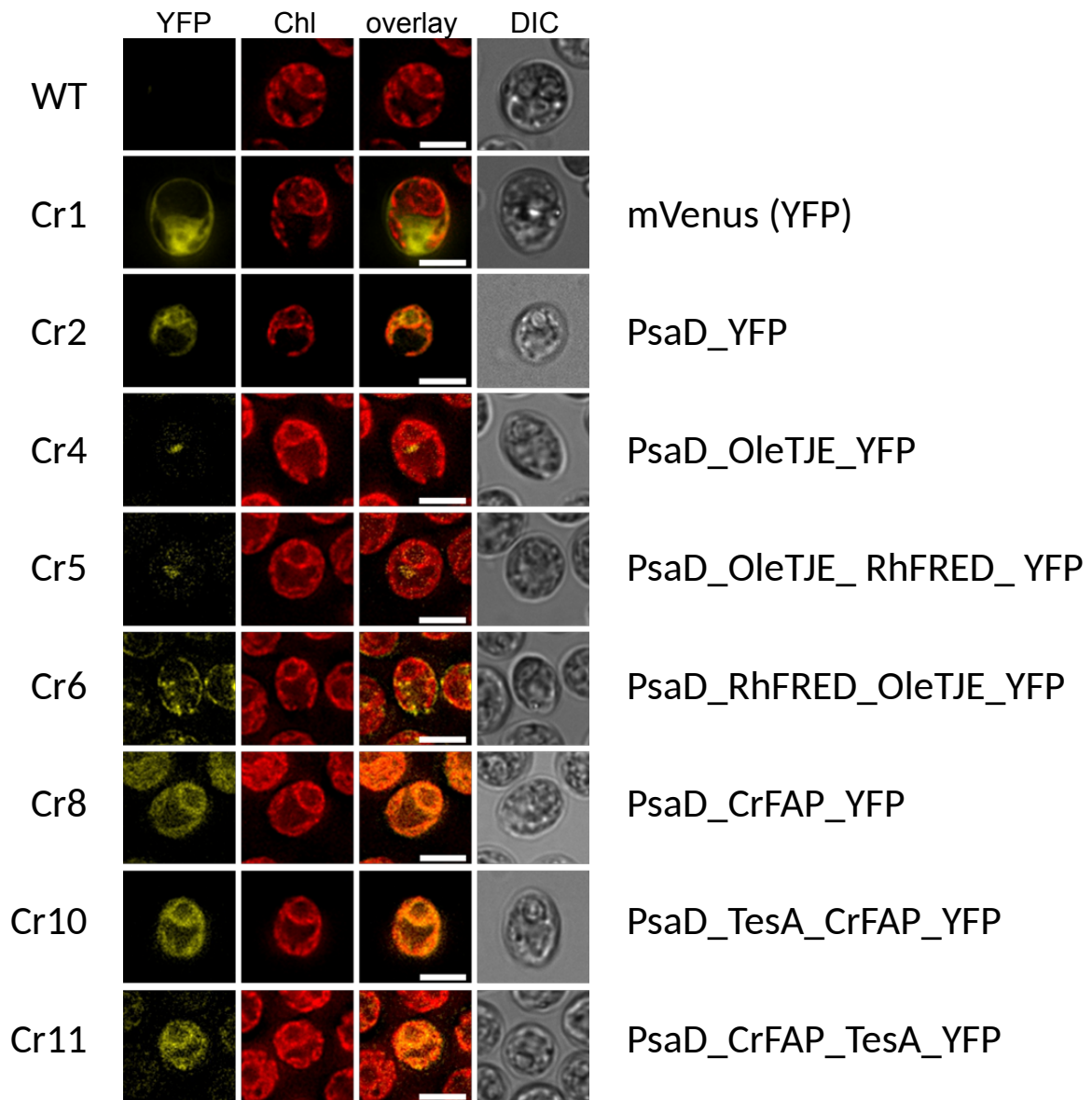
Supplementary Figure 7. GC-MS chromatogram for 7-heptadecene in wild-type *Chlamydomonas.* Identification of 7-heptadecene (7-h) and product partitioning between cells, culture medium (supernatant), and a dodecane overlay. **A** *Chlamydomonas reinhardtii* strain UVM4 was cultivated in TAP medium for 5 d in constant light with and without an overlay of 5% dodecane. (A) Only traces of the product were detectible in the overlay, and the major fraction was found in the cell pellet (middle panel and C). α-humulene served as internal standard (IS). 7-heptadecene was detected at earlier retention times than the commercial 1-heptadecene (1-h) standard (lower panel), however, it showed (B) similar mass fractionation pattern. (C) Presence (+d) or absence (-d) of the dodecane overlay did not affect total product yields. Error bars represent 95% confidence intervals of strains cultivated in biological triplicate.
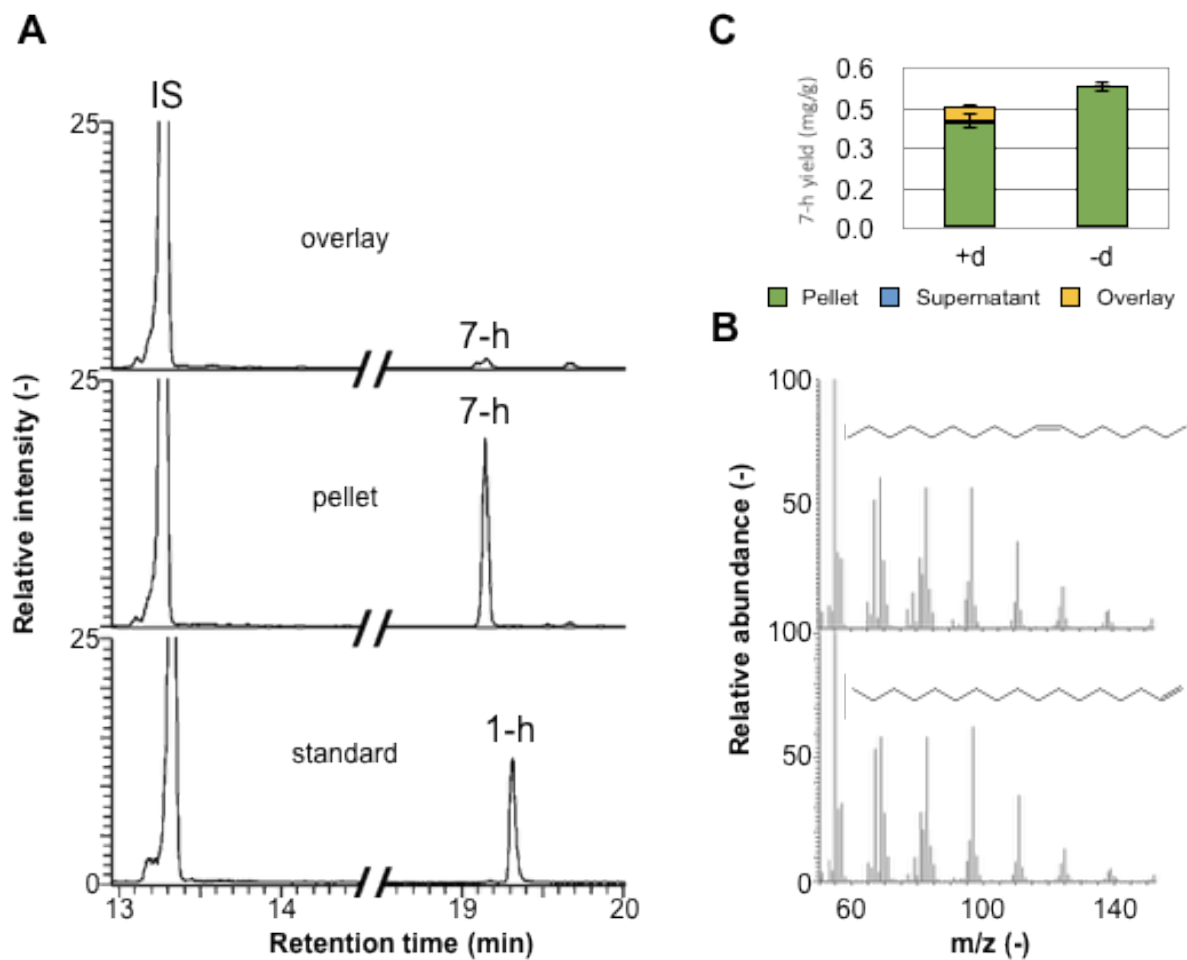
Supplementary Figure 8. Unidentified alkenes accumulating in the 6803-Δ*aas*-'TesA-FAP strain. Two unique (relative to wild-type) products that accumulated in the 6803-Δ*aas*-'TesA-'FAP strain are most likely also alkenes based on NIST mass spectrometry (2.2) analysis of individual peaks eluting at 6.86 ((A) 6,9-heptadecadiene) and 6.88 minutes ((B) 8-heptadecene), respectively (indicated by the arrow).