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Metabolic phenotyping of the cyanobacterium *Synechocystis* 6803 engineered for production of alkanes and free fatty acids

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h i g h l i g h t s

- ***Synechocystis* 6803 was engineered for enhanced photosynthetic conversion of CO₂ to alkanes.
- ***Synechocystis* 6803 was engineered for accumulation of free fatty acids.
- **Single-cell metabolic phenotyping was performed using SR-FTIR spectromicroscopy.
- **Multivariate analysis of SR-FTIR data revealed biochemical shifts in engineered cells.
- **SR-FTIR spectromicroscopy provides a high-throughput tool for screening engineered cells.

a b s t r a c t

Keywords:
Alkanes
Cyanobacteria
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FTIR
Metabolic engineering
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We demonstrate a simple high-throughput single-cell approach that exploits the ultrahigh brightness and non-invasive nature of synchrotron infrared beam to characterize strains of the cyanobacterium *Synechocystis* 6803 (*S. 6803*) constructed with altered metabolic traits affecting the acyl-CoA pool. Their metabolic responses to the modified traits were phenotyped by single-cell synchrotron radiation Fourier transform infrared (SR-FTIR) spectromicroscopy and multivariate analysis. SR-FTIR difference spectra and cluster vector plots segregated the strains as phenotypic populations based on signals in the hydrocarbon and biomolecular fingerprint regions, although each population incorporated a stochastic distribution of cells with different metabolic properties. All engineered strains exhibited an increase in FTIR features attributed to functional groups in hydrocarbon, fatty acid (FA), and/or FA ester chains, and a decrease in polysaccharide features. The metabolic signatures obtained by SR-FTIR were consistent with detailed qualitative and quantitative metabolic information provided in GC/MS/NMR data. A strain with extra copies of the FAR and FAD genes, encoding, respectively, the fatty acyl-ACP reductase and fatty aldehyde decarboxylase enzymes in the alkane biosynthesis pathway, showed up to a fivefold increase in the intracellular levels of heptadecane, a threefold increase in 9-heptadecene, and a significant increase in secreted 16:0 and 18:0 free FAs (FFAs). Inactivation of the AAS gene, encoding acyl-ACP synthetase, prevented re-thioesterification of FFAs generated from membrane lipid recycling and led to elevated levels and of intracellular FFAs of an altered composition, and a decrease in heptadecane and secreted FFAs. Introduction of a FatB gene, encoding a thioesterase (TE), which catalyzes the liberation of FFAs from acyl-ACP, yielded little effect in itself. However, the activity of the TE enzyme was clearly manifested in combination with AAS inactivation; A TE-containing strain lacking AAS showed a dramatic (30-fold) increase in intracellular FFAs (with the majority being 16:0) and increases in heptadecane and secreted FFAs.

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1. Introduction

Cyanobacteria, like algae and plants can use solar power to capture CO₂ via the Calvin–Benson–Bassham (CBB) cycle and convert it to a suite of organic compounds.

As opposed to microalgae that can accumulate large amounts of triacylglycerols (TAGs) as storage lipids, the cyanobacteria studied to date produce little or no TAGs, but their FAs are directly shuttled to membrane lipid synthesis. Conversely, cyanobacteria, which are Gram-negative bacteria, are well suited for synthetic biology and metabolic engineering approaches aimed at redirecting carbon flux in lipid metabolism to specific biofuel molecules, including ethanol, butanol, biodiesel, and hydrocarbon biofuels. First, whereas in plants and algae, including microalgae, lipid metabolism involves several different cellular compartments, in cyanobacteria, lipid metabolism occurs via soluble or membrane-bound enzymes in the cytosol. Second, being bacteria, cyanobacteria are amenable to homologous recombination, which allows rapid site-directed mutagenesis, gene insertions, replacements, and deletions in a precise, targeted and predictable manner [1–6]. Phototrophic biosynthesis of high-density liquid biofuels in cyanobacteria could augment microbial production of biodiesel and hydrocarbons in heterotrophic bacteria such as *Escherichia coli*.

Fatty acid (FA) biosynthesis in bacteria is accomplished by a type II FA synthase (FASII), a multienzyme system utilizing a freely dissociable acyl carrier protein ACP [2,7]. The products of FASII are released as acyl-ACPs and may be directly incorporated into membrane lipids by two acyltransferases, glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), and 1-Acylglycerol-3-phosphate acyltransferase (AGPAT; EC 2.3.1.51) that each attaches a FA to the glycerol 3-phosphate (G3P) backbone to form the key intermediate, phosphatidic acid (PA) [8,9]. In plants and algae, *de novo* FA synthesis occurs in the plastids, which also exhibit the FASII machinery. In the plastids, acyl-ACPs are hydrolyzed by Acyl-ACP thioesterases (TE; EC 3.1.2.14, e.g. FatB in *Arabidopsis*) to yield free FAs for transport across the plastid envelope. Upon arrival at the outer plastid surface, the free FAs are re-activated by Acyl-CoA synthetase (FadD; EC 6.2.1.3) to form acyl-CoA. Acyl-CoA is the starting substrate for synthesis of triacylglycerides (TAGs), but can also be used for β -oxidation and for biosynthesis of membrane lipids. Cyanobacteria like many other bacteria lack TE enzymes that act on FA-ACPs, and formation of free FAs mainly occurs during recycling of membrane lipids or degradation of acylated proteins [10].

The fatty acyl-ACP product from FA synthesis is a crossroad metabolite for the potential photosynthetic production of lipid-based biofuels in cyanobacteria (Fig. 1). As previously demonstrated [4], by the introduction of TE genes acyl-ACP can be hydrolyzed to free FAs (FFAs) for downstream chemical processing to fuels. As has been shown for *E. coli* [11], the possibility also exists to convert FFAs directly to FA methyl esters (FAMES), i.e., biodiesel, *in vivo* by endowing cyanobacteria with genes for a suitable FA methyltransferase (FAMTase). Further, acyl-ACP in cyanobacteria is the substrate for alkane biosynthesis in a two-step reaction involving the enzymes fatty acyl-ACP reductase (FAR) and fatty aldehyde decarbonylase (FAD) [2,3,12] (Fig. 1).

The purpose of the work described in this paper was to demonstrate the simple single-cell synchrotron radiation SR-FTIR spectromicroscopy approach with multivariate analysis as a high-throughput diagnostic tool to study metabolic shifts in the cyanobacterium *Synechocystis* 6803 (*S. 6803*) engineered for increased accumulation of FFAs and alkanes. Because biomolecules interact non-destructively with infrared in the mid-infrared region (\sim 2.5–12.5 μ m wavelength, or \sim 4000–800 cm⁻¹ wavenumber) [13], and because mid-infrared photons emitted from a synchrotron source can easily be focused onto a measurement area with a micrometer

accuracy [14], we have developed SR-FTIR spectromicroscopy to study the chemistry changes in single or in small groups of several individual live microbial cells in real time [14,15]. To overcome the challenge of understanding a large and complex data set from the intricate biological systems, such as relating the functional metabolism features to the vibrational frequencies (wavenumbers) of the molecules, we used multivariate approaches including principal component analysis (PCA) and linear discriminant analysis (LDA). These data reduction methodologies allow identification of both biochemical distinctions and heterogeneity within cellular systems, which can then be related to discriminating vibrational frequencies in the infrared spectra [16]. The amount and detailed composition of FFAs, alkanes and other metabolites in the strains were determined by GC/MS and NMR spectroscopy, which also confirmed the validity of the SR-FTIR approach.

2. Material and methods

2.1. Chemicals and reagents

Chemicals, kits, primers, and reagents were from common commercial vendors.

2.2. Cyanobacterial strains, growth conditions, and transformation

S. 6803 was obtained from The Pasteur Culture Collection (PCC; <http://www.pasteur.fr/ip/easysite/go/03b-000012-00g/collection-of-cyanobacteria-pcc>). Cells were grown on solid or liquid BG11 media as described [17], with the exceptions that liquid cultures were grown in a shaking incubator at ambient CO₂ concentrations and antibiotics were added at the concentrations of 25 μ g·ml⁻¹ for kanamycin (km) and 120 μ g·ml⁻¹ for spectinomycin (Spc). Under these, non-CO₂-enriched conditions, the mean generation time was 32, 49, 45, 33, and 61 h for the F0, F3, F16, F3;16, and F30 strains, respectively.

Transformation of *S. 6803* cells and propagation of transformants on selective media were as described [17].

The concentration of assayed metabolites is expressed in % DW. Conversion between OD₇₅₀ and cell DW was performed by regression analysis (Fig. S3).

2.3. Plasmids and gene constructs

Plasmids pUC4K [18], and pKRP13 [19] were kind gifts from Marcelo Tolmasky, CSU Fullerton, and Gregory Phillips, Iowa State University, respectively. Plasmids pUC57 and pUC57-simple were obtained from GenScript (GenScript Inc., USA). DNA constructs were designed using the MacVector software (MacVector Inc., USA) and synthesized by GenScript.

Plasmids (Figs. S4–S6) were constructed as described below. Codon optimization of heterologous genes for expression in *S. 6803* were performed using the GenScript algorithms. Relevant plasmid sequences are shown in Figs. S7–S9. The introduced genes are controlled by strong *S. 6803* promoters, P-*psbA2* for *FatB* and P-*rbcl* for the FAR-FAD operon. We used the *psbA2* gene as an integrative platform in the *S. 6803* genome for both the *FatB* and FAR-FAD constructs. The *psbA2* gene encodes the photosystem II reaction center protein D1. We have previously shown [17,20] that insertional inactivation of *S. 6803 psbA2* upregulates a second *psbA* gene, *psbA3*, so as to restore D1 synthesis.

pFUEL3d: A 586 bp 5' portion of the *S. 6803 psbA2* (locus *slr1311*; NCBI ID: 951890) gene, up to and including the start ATG codon, followed by the coding sequence (CDS) of the *FatB* gene from *Arabidopsis thaliana* (Accession NP_172327; NCBI ID: 837372)

Trait	Trait description	Gene constructs
3	TE with high activity for 16:0 and 18:0 FA-ACPs	<i>FatB</i> from <i>Arabidopsis thaliana</i> (GenBank: NP_172327)
16	Inactivation of AAS activity	Insertional inactivation of <i>S. 6803</i> AAS gene (locus <i>slr1609</i>)
30	Enhanced activity of FAR and FAD	Operon with <i>S. 6803</i> FAR and FAD genes (loci <i>slI0208</i> and <i>slI0209</i> , respectively)

AAS: Acyl-ACP synthetase
ACC: Acetyl-CoA carboxylase
ACP: Acyl carrier protein
AGPAT: Acylglycerol-3-phosphate acyltransferase
CBB cycle: Calvin-Benson-Bassham cycle
DHAP: Dihydroxy-acetone phosphate
FA acyl-ACP: Fatty acyl-ACP
FAD: Fatty aldehyde decarboxylase
FAR: Fatty acyl-ACP reductase
FDH: Formate dehydrogenase
FFA: Free fatty acids
GPAT: Glycerol-3-phosphate acyltransferase
PHAS: Polyhydroxyalkanoate synthase
PHB: Polyhydroxybutyrate
TE: Thioesterase

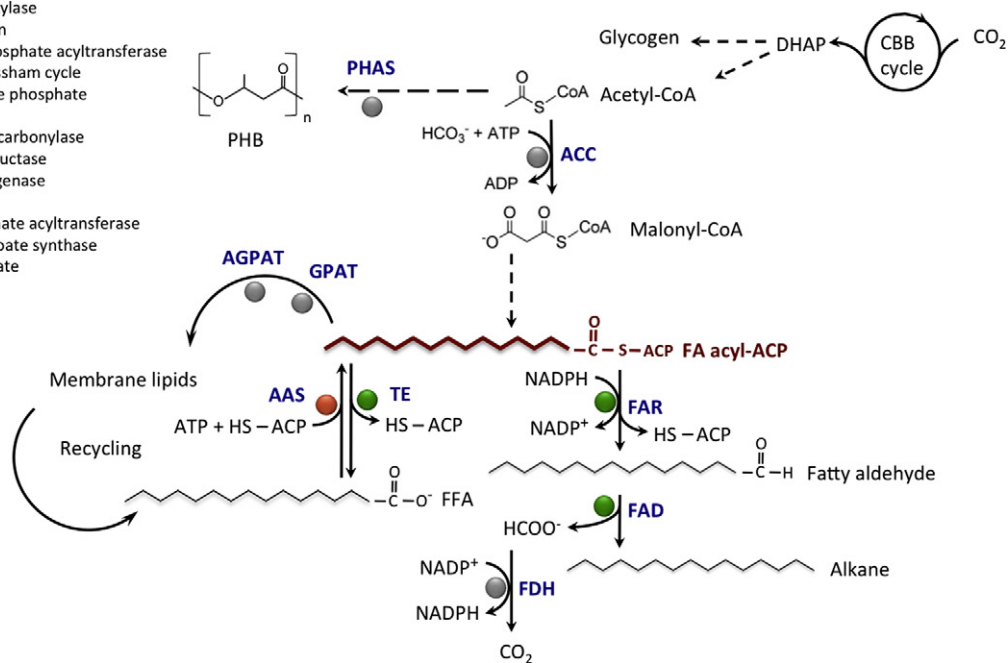


Fig. 1. Lipid metabolism in *S. 6803*-FUEL strains. Key enzymes affecting FA and alkane biosynthesis are shown (spheres). Enzyme activities targeted in this study are indicated with green for introduced/enhanced activities and red for blocked activity. Traits used to designate the strains are shown. The crossroad position of FA acyl-ACP in FA and alkane biosynthesis is indicated.

with the *S. 6803* codon preference (e.g. <http://exon.gatech.edu/metagenome/CodonUsageDatabase/?&page=16>), and the 720 bp 3' flanking region of *S. 6803* *psbA2*, were designed as a contiguous sequence. An NdeI site encompassing the *psbA2* ATG start codon in the *psbA2*-*FatB* junction, a PstI site at the 5' end, and a KpnI site at the 3' end of the construct were added. The sequence was synthesized as an EcoRV fragment and blunt end cloned into pUC57-simple (GenScript). The resulting plasmid is referred to as pFUEL2. The km resistance gene, *nptII*, from pUC4K, including its own promoter, was released as a Sall fragment and cloned into Sall-digested pFUEL2, generating plasmid pFUEL3. To remove the endogenous NdeI site in the vector backbone, pFUEL3 was digested with ZraI and BstAPI, blunt ended with mung bean nuclease, and religated. This generated plasmid pFUEL3d.

pFUEL16: The *S. 6803* AAS gene (locus *slr1609*; NCBI ID: 953643) was PCR amplified from genomic *S. 6803* DNA using primers with Gateway-compatible extension (Invitrogen, USA) and cloned into the Gateway donor vector pDONR221 (Invitrogen). This resulted in plasmid pFUEL15. An XmaI fragment from pKRP13, containing the streptomycin/spectinomycin resistance cassette, was cloned into XmaI-digested pFUEL15, generating pFUEL16.

pFUEL30: The Gateway Frame A Cassette was inserted into Sfol-digested pFUEL3d, generating pFUEL29. The *slI0208* (NCBI ID: 952286) and *slI0209* (NCBI ID: 952637) loci in *S. 6803*, encoding the FAD and FAR enzymes, respectively, were assembled as an operon by utilizing the 5' untranslated region (UTR) containing the -35 and -10 promoter signals, the first and third ribosome binding sites (RBSs), and the transcription termination signals of the *S. 6803* *rbclSX* operon. NotI, PacI, FseI, and SpeI sites were added. The

construct was cloned as an EcoRV fragment in pUC57-simple. This plasmid is referred to as pFUEL23. The *slI0208*-*slI0209* operon from pFUEL23 was PCR amplified with Gateway primers and cloned into pFUEL29, replacing the Gateway cassette. This resulted in plasmid pFUEL30.

2.4. PCR

Phusion and OneTag DNA polymerases were used for cloning and colony PCR respectively. PCR reactions were carried out according to standard protocols.

2.5. Preparation of cells for single-cell SR-FTIR spectromicroscopy

Gold-coated glass slides were cut into 1.5 × 0.5 cm pieces using a diamond knife. The gold-coated slides were submerged in a series of washes for 5 min each: acetone, followed by sterile deionized water, 95% ethanol, and sterile deionized water. To ensure sterility, the gold-coated slides were autoclaved (121 °C for 20 min) three times, allowing a day between autoclaving to allow spores to germinate. The slides were functionalized with poly-L-lysine to facilitate cell adherence to the slide (100 µl of a 1/10 dilution of poly-L-lysine solution (P8920-100ML; Sigma Aldrich, USA) was applied to the slide immediately before use). The slides were coated for 1 h, and then rinsed three times with sterile deionized water.

Cells for SR-FTIR measurements were prepared using BG 11 medium and appropriate antibiotics. 1 ml of cell cultures in exponential phase was sampled and spun down twice for five minutes at 5000 rpm with fresh media to remove dead cells. Cells were then

resuspended in 300 μl fresh media and roughly 100 μl were dispersed onto the gold-coated slides and allowed to attach to the functionalized surface overnight at 30 °C with constant fluorescent light and ambient CO₂ concentrations and subsequently flooded with fresh medium. Immediately before the SR-FTIR spectromicroscopy measurements, cells (on the gold-coated slides) were first rinsed with Hanks' Buffered Saline Solution (HBSS) and then excess free-flowing HBSS was wicked away. Cells were then measured non-invasively and analyzed for their chemical composition.

2.6. SR-FTIR spectromicroscopy

All measurements were performed using a Nicolet Magna 760 FTIR bench and a Nicolet Nic-Plan IR microscope (Thermo Scientific, MA, USA) equipped with a microscope stage chamber at the infrared beamline of the Advanced Light Source (Lawrence Berkeley National Laboratory, CA, USA; <http://infrared.als.lbl.gov/>). The numerical aperture of the objective of the infrared microscope was 0.65. The location of the synchrotron infrared beam within the field of the microscope was fiducialized to a 1- μm accuracy by mapping a titanium on a silicon calibration target. During the measurements, photons emitted over a mid-infrared wavenumber range of 4000–800 cm^{-1} from the synchrotron were focused by the all-reflective optics infrared microscope through the cells onto the gold-coated reflective surface. We visually identified and marked the locations of the cells before raster scanning, collecting full SR-FTIR transmittance spectra at each position using a single-element MCT detector. In transmittance, the synchrotron infrared beam transmitted through the cells, reflected off the gold-coated surface, and then transmitted through the sample a second time before reaching the detector. Each spectrum was collected at a spectral resolution of 4 cm^{-1} with eight co-added scans and a peak position accuracy of 1/100 cm^{-1} . Background spectra were acquired from neighboring locations without any cells, and used as reference spectra for both samples and standards to remove background H₂O and CO₂ absorptions. The following data processing and analysis were used to interrogate metabolic alternations in engineered *S. 6803* for biosynthesis of free FA.

2.7. SR-FTIR data processing and multivariate analysis

All SR-FTIR transmittance spectra were subjected to an array of data preprocessing and processing calculations using Thermo Electron's Omnic version 7.3. The processing includes the computation conversion of transmittance to absorbance, spectrum baseline removal, and statistical analysis. The absorption spectra show clear signatures of free fatty acids, proteins, lipids, and polysaccharides (Fig. 2).

Baseline corrected and vector-normalized spectra in the biochemical fingerprint region between 4000 and 1000 cm^{-1} (Fig. S10) were then subjected to the multivariate principal component analysis (PCA) and then linear discriminant analysis (LDA) using MathLab (7.0). PCA and LDA were used to generate new variables (factors) that were linear combinations (i.e. weighted sum) of the original variables (wavenumbers). PCA was applied to the spectra first to reduce the hundreds of absorbance intensities at different wavenumbers to just a few factors that could capture more than 95% of the variance. We typically selected seven components based on the 95% percentage of variance explained and on the spectral features of the loading plot. LDA was then applied to maximize the "inter-class" variance over the "intra-class" variance of the factors. We visualized the multivariate analysis results in the form of score plots and cluster vector plots (Fig. 2). In this study, score plots were 2- and 3-dimensional plots where the first three PC-LDA components were the *x*-, *y*- and *z*-axes; the nearness between classes (clusters) indicates the similarity, whereas the dis-

tance between classes implies dissimilarity. The cluster vectors plots [21] were 2D plots where the *x*-axis is in wave number units, and the *y*-axis depicts the cluster vectors coefficient values of engineered strains relative to the F0 control strain. The PC-LDA loadings determined the wavenumbers (modes of vibrations) responsible for segregation of classes. The loadings plots were 2D plots where the *x*-axis is in wave number units, and the *y*-axis depicts the loadings coefficient values (Fig. S1).

2.8. GC/MS analyses

Free Fatty Acids (FFAs), phospholipids (PLs) and alkanes were quantified by extraction of pellets from cells harvested at OD₇₅₀ = 0.6 by the Bligh–Dyer method [22–25]. Briefly, 10 ml of a 10:5:4 mixture of methanol:chloroform:pH 7 phosphate buffer was combined with the samples. Three internal standards, 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, Alabama), dodecanoic acid, and eicosane (Sigma Aldrich, St. Louis, MO) were added as controls. The mixture was vortexed, sonicated for 2 min and extracted at room temperature in the dark for 3 h. Phases were separated with the addition of 2 ml of chloroform and 2 ml of water, vortexed, and centrifuged at 2000 rpm for 15 min to separate the organic and aqueous layers. The organic layer was removed and dried under N₂ and the FFAs and alkanes were separated from the PLs on a C-18 silica column (Sigma Chemicals, St. Louis, MO) by elution first with chloroform with 50 μL of glacial acetic acid added to collect the free fatty acids followed by methanol to elute PLs. Both fractions were dried under N₂ and subjected to an acid hydrolysis by resuspending with 10:1:1 methanol:chloroform:concentrated HCl, vortexed for 2 min and incubated in a water bath at 60 °C for 15 h. This procedure methylates both the FFA and PL to fatty acid methyl esters (FAMES). The resulting FAME compounds were extracted with 3 \times 2 ml of hexane, and dried under N₂. A 50 μL of 46.2 mg/L methyl undecanoate (Sigma Chemicals, St. Louis, MO) was added to the dried extracts as an external standard. The resultant FAME and alkane compounds were detected on an Agilent 6890 GC/MS.

2.9. NMR analysis

Results were obtained on the methanol:water (5:4 v/v) fraction from the cell extraction protocol (stored at –20 °C). The top 6 ml of each sample were placed in a 50 ml Falcon tube, and 14 ml of water were added prior to lyophilizing the sample (to prevent methanol boil off). The samples were lyophilized for approximately 24 h, and subsequently dissolved in a mixture of 500 μl of deuterated methanol and 400 μl deuterium oxide. Each sample was vortexed and centrifuged briefly. 700 μl of this resuspended sample was then transferred into an eppendorf tube, and 20 μl of a 0.42 mM trimethylsilyl propanoic acid (TSP) was added for reference. Finally, 600 μl of the final mixture were placed into a 5 mm NMR tube. All of the spectra shown here were acquired on an 800 MHz ¹H frequency Bruker NMR spectrometer.

3. Results

We have generated a suite of *S. 6803* strains aimed for FFA and alkane production. Here we describe four of these *S. 6803*-FUEL strains, F3, F16, F3;16, and F30 (Fig. 1). Strain F3 contains a codon-optimized *TE* gene encoding the Arabidopsis *FatB* sequence. The Arabidopsis *FatB* TE shows preference for 16:0 and 18:0 FA acyl-ACPs, and thus would be expected to yield *S. 6803* cells producing 16:0 and 18:0 FFAs. Additionally, the concomitant decrease in acyl-ACP levels should also relieve the rigorous feedback inhibition of acetyl-CoA carboxylase (ACC; EC 6.4.1.2) (and other

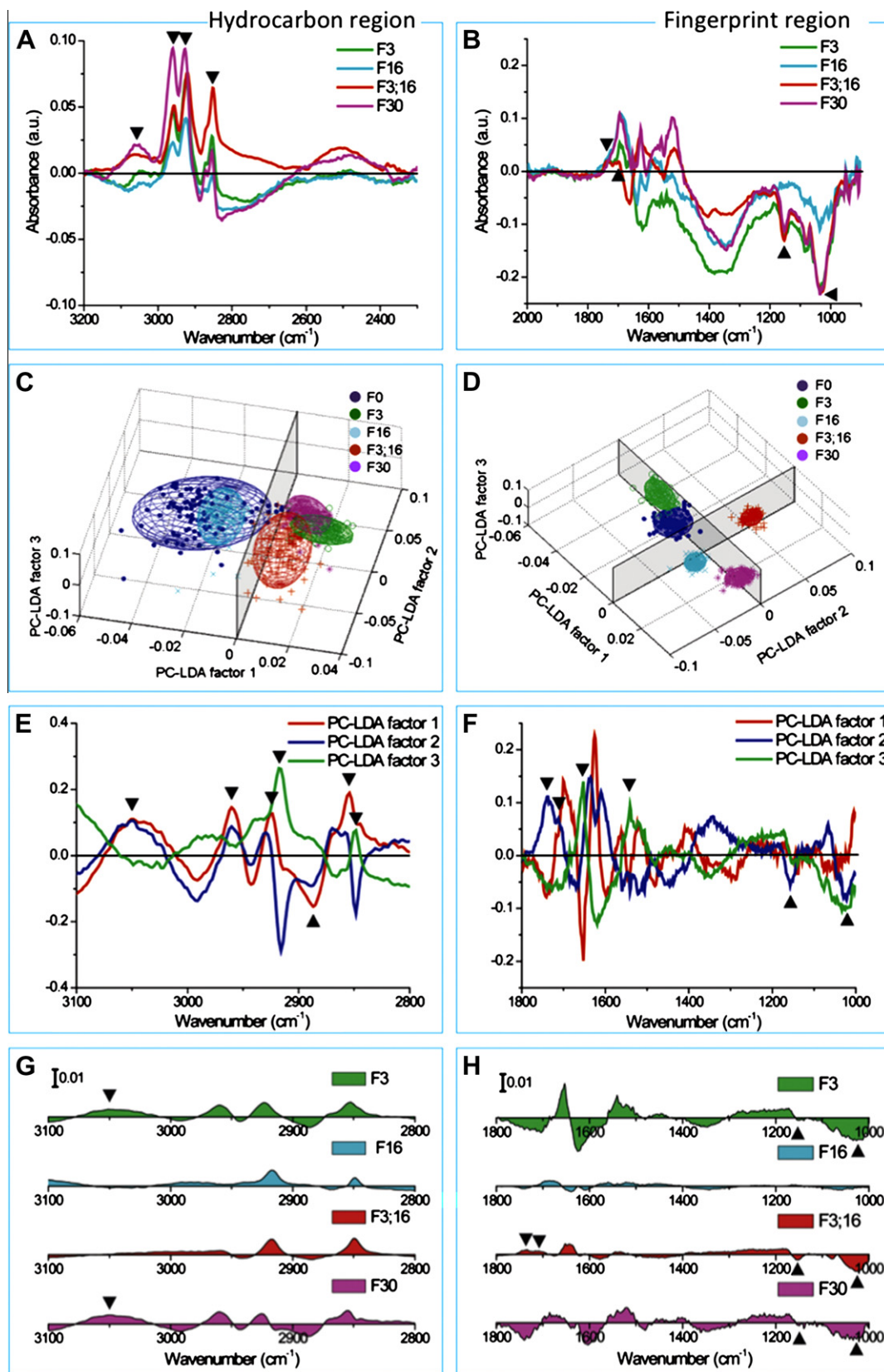


Fig. 2. Infrared spectroscopy analysis of phototrophic productions of biofuels in Cyanobacteria in the hydrocarbon and the fingerprint spectral regions. (A and B) The mean difference spectrum between the engineered strains and the F0 control in (A) the hydrocarbon region (3100–2800 cm⁻¹) and (B) the biomolecular fingerprint region. (C and D) Exploratory 3D PC-LDA of populations of the strains in the two spectral regions. The partitions are for improved viewing. Two-by-two 2D comparisons of the PC-LDA plots are shown in Fig. S1. (E and F) The first three PC-LDA loadings of the complete data set in the two spectral regions. (G and H) Cluster vector plots (F0 as comparator) for the two spectral regions. Arrows: markers of spectral features for metabolic shifts and productions of biofuels. See text for peak locations.

FA-biosynthesis enzymes) exerted by this end product [2]. In strain F16, the *S. 6803 AAS* gene, encoding the enzyme acyl-ACP synthase is inactivated. The purpose with this maneuver is to prevent re-thioesterification of FFAs to FA acyl-ACPs. Thus FFAs formed via recycling of membrane lipids will not find their way into the acyl-ACP pool. Strain F3;16 contains both the 3 and 16 trait. Strain F30 contains one extra copy each of the *S. 6803 FAR* and *FAD* genes assembled as an operon. The increased activity of the *FAR* and *FAD* enzymes would be expected to result in enhanced alkane production. This may also provide a stronger sink for FA acyl-ACPs and, hence, further increase the carbon flux from acetyl-CoA toward FFAs. The control strain of *S. 6803* is referred to as F0.

3.1. Single-cell SR-FTIR metabolic fingerprinting

Alterations in metabolism of the *S. 6803-FUEL* strains were investigated using SR-FTIR spectromicroscopy. The aim here was to exploit the high-resolution SR-FTIR spectromicroscopy as a high-throughput (150 cells in 20 min) analytical tool for collecting multiplexed metabolic information from individual engineered cells. The spectral information was stored and subsequently deconvoluted and dissected in greater detail. In this study, we focused on key absorption bands throughout two spectral regions: the hydrocarbon region (3100–2800 cm^{-1}) and the biomolecular fingerprint region (1800–1000 cm^{-1}). Individual mean and difference absorption spectra are shown in Figs. 2A and B and S1. Their molecular assignments are summarized in Table S1. At least 100 single cells were measured for each strain. The multivariate principal component and linear discriminant analysis (PC-LDA) of individual spectra for each cell reveal the following molecular information.

1. In the hydrocarbon region (3100–2800 cm^{-1}), the three-dimensional score plot revealed that F3, F3;16, and F30 are separated from F16 and F0 by the first PC-LDA factor (Figs. 2C and S1-A). The loadings of this factor (the red trace in Fig. 2E) reveal that the positive features near 3049 cm^{-1} , 2960 cm^{-1} , 2924 cm^{-1} and 2854 cm^{-1} , and the negative feature near 2887 cm^{-1} are responsible for the separation. When compared with the difference spectrum for each strain (Fig. 2A), the loading features near 3049 cm^{-1} co-located with the absorption band that can be assigned to the CH vibration in alkenes of hydrocarbons (Table S1); whereas the loading features near 2960 cm^{-1} , 2924 cm^{-1} and 2854 cm^{-1} co-located with absorption bands that can be assigned to CH_2 and CH_3 stretching vibrations of saturated hydrocarbon chains. The overall PC-LDA1 scores of the F3, F3;16, and F30 strains are positive (Fig. 2C), reflecting a relative abundance in hydrocarbons compared to the F16 and F0 strains. The cluster vector plots for each engineered strain (Fig. 2G), which reflect its difference from F0, show that only the F30 and F3 strains exhibit increasing absorption near 3049 cm^{-1} that could be assigned to alkene-like hydrocarbons (Table S1).
2. In the biomolecular fingerprint region (1800–1000 cm^{-1}), the 3D score plot shows a more intricate clustering pattern (Fig. 2D). The first PC-LDA factor separates strains F0 and F3 from F3;16, F16, and F30; the second PC-LDA factor further separates strain F3;16 from F16 and F30; while the third PC-LDA factor separates F0 from F3 (Figs. 2D and S1B). The loadings of the first factor (red¹ trace in Fig. 2F) show that the protein structures amide I (between 1700–1600 cm^{-1}) and amide II (between 1580–1510 cm^{-1}) are responsible for the separation. The loadings of the second factor (blue trace in Fig. 2F) show that the posi-

tive features near 1738 cm^{-1} and 1712 cm^{-1} in the ester and FFA carbonyl region (1700–1780 cm^{-1}), respectively, play a key role in discriminating F3;16 from F16 and F30. The loadings of the third factor show that the negative feature near 1157 cm^{-1} and 1022 cm^{-1} in the region for glycogen and other polysaccharides is responsible for the separation. Similarly, the cluster vector plots (Fig. 2H) together with the difference spectra (Fig. 2B) reveal that the positive feature, which is present only in F3;16, could arise from the vibrations of the C=O near 1738 cm^{-1} in PHAs [26] and the C=O near 1712 cm^{-1} in FFA [27]. In contrast, the negative features near 1157 cm^{-1} and 1022 cm^{-1} arise from the vibrations of glycosidic bonds and the C–O–C and C–O–P bonds of glycogen [28,29] (Table S1). A close comparative examination of the cluster vector plots shows that the overall polysaccharide features in all engineered strains decreased relative to the F0.

3.2. Bulk measurements by GC/MS/NMR

3.2.1. Alkane biosynthesis

The pathway for alkane synthesis in cyanobacteria seems to proceed via decarbonylation of fatty aldehydes [12], the major route for alkane synthesis in most organisms [30]. Gene sequences for *FAR* and *FAD* have recently been identified from several cyanobacteria [12]. Interestingly, the decarbonylation step in cyanobacterial alkane biosynthesis may involve the release of formate (HCOO^-) rather than CO, which has been the assumed coproduct in alkane biosynthesis [31] (Fig. 1). The physiological role(s) of alkanes in cyanobacteria is unknown. Not all cyanobacteria synthesize alkanes and in those that do, alkanes accumulate in very small amounts. It is possible that alkanes are required for proper membrane fluidity or function. Although heptadecane (C17) is the predominant *n*-alkane among cyanobacteria, many strains synthesize a wide array of linear, branched, and cyclic alkanes, some of which (e.g. branched methyl- and ethylalkanes) may be unique to cyanobacteria [2,32]. For example, the cyanobacterium *Microcoleus* produces four *n*-alkanes and more than 60 different branched alkanes [32].

Analysis of cellular extracts from strain F0 showed that *S. 6803* produces heptadecane and, to a much lesser extent, the alkene 9-heptadecene (Fig. 3A). The levels of both heptadecane and 9-heptadecene were significantly enhanced in strain F30 containing the *FAR-FAD* operon. This holds promise for further optimizing alkane yield in cyanobacteria by continued pathway engineering, e.g. increasing carbon flux upstream of FA acyl-ACP.

Also the F3;16 strain showed enhanced levels of heptadecane. This finding is counterintuitive since both the “3” and “16” traits would be expected to decrease the size of the acyl-ACP pool (Fig. 1), a notion that is reinforced by the results from the F3 and F16 strains, where the heptadecane levels remained unaffected, or were slightly decreased. Why the F3;16 strain had increased accumulation of heptadecane is not obvious. It should be cautioned, however, that this strain exhibited great variability in heptadecane levels between samples, and thus attempts at finding a possible explanation may be premature.

To reveal a possible bottleneck in the flux of carbon through the *FAR* and *FAD* enzymes, we looked for accumulation of octadecanal, the FA aldehyde substrate for *FAD*, in the F30 strain. No such compound was detected in any of the strains, although low levels of octadecenal species were found in F3;16 but were virtually undetectable in the other strains (Fig. 3B).

3.2.2. Intracellular FFAs

Contrary to expectation, strain F3 showed no increase in the amounts of intracellular FFA (Fig. 4). Strain F16 showed a modest but notable increase, and strain F3;16 demonstrated a dramatic

¹ For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

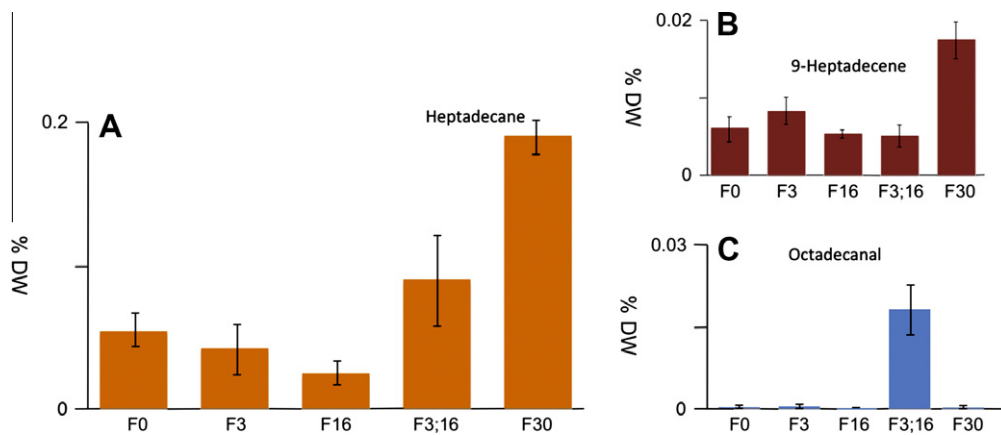


Fig. 3. Hydrocarbon (A) and aldehyde (B) levels in cell pellets from *S. 6803* cultures. Data were obtained from biological triplicates.

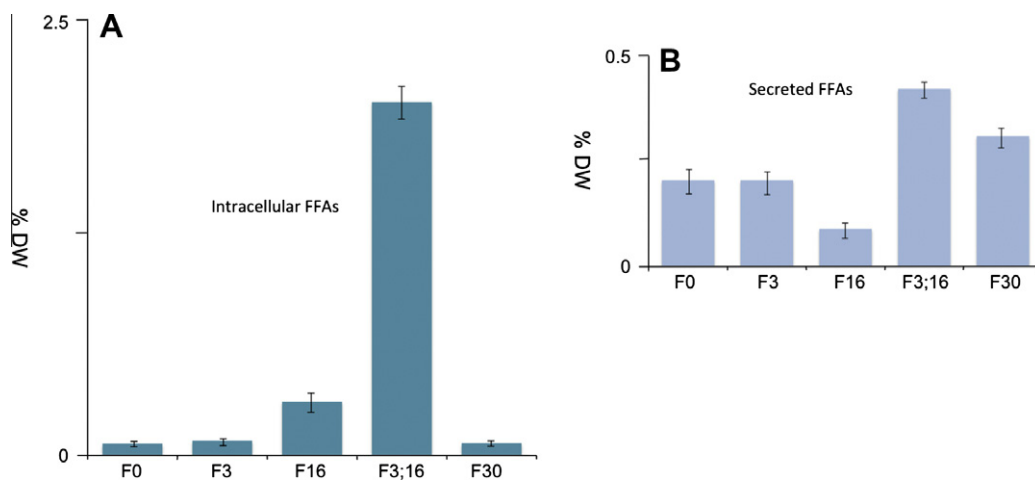


Fig. 4. Levels of total intracellular or secreted FFAs from *S. 6803* cultures. Data were obtained from biological triplicates.

30-fold rise in FFA levels. No increase in FFAs was observed in the F30 strain. The reason for the low levels of FFAs in F3 could be because of one or more of three possibilities; (i) the AAS enzyme is efficient enough to compensate for the introduced TE activity, (ii) the majority of the FFAs was secreted to the culture medium, or, (iii) the introduced TE enzyme is non-functional. The latter alternative is less likely, given the strikingly different phenotypes between the F3 and F3;16 strains. Also, although the amount of FFAs found in the culture medium of the F3 strain was approximately twice that which remained in the cells, the same was true for the F0 strain (see below). Thus this also does not seem to explain the lack of increase in FFAs levels in the F3 strain. Rather, we are left with the conclusion that a plausible reason for the lack of increased FFA levels in the F3 strain is because a high activity of the AAS enzyme masks the liberation of FFAs from the acyl-ACP pool. In other studies on *S. 6803* it was reported that most of the FFAs produced by the presence of a TE and/or absence of the AAS were secreted, and that secretion of FFAs in the control strains was insignificant [4] and Kaczmarzyk and Fulda [10]. Our results differ in this respect since the F0 strain showed a roughly equal distribution of intracellular and secreted FFAs. Also, although the F3;16 strain secreted twice as much FFAs as F0, the proportion of intracellular/secreted FFAs was higher in F3;16 than in F0, due to the large total increase in FFAs. The reason for this discrepancy between studies could be different culture conditions (we used gentle shaking with ambient CO₂), or because of different variants of

the *S. 6803* strain (we used the non-G strain [33]). In agreement with the conclusion above, the difference in FFA production between F3;16 and F16 is indicative of the TE activity, while the difference between F3;16 and F3 suggests a high AAS activity.

An inspection of the intracellular FFAs in the F3;16 strain shows a predominance of stearic acid (octadecanoate; 18:0) and palmitic acid (hexadecanoate; 16:0), with the remainder being mostly mono-unsaturated octadecanoates, primarily oleic acid (18:1 Ω9), vaccenic acid (18:1 Ω7) and euric acid (22:1 Ω9) (Fig. 5). Cyanobacterial acyl-ACPs are all saturated and desaturases in cyanobacteria act exclusively on lipids [34], and, therefore, unsaturated FFAs are likely to be derived from recycling of membrane lipids. This would explain the different FFA profiles between F3;16 on one hand, and F0, F3 and F30 on the other; in F3;16, where the AAS gene has been inactivated, the conversion of recycled FFAs from membrane lipids to acyl-ACPs has been disrupted, resulting in an accumulation of unsaturated FFAs. It must be emphasized that the FFA distribution displayed in Fig. 5B only serves to show relative amounts for a specific strain; as is shown in figures 3 and 4A, the total amounts of FFAs in F0, F3 and F30 are very small. The high levels of 16:0 and 18:0 FFAs in F3;16 are due to the presence of the *Arabidopsis* FatB TE, which has a substrate preference for 16:0 and 18:0 FA acyl-ACPs. The FatB TE exhibits the strongest affinity for 16:0 FA acyl-ACPs, which is reflected in the higher proportion of 16:0 over 18:0 FFAs in F3. On the other hand, the lack of noticeable 16:0 FFAs in F3 again points

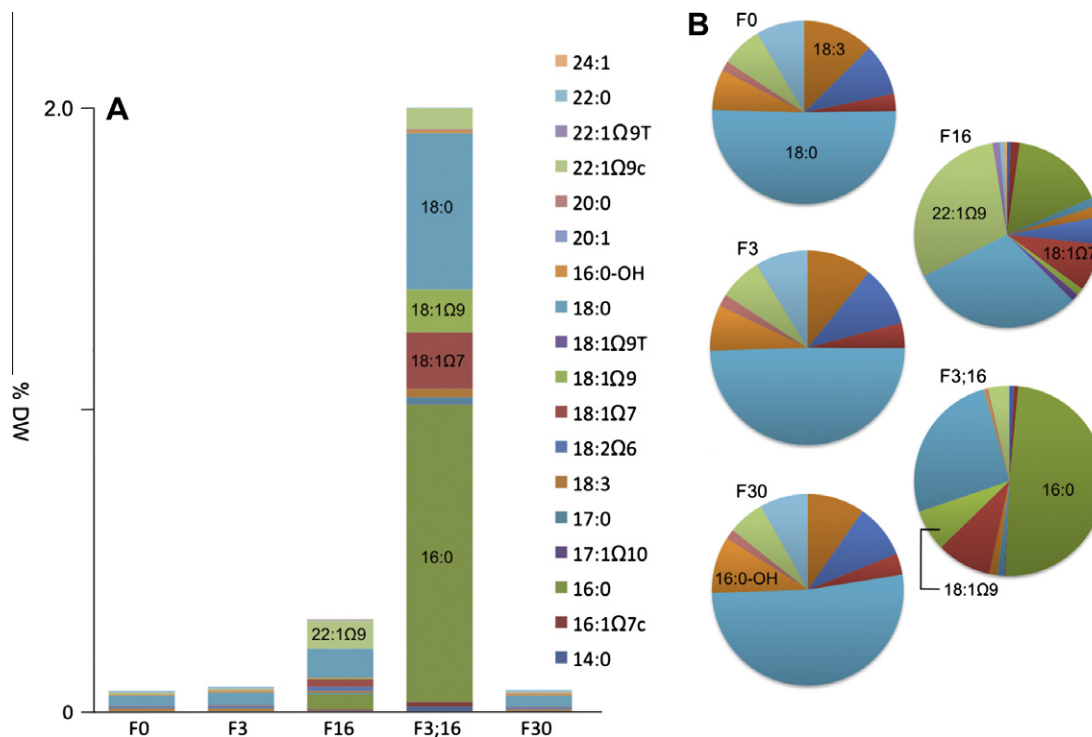


Fig. 5. Composition of the intracellular FFA pool. (A) The relative amounts of FFAs between the different strains, (B) the relative amounts of different FFAs in each strain. Data were obtained from biological triplicates.

to a high activity of the AAS enzyme, which apparently very efficiently re-thioesterifies FFAs generated by the introduced TE enzyme.

3.2.3. Secreted FFAs

The composition of FFAs secreted by the F3;16 cells differed significantly from the intracellular pool, with the majority being unsaturated, chiefly linoleic acid (18:2 Ω9), palmitoleic acid (16:1 Ω7), vaccenic acid (18:1 Ω7) and α -linolenic acid (18:3 Ω3) derived from membrane lipid degradation (Fig. 6). Similar to the intracellular pool of FFAs in F3;16, the activity of the TE enzyme manifested itself in the over 4-fold higher amount of secreted FFAs for the F3;16 strain compared to the F16 strain. The reason for the

higher unsaturated/saturated FFA ratio in the secretion products, which is also evident for the F0, F3 and F16 strains, is not clear but may be due to a preference in the secretion process for unsaturated over saturated FAs [10]. The F30 strain deviated from the general observation of a high proportion of unsaturated FA in the secreted FFA pool by showing relatively high amounts of stearic and palmitic acids, which contributed to the high levels of total FFAs secreted from this strain (Fig. 4). We find it difficult to reconcile these results with the enhanced FAR and FAD activities in F30, which increased the flux of 18:0 acyl-ACP toward heptadecane biosynthesis.

3.2.4. Hydroxylated FFAs

Selected compounds detected in the phospholipid fraction of the *S. 6803* strains are listed in Table S2. In accordance with the study by Murata and colleagues, e.g., [35], our results show that lipids of *S. 6803* have a high 16:0/18:0 FA ratio. One notable finding from the phospholipid analysis is the elevated levels of two hydroxylated FFAs, 3-OH stearic acid (3-OH 18:0) and 3-OH myristic acid (3-OH 14:0) in the F16 and F3;16 strains (Fig. 7A). These FFAs are known constituents of lipid A, a component of the lipopolysaccharide (LPS, also referred to as “endotoxin”) of the outer membrane of Gram-negative bacteria [36]. Hydroxylated FFAs are rarely found in any other lipids in Gram-negative bacteria and we assume that the 3-OH 18:0 and 3-OH 14:0 detected in the *S. 6803* strains were extracted from the outer membrane. The higher amounts of these FFAs in the F16 and F3;16 strains compared to F0 may either be indicative of an increased *de novo* synthesis of 3-OH stearic acid and 3-OH myristic acid and possibly increased formation of lipid A, or an accumulation of 3-OH stearic acid and 3-OH myristic acid as partly degraded outer membranes. The levels of 3-OH stearic acid were higher in F3;16 than in F16, suggesting the release of 18:0 FFAs from the acyl-ACP pool by the TE enzyme with subsequent hydroxylation. This link, between production of FFAs and accumulation of 3-OH FFAs, in turn, indicate that *de novo*

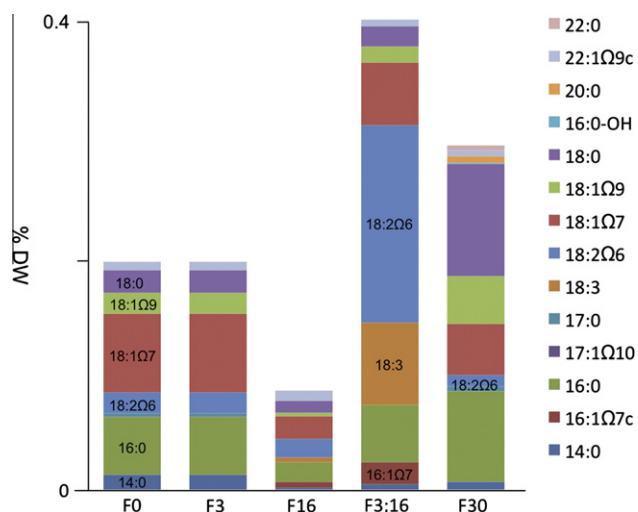


Fig. 6. Secreted FFAs. The relative amounts of the FFAs between different strains are shown. Data were obtained from biological triplicates.

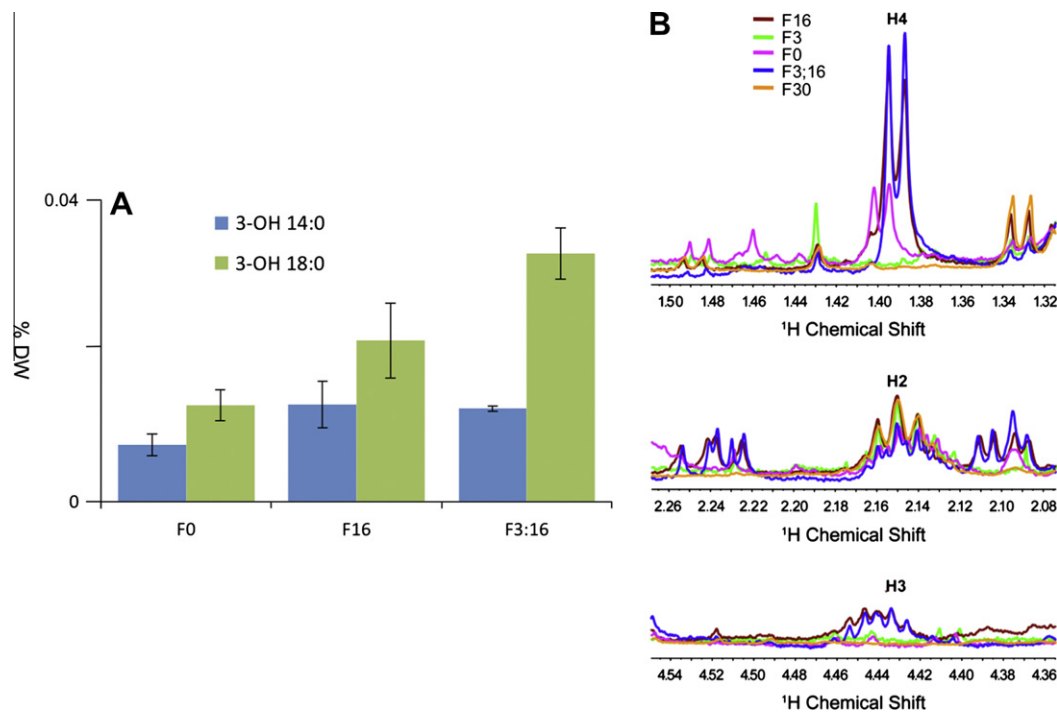


Fig. 7. Levels of 3-OH FAs as determined by GC/MS (A) and NMR (B). (A) Amounts of 3-OH stearic and 3-OH myristic acid. (B) Expansions of different regions of the ¹H 1D NMR spectra showing the presence of 3-OH butyric acid. Data were obtained from biological triplicates.

synthesis of 3-OH stearic and myristic acids at least partially accounts for their increased amounts in the F3;16 strain.

Another 3-OH FA, 3-OH butyrate (3-OH 4:0; β-OH butyrate), was detected as a significant signal in 1-dimensional ¹H NMR spectra in extracts from the F16 and F3;16 strains but was below detection limits in the other strains (Fig. 7). The identity of the compound as 3-OH butyrate was confirmed with 2D spectra (Fig. S2). A distinguishing feature of 3-OH butyrate is as a precursor in the biosynthesis of polyhydroxybutyrate (PHB), a polyhydroxyalkanoate (PHA) produced by *S. 6803* and several other bacteria as an energy and carbon storage compound.

4. Discussion

Our SR-FTIR observations in the hydrocarbon region clearly demonstrate an increase in the accumulation of hydrocarbons in strains F3, F3;16 and F30 strains in comparison to F0 (Fig. 2A, C, E, and G). This agrees with the accumulation of heptadecane and 9-heptadecene in F30 (Fig. 3), the elevated levels of 9-heptadecene in F3 (Fig. 3), and the accumulation of FFAs and the elevated levels of heptadecane and octadecenal in F3;16 (Figs. 3 and 4). It is noteworthy that the distinct phenotypic separation between F3 and F0 in the PC-LDA1 scores (Figs. 2C and S1A) corresponds to only minor observable differences in the GC/MS analyses, i.e., a slightly higher amount of 9-heptadecene and a slightly lower amount of heptadecane in F3 (Fig. 3). In fact, from the GC/MS analyses, the properties of F3 are mainly revealed in the context of the F3;16 background.

Also in the SR-FTIR fingerprint region, the F3 strain clearly segregates from the F0 strain, mainly due to the decrease in glycosidic bonds (Figs. 2D, H and S1B). Glycosidic bonds are decreased also in the F3;16 and F30 and, to a lesser extent, F16 strains (Fig. 2B and H). Polysaccharides such as glycogen and exopolysaccharide substances (EPSs) are prominent compounds in cyanobacteria, and the data suggest that portions of the carbon flux in the engineered

strains has shifted from polysaccharide biosynthesis in favor of being shunted to acetyl-CoA (Fig. 1). Although exhibiting similar negative peaks for polysaccharides in the difference spectra (Fig. 2B), F3;16 and F30 were clearly phenotypically separated by the second PC-LDA factors (Fig. 7D). This reflects the large increase in F3;16 of carbonyl groups in FAs and PHA (Fig. 2B and H; Table S1). The increase in FAs is consistent with the accumulation of FFAs (Fig. 4) and 3-OH stearic and 3-OH butyric acid (Fig. 7). The presence of carbonyl groups as carboxyl esters in PHA implies that the 3-OH butyric acid was used for PHA biosynthesis. Although we do not know the mechanisms, it is conceivable that the accumulation of FFAs in F3;16 signals a metabolic imbalance that triggers a shift in the utilization of acetyl-CoA from FA synthesis to formation of PHB. No PHA was detected in the F16 strain, although it also accumulated 3-OH butyric acid (Figs. 2C and 7). The separation of strains by the first PC-LDA factor is to a large extent caused by differences in the amount and nature of protein secondary structures and can be attributed to the altered composition of enzymes that results from the engineering steps.

Each data point in the PC-LDA plots in Fig. 2C and D represents one cell. Thus although the strains segregate in phenotypic populations, a great deal of metabolic heterogeneity exists within each strain, including the F0 control. It is becoming increasingly clear that such cell-to-cell variation in isogenic microbial populations is stochastic in nature [37–39], and that this heterogeneity to a major extent dictates how the population responds to perturbations such as environmental stress or to metabolic engineering.

As pointed out by Lidstrom and Konopka [37], if only a small subset of cells in a population of an engineered strain exhibits a certain trait, such as high-yield production of a desirable metabolite, simply overexpressing the target pathway in the bulk population is unlikely to increase the proportion of high producers. Rather, by appreciating that the monitored trait of the population (i.e., high productivity), is dominated by a subpopulation, we have a better chance at increasing the number of high producers by understanding the mechanisms by which these cells differ from

the rest in the population. For example, FTIR analysis is consistent with the GC/MS measurements in showing that the bulk of the F0 population contains low amounts of hydrocarbons. Moreover, the PC-LDA scores also reveal that some F0 cells exhibit higher hydrocarbon levels (Fig. 2C). Indeed, some cells overlap with the lower end of the F30 population. As another example, the F30 and F3;16 populations harbor cells with positions at the lower end of the 2nd dimension in Fig. 2C, probably indicating deviation in the details of the hydrocarbon structures, such as branching.

5. Conclusions

We have demonstrated a simple high-throughput single-cell SR-FTIR approach for rapidly examining the metabolic profiles of *S. 6803* strains engineered for enhanced phototrophic production of alkanes and FFAs. SR-FTIR data showed accumulation of functional groups in agreement with the GC/MS/NMR results. SR-FTIR data also could demonstrate a shift in carbon utilization from polysaccharide to lipid-based metabolism and to PHB biosynthesis. Finally, multivariate analysis of FTIR spectra revealed that the different strains were phenotypically segregated, but that the stochasticity in the populations gave rise to a high degree of cell heterogeneity within the population for each strain. These findings point to the importance of single-cell approaches to unravel the mechanisms that control specific traits in individual cells. Since SR-FTIR spectromicroscopy is a non-invasive tool, cells can be selected for downstream processing and omic investigation based on FTIR data. We suggest that our results demonstrate the applicability of SR-FTIR spectromicroscopy as a powerful means for metabolic screening and phenotyping of live individual cells.

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