Fatty acid production in genetically modified cyanobacteria

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To avoid costly biomass recovery in photosynthetic microbial biofuel production, we genetically modified cyanobacteria to produce and secrete fatty acids. Starting with introducing an acyl–acyl carrier protein thioesterase gene, we made six successive generations of genetic modifications of cyanobacterium *Synechocystis* sp. PCC6803 wild type (SD100). The fatty acid secretion yield was increased to 197 ± 14 mg/L of culture in one improved strain at a cell density of 1.0×10^9 cells/mL by adding codon-optimized thioesterase genes and weakening polar cell wall layers. Although these strains exhibited damaged cell membranes at low cell densities, they grew more rapidly at high cell densities in late exponential and stationary phase and exhibited less cell damage than cells in wild-type cultures. Our results suggest that fatty acid secreting cyanobacteria are a promising technology for renewable biofuel production.

Development of clean sustainable biofuels has gained significant support owing to global climate change, energy shortage, and petroleum supply. Photosynthetic microorganisms, including microalgae and cyanobacteria, are efficient at converting solar energy and recycling CO_2 into fuels, and they do not compete with the food industry for starch stocks and arable land (1). Currently, oleaginous algae are most popular in the microbial biofuel field because they have the ability to produce substantial amounts of triacylglycerols (TAG) as a storage lipid (2). However, production of TAG by microalgae requires environmental stresses, which makes the process complicated and costly (3). Although cyanobacteria usually do not accumulate neutral lipids, their photosynthetic membranes are made of diacylglyerol lipids and they have a robust lipid biosynthetic metabolism (4). Additionally, cyanobacteria are much more genetically manipulatable than algae (5).

In the field of algal or cyanobacterial fuels, biomass serves as a criterion for their potential biofuel productivity (6). Typically, cells with lipids need to be harvested, dried, and then extracted by solvents. Biomass extraction processes are energy intensive, usually accounting for 70–80% of the total cost of biofuel production (7). To skip these steps, we genetically engineered cyanobacteria to continuously secrete free fatty acids (FFA), which can be directly collected from the culture medium. In this scheme, the cyanobacteria are not the biomass that must be processed; they are cell factories that convert solar energy and CO_2 into biofuel precursors. As Ramachandra et al. said, "We do not harvest milk from cows by grinding them up and extracting the milk. Instead, we let them secrete the milk at their own pace, and selectively breed cattle and alter their environment to maximize the rate of milk secretion" (8).

Our FFA overproduction and secretion approach is based on Cho and Cronan's research on bacterial fatty acid synthesis (FAS) pathways of *Escherichia coli* (9). *E. coli* acyl–acyl carrier protein (ACP) thioesterase (TE) I (encoded by the *tesA* gene) is normally a periplasmic enzyme, but producing the mutant protein (called *TesA) without the signal sequence peptide redirects FAS to FFA secretion to the culture medium (9). This concept is being industrialized for biofuel production by the bioenergy company LS9 using *E. coli* (10). When applying this concept to cyanobacteria, a big advantage arises because cyanobacteria do not need an additional carbon feed because the precursor for FAS, acetyl-CoA, comes directly from the Calvin-Benson-Bassham cycle (11) (Fig. 1). Compared with *E. coli* FFA overproduction by using sugar, cyanobacterial FFA overproduction relies on photosynthetic CO_2 fixation streamlining metabolic pathways avoiding sugar synthesis and thus increases solar energy efficacy.

Cyanobacterial FAS type II (12) provides fatty acid substrates for membrane lipids (4). In FASII, long-chain acyl-ACP molecules are important feedback inhibitors of the activity of FAS enzymes (13), such as acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the conversion of acetyl-CoA into malonyl-CoA (14); FabH, which catalyzes the first step of FASII (15); and FabI, which catalyzes the completion of acyl-ACP elongation (16). Overproduction of TE reduces the cellular acyl-ACP concentrations, thus stimulating the FAS flow by decreasing feedback inhibition (13). Bacterial FFA secretion is the result of intracellular FFA overproduction, and this overproduction channels the products into the extracellular metabolic sink (17).

Besides bacterial TEs, plant TEs are able to hydrolyze acyl-ACP thioester bonds. The known plant TEs can be divided into two main classes (18). The "FatA" type of plant TE has preferential activities on oleoyl-ACP (C18:1). The "FatB" type of plant TE has activity on saturated acyl-ACPs. For example, when overproduced in *E. coli*, Cc FatB1 from *Cinnamomum camphorum* increased myristate (14:0) (19), Uc FatB1 from *Umbellularia californica* accumulated laurate (12:0) (20), and Ch FatB2 from *Cuphea hookeriana* provided TE activity for 8:0- and 10:0-ACP substrates (21).

In this study, we applied the fatty acid uncoupling strategy in cyanobacterium *Synechocystis* sp. PCC6803 strain SD100 (22) to overproduce and secrete FFAs. We also tested multiple means to improve the quantity and quality of FFA produced.

Results

Construction of Sun Devil (SD) Strains. We constructed six generations of SD100-derived strains for FFA secretion (Fig. 2 and Table S1). Insertions were always introduced in place of genes with either negative or competing consequences for FFA production. All strains are fully segregated and genotypically pure, as shown in Fig. S1 for the strain SD249. In the first-generation SD strains, the *E. coli* TE gene *'tesA* (9) was expressed using two different strategies (one in SD215 and one in SD216) (Fig. 2). In strain SD215, *'tesA* was controlled by a Ni^{2+} inducible regulator, which was turned on by addition of 7 μM Ni^{2+} in the medium (22). In strain SD216, *'tesA* was constitutively expressed at high level by the promoter P_{psbA2} (23). Also in SD216, the fatty acid activation gene *aas* (*slr1609*), encoding an acyl-ACP synthetase

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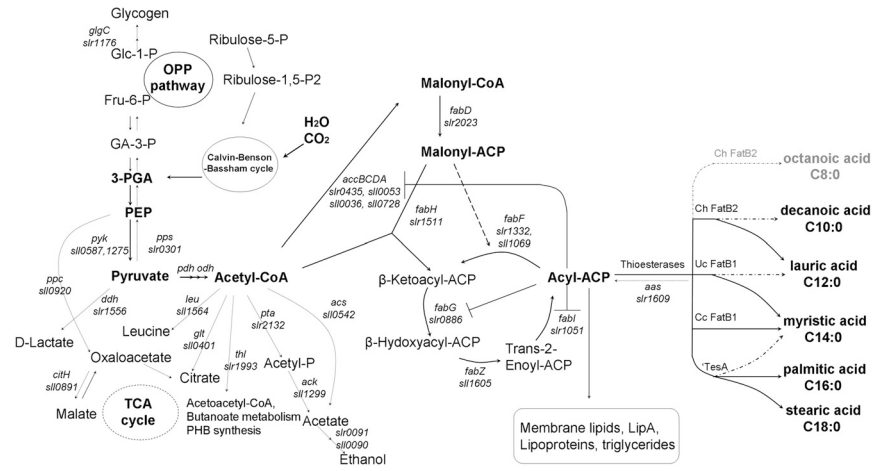

Fig. 1. The *Synechocystis* FAS pathways and modifications for FFA overproduction. The molecules and reactions in primary pathways toward FFA overproduction are indicated as bold text, whereas those in the competing pathways that uncouple the carbon flux from FFA overproduction are indicated as regular unbolded text. OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; GA-3-P, glyceraldehyde-3-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvic acid; Ch FatB2, TE from *C. hookeriana*; Uc FatB2, TE from *U. californica*; Cc FatB1, TE from *C. camphorum*.

(24), was deleted by inserting the P_{psbA2} *'tesA* cassette in place of the *slr1609* gene.

ACC is considered the rate-controlling enzyme in FAS (25). For second-generation strains (SD223 and SD225), an artificial operon P_{cpc} *accB accC* P_{rbc} *accD accA* was introduced into SD215 and SD216 to overproduce ACC subunits in relatively equimolar amounts (Fig. 2). The P_{cpc} promoter of the *cpc* operon, which encodes the photosynthesis antenna protein phycocyanin (26), and the P_{rbc} promoter of the *rbc* operon, which encodes ribulose 1,5-bisphosphate carboxylase (27), both drive highly expressed genes in SD100. In SD223 and SD225, two poly-3-hydroxybutyrate (PHB) synthesis genes (*slr1993* and *slr1994*) were deleted.

In the third-generation strain SD232, Uc *fatB1*, a 12:0 acyl-ACP TE encoding gene from *U. californica* (20) and Ch *fatB2*, an 8:0 and 10:0 acyl-ACP TE encoding gene from *C. hookeriana* (21) were synthesized in an artificial operon $^{*}P_{psbA2}$ Uc *fatB1* P_{rbc} Ch *fatB2* and inserted to knock out *sll1951* (Fig. 2), which encodes a hemolysin-like surface layer protein (28) with repeat in toxin motifs as found in other bacterial surface layer proteins (29, 30). In the fourth-generation strain SD243, Ch*fatB2* was synthesized and the $^{*}P_{psbA2}$ Ch *fatB2* cassette inserted to eliminate *slr2001* and *slr2002* (Fig. 2), which encode cyanophycin synthetases (31). In the fifth-generation strain SD249, *Cc fatB1* from *C. camphorum* (19) was synthesized and the $^{*}P_{psbA2}$ Cc *fatB1* cassette inserted to replace *slr1710*, a penicillin-binding protein (PBP2) gene responsible for peptidoglycan layer assembly (32). $^{*}P_{psbA2}$ is a modified promoter sequence of *psbA2*, in which the ATG box (9–18 bp upstream from the ATG start codon) was removed from $^{*}P_{psbA2}$ to enhance mRNA stability under dark conditions (23). In the sixth-generation strain SD277, an artificial operon P_{trc} *tesA137* was inserted to knock out *slr2132*, a *pta* gene coding for phosphotransacetylase (33). The plant TE genes and *E. coli tesA137* were synthesized after sequence optimization to enhance translation and mRNA stability.

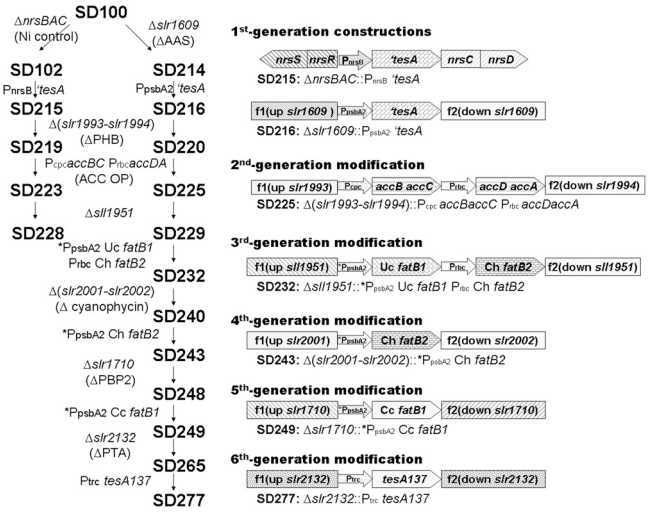

Fig. 2. The genetic modifications in SD strains for FFA secretion. The genealogy of sequential modifications is shown on the left, and detailed modifications are shown on the right. As shown for SD215, P_{nrsB} is the nickel-inducible promoter that serves as the upstream flanking region (f1), whereas *nrsCD* is the downstream flanking region (f2). In SD216, f1 and f2 are the flanking regions for deletion of *slr1609* (*aas*) and insertion of the P_{psbA2} *'tesA* cassette, of which, f1 contains the residual promoter of *slr1609* (P_{aas}). In SD225, f1 and f2 are the flanking regions for deletion of *slr1993* and *slr1994*; P_{cpc} and P_{rbc} are the promoters for the ACC genes (*accB*, *accC*, *accD*, and *accA*). In SD232, f1 and f2 are the flanking regions for deletion of *sll1951*; $^{*}P_{psbA2}$ is an improved promoter from P_{psbA2}; Uc *fatB1* is a TE gene from *U. californica*; and Ch *fatB2* is a TE gene from *C. hookeriana*. In SD243, f1 and f2 are the flanking regions for deletion of *slr2001* and *slr2002*. In SD249, f1 and f2 are the flanking regions for deletion of *slr1710*; Cc *fatB1* is a TE gene from *C. camphorum*. In SD277, f1 and f2 are the flanking regions for deletion of *slr2132*; *tesA137* is a *'tesA* gene with codon optimization.

Growth of the SD strains. We observed that successive modifications of FFA-secretion strains caused slower growth and cell damage at low cell density (Fig. 3). For example, when 4×10^8 SD232 cells grown in a glass tube (Fig. S2*C*) were inoculated into 200 mL BG-11 medium with 100 mL/min aeration of 1% CO_2-enriched air, the culture showed 14.7% permeable cells on the first day and 33.7% permeable cells on the second day revealed by SYTOX green staining (34) (Fig. S2 *E* and *F*). Our experiment showed that only 1% of the permeable cells (sorted by flow cytometry for green fluorescence) were able to form colonies on BG-11 plates, suggesting that CO_2 bubbling damaged cells at low cell densities (below 10^7 cells/mL). To avoid cell damage, we always maintain cell densities above 10^7 cfu/mL by stepwise scaling up the culture (*SI Text*). The cell damage caused by CO_2 to wild-type cells before stationary growth phase was achieved was much less than that for SD232 (below 2%, Fig. 3). Genetic tests using PCR analysis of single colonies derived from single cells showed that genetic modifications were stable after prolonged growth of cultures after lag phrase growth (Fig. S3), suggesting that no significant genetic changes occurred during the prolonged lag phrase.

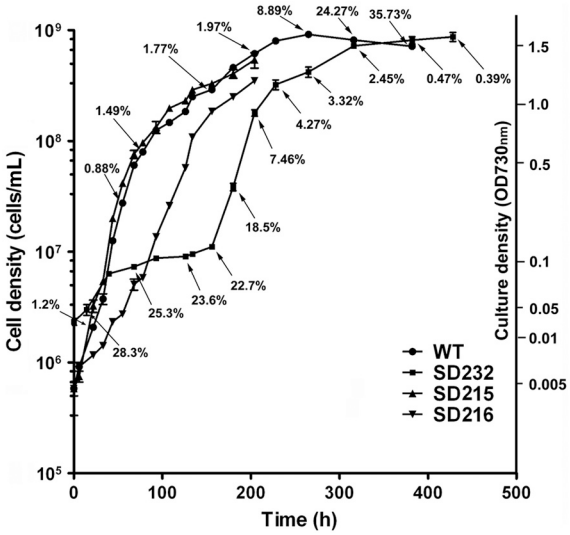

Fig. 3. Growth curves for SD strains. Cultures were grown at 30 °C in BG-11 medium and bubbled with 1% CO_2-enriched air. Cell density was transformed from culture optical density according to Fig. S5. The numbers pointed out by arrows are the damaged cell percentages in the SD232 and WT cultures at the specified times.

FFA Overproduction and Secretion. FFA secretion was observed and determined by GC, for the constitutively *'tesA* expressing strains (Fig. 4). Except for SD225 (ACC overproduction addition), most genetic modifications resulted in increased FFA secretion compared to the parent strain, but the intracellular FFA amount did not increase (Table 1). In SD277, insertion of the codon-optimized *tesA137* gene driven by the artificial promoter P_{trc} not only increased FFA secretion from 14.3×10^{-11} to 20.0×10^{-11} mg/cell, but also restored growth rate (Table 1). The secreted FFA accounts for about 13% of the biomass (the dry weight of an SD100 cell is approximately 150×10^{-11} mg). However, we did not observe a significant FFA increase from SD249 to SD265 (with *pta* gene deletion without P_{trc} *tesA137* insertion). We postulate that the FFA increase in SD277 is mainly from P_{trc} *tesA137*.

Chemical Composition of the FFAs. The chemical composition of the intracellular and secreted FFAs was analyzed by GC (Table 2). First, the percentage of C10:0-C14:0 FFAs increased with successive generations of strain constructions. For example, C14:0 and C12:0 increased from 16.5% (SD216 secretions) to 47.3% (SD249 secretions), meaning we made the overall FFA chain length shorter (fit the jet fuel carbon range) by introducing short-chain acyl-ACP TE genes from plants. Second, we observed that unsaturated FFAs almost disappeared in FFA-overproducing strains, which is favorable for biofuel quality. Third, we observed that the percentage of middle-chain FFAs was higher in the secreted extracts than in the intracellular unsecreted extracts (Table 2), suggesting that middle-chain FFAs are easier to secrete than longer chain FFAs.

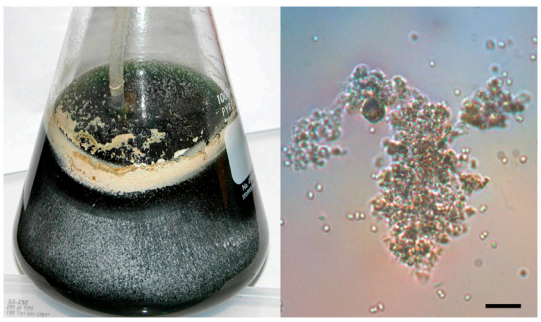

Fig. 4. The secreted FFAs (white deposit) from an SD232 culture. Picture on the left shows an 800 mL culture of SD232 grown in an aeration flask for 4 d. Notice the secreted FFAs precipitated out of the culture medium as a granular ring on the flask wall above the aqueous phase. GC analysis indicates that the white precipitate contains about 60% water with half of the 40% dry weight being FFAs. Picture on the right shows microscopy of FFA secretion. (Scale bar: 10 μm.)

Weakening Cell Walls to Facilitate FFA Secretion. Cyanobacterial cell walls include surface layers (35) composed of regularly arrayed proteins that provide a protective barrier, outer membranes, and peptidoglycan layers (36). We noticed that deleting the possible surface protein (Sll1951) from the cell envelopes as done in SD232 caused FFA secretion increase compared to the parent SD225. To test the hypothesis that weakening the peptidoglycan layers would facilitate the secretion of FFAs, we attenuated the peptidoglycan layers of the controllable *'tesA* strain SD215 by addition of ampicillin (Amp). After induction of *'tesA*, the secretion efficiency increased with Amp dose before cell lysis (Table S2). On the basis of these observations, we further genetically weakened peptidoglycan layers by deleting a peptidoglycan assembly protein (PBP2) in SD249, which increased FFA secretion (Table 1). The intracellular FFA amount decreased with *sll1951* deletion (SD232) and peptidoglycan weakening (SD249, Table 1), indicating that removal of the hydrophilic cell wall barrier did facilitate FFA secretion and, by decreasing feedback inhibition of enzymes involved in synthesis of fatty acid precursors, increased overall FFA production.

Discussion

Our cyanobacterial FFA-secreting strategy emulates the FAS in plants. In plants, acyl-ACP TEs are nuclear-encoded, plastid-targeted proteins that terminate the FAS (18). The resulting FFAs are "secreted" into the cytosol where they are esterified to CoA and further metabolized into membrane lipids and/or TAGs. Cyanobacteria are believed to be the evolutionary ancestors of plant plastids, and the FAS machinery of both plant plastids and cyanobacteria are similar (37). By genetic modification, we demonstrate that cyanobacteria have great potential to produce and export FFAs like plant plastids.

In designing genetic modifications for FFA production, we deleted SD100 genes based on whether they divert energy into production of substantial by-products that would compete with production of FFAs (e.g., PHB and cyanophycin synthesis genes), or to channel needed substrates for synthesis of FFAs into divergent or reverse pathways (e.g., *aas* and *pta* genes), or to enhance secretion of FFAs (e.g., surface layer and PBP genes). In terms of insertions, we selected genes to increase production of FFA and to modify the chain length of FFAs for better fuel quality.

We found both disadvantages and advantages of FFA overproduction on growth of SD strains. The disadvantage was the fragility of SD cells with CO_2 aeration at low cell density, which caused a long lag phase for FFA-secreting SD cultures. In addition, surface layer elimination contributed to cell fragility. Proper cell density is therefore important for SD cultures with multiple gene alterations to grow in a healthy manner with added CO_2 aeration as described in *SI Text*. However, the increased cell fragility may cause growth problems and low practical yields under industrial conditions. We believe that the industrial working strains need hyper and endurant biofuel productivity, robust cell growth, and cell rigidity. Based on the lessons we learned from the pilot strains, we will avoid modifications that sacrifice cell growth and rigidity in optimizing and reconstructing FFA strains for industrial use.

An advantage is that FFA-overproduction strains exhibit less cell damage than wild-type cells in stationary phase (Fig. 3) with the slight damage likely caused by excess electrons from photosynthesis when no significant NADPH consumption is required

Table 1. The secreted FFA and intracellular FFA of SD strains

Strains*	Doubling time,† Hour	Final cell density,‡ cells/mL	FFA-secretion yields,§ mg/L	Secreted FFA,¶ mg/cell	Intracellular lipids/FFA,‖ mg/cell	Genetic modifications
SD100	7.4	3.2×10^9	1.8 ± 0.06	0.05×10^{-11}	0.16×10^{-11}	wild type
SD216	11.9	8.3×10^8	83.6 ± 8.3	8.0×10^{-11}	3.01×10^{-11}	*'tesA* overexpression and *aas* deletion (Δ*slr1609*::P_{psbA2} *'tesA*)
SD225	12.1	8.3×10^8	83.6 ± 11.4	8.0×10^{-11}	2.38×10^{-11}	PHB synthesis gene deletion ACC overproduction Δ(*slr1993-slr1994*):: P_{cpc} *accBC* P_{rbc} *accDA*)
SD232	12.4	7.5×10^8	90.5 ± 6.4	9.4×10^{-11}	1.89×10^{-11}	*sll1951* deletion C10:0 C12:0 TEs (Δ*sll1951*:: $^*P_{psbA2}$ Uc *fatB1* P_{rbc} Ch *fatB2*)
SD243	14.3	1.0×10^9	92.9 ± 3.0	10.2×10^{-11}	1.24×10^{-11}	cyanophycin synthesis gene deletion C8:0 C10:0 TE [Δ(*slr2001-slr2002*):: $^*P_{psbA2}$ Ch *fatB2*]
SD249	16.7	1.3×10^9	146 ± 21	14.3×10^{-11}	1.05×10^{-11}	PBP2 gene deletion C14:0 TE (Δ*slr1710*:: $^*P_{psbA2}$ Cc *fatB1*)
SD277	14.1	1.0×10^9	197 ± 14	20.0×10^{-11}	1.12×10^{-11}	phosphotransacetylase gene deletion and codon-optimized *tesA137* gene (Δ*slr2132*:: P_{trc} *tesA137*)

*The genotypes and constructions of strains were described in Table S1.
†The doubling times were determined for exponential growing cultures (density below 10^8 cells/mL) in separate experiments.
‡The final cell density was determined by cfu.
§Twenty milliliter cultures were extracted by hexane and analyzed by GC for FFA amounts.
¶For each strain, the secreted FFA per cell was calculated based on the final cell density and the FFA-secretion yield of a single culture.
‖The intracellular unsecreted FFAs were extracted by the Folch method and calculated concentrations based on final cell densities.

(3). The accumulated electrons may induce overproduction of reactive oxygen species, which damage the membranes. We observed a much lower cell damage percentage (0.39%) in SD232 compared to wild-type cultures in the stationary phase of growth (Fig. S4 *G* and *I*), which suggested that FFA secretion might be able to relax the overreduced photosynthetic electron transport chain and make the cells healthier in stationary growth phase. This advantage is beneficial for continuous FFA production using stationary-phase cyanobacterial cultures.

We showed that two factors significantly increased FFA-secretion quality and quantity. One factor is increasing cytosol TE activities. Our data have shown that introducing extra TEs into SD100 did increase FFA production (Table 1) and optimize the FFA chain length (Table 2). However, the product chain length of plant TEs in SD strains did not totally match their substrate preference in plants or *E. coli*. For example, Ch FatB2 produced a substantial amount of C12:0 in SD243 (Table 2), whereas it accumulated C10:0 and C8:0 in *C. hookeriana* and recombinant *E. coli* (21). Introducing TEs also increased the fraction of fully saturated FFAs (Table 2). We hypothesize that the SD100 fatty acid dehydrogenases are located in the diglycerolipid membranes. When the FFAs were released from acyl-ACP, they would not be incorporated into membrane lipids for further desaturation. This phenomenon is beneficial for biofuel production, because unsaturated carbon chains result in a lower octane rating, and they are less stable and could potentially compromise storage. Another important factor is weakening cell envelopes to facilitate FFA secretion. As Hamilton has shown, long-chain fatty acids perform fast free diffusion by "flip-flopping" in the phospholipid bilayer (38). Because membranes are not substantial barriers to FFA secretion, we focused on compromising the layers made of polar molecules (39), i.e., the surface proteins (35) and peptidoglycan layers (36). Removal of FFA will increase the intracellular FFA production, because removing the final products from a reaction will push the equilibrium toward products (17) as well as reduce feedback inhibition.

We are continuing to investigate a diversity of means for further genetic improvements in our strains to give increased production and secretion of fatty acids. For example, we will enhance the primary FFA pathway genes and eliminate or down-regulate the competing pathway genes (Fig. 1). We will also make further improvements of growth conditions (CO_2 concentration, culture medium, temperature, illumination, pH, and cell density) to enhance FFA yields. We also developed a Green Recovery strategy

Table 2. GC analysis of the FFA profile of the SD strains

FFA type*	Fatty acid weight percentage,† %													
	SD100			SD216		SD225		SD232		SD243		SD249		SD277
	Lipids‡	Secr§	Cell¶	Secr	Cell	Secr	Cell	Secr	Cell	Secr	Cell	Secr	Cell	Secr
10:0	*t*	ND	ND	ND	ND	ND	ND	ND	ND	2.3	ND	1.6	ND	0.6
12:0	*t*	4.7	3.3	2.5	8.2	2.3	14.3	19.5	10.6	20.5	10.2	22.3	8.3	19.9
14:0	*t*	5.2	3.1	14,0	13.4	11.9	13.3	10.9	13.7	11.7	14.8	25.0	19.5	20.9
16:1	3	11.8	7.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:0	52	38.3	46.8	68.2	60.5	66.6	54.8	51.3	54.1	48.7	48.0	40.6	43.8	43.3
18:3	29	8.7	11.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.4
18:2	11	3.7	6.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.6	1.1
18:1	5	5.3	5.4	ND	ND	ND	ND	ND	ND	ND	9.6	ND	9.6	1.5
18:0	*t*	22.3	16.0	15.3	17,9	19.2	17.7	18.3	21.6	16.8	17.4	10.5	11.2	11.3
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100

ND, not detected; *t*, trace amount (less than 4%) (4).
*The number of FFA carbons: double bonds.
†The fatty acid percentages were based on each FFA species measured by GC for each sample.
‡The fatty acid percentages in SD100 membrane lipids were obtained from Wada and Murata (4).
§Secr represents mean secreted FFAs in the culture medium. Secr samples were prepared by extracting the whole culture with hexane without disrupting cells.
¶Cell samples represent the unsecreted FFAs remaining inside cells. Cell samples were prepared by extracting sedimented cells by the Folch method.

that enables recovery of membrane lipids in the spent cyanobacterial biomass (40). The cyanobacteria-based FFAs are ready biofuel precursors, which can be converted into biodiesel by esterification or jet fuel by decarboxylation. We are optimistic that further modifications to SD strains will result in economical biofuel production.

Materials and Methods

Bacterial Strains, Media and Growth Conditions. All SD strains are derived from *Synechocystis* sp. PCC6803 (SD100). SD strains were grown at 30 °C in BG-11 medium (41) under continuous illumination (140 μmol photons m^{-2} s^{-1}) and bubbled with 1% CO_2-enriched air. The details for growing an SD culture from a colony descended from a single cell are described in the *SI Text*. For plating and transformant selection, 50 μg/mL kanamycin or 4.5% (wt/vol) sucrose was added to 1.5% agar plates (wt/vol), which were incubated under continuous illumination (50 μmol photons m^{-2} s^{-1}). All strains were maintained as concentrated cultures in BG-11 medium with 20% glycerol and stored at −80 °C.

Growth and Cell Damage Measurement. Bacterial growth in liquid culture was monitored spectrophotometrically or by flow cytometry and/or by plating. The relationship between SD100 culture optical density and cell density is used for conversion (Fig. S5). Staining with 5 μM SYTOX green nucleic acid stain (Invitrogen Molecular Probes, Inc.) (34) for 5 min was used to detect damaged cells. Cells were observed under an Axioskop 40 fluorescence microscope (Zeiss). Green cells are sorted in a FACSAria flow cytometer (BD Biosciences) and counted as damaged.

Synthetic Molecular Procedures. Methods for DNA manipulation are standard (42). The primers for constructions and genotype verifications are listed in Table S3. Gene segments are synthesized at Genscript. The nucleic acid sequences of foreign genes were redesigned by codon optimization on the basis of the codon frequencies of highly expressed *Synechocystis* genes (Table S4). Also stem-loop hairpins in the predicted mRNA secondary structure were removed to stabilize mRNA by prolonging its half-life (43).

Deletion and Insertion of Genes into SD Strains. Multiple gene modifications of SD strains were achieved by using a *sacB*-Km^R cassette (22) as detailed in *SI Text*.

FFA Separation and Measurement. Secreted FFAs were quantitatively separated from the culture medium by hexane, which is unable to release FFAs and other lipids from intact SD100 cells. Twenty milliliters of culture was acidified by 0.4 mL H_3PO_4 (1M) containing 0.4 g NaCl, and extracted with 10 mL hexane. Being aware of hydrolysis caused by acid, we limited extraction times to 30 min, and used wild-type and parent strains for controls. For the unsecreted intracellular FFAs and lipids, the cells were extracted by the Folch method (44) for total lipids. The FFA samples were analyzed by GC (45) (Fig. S6).

To measure FFA secretion by constitutively producing strains, the accumulations of FFAs were measured for late-log phase cultures with densities of about 10^9 cells/mL. Briefly, during the continuous cultivation of a 50-mL culture, aeration was switched from air to 1% CO_2-enriched air when culture density reached about 1.5×10^8 cells/mL. After the cell density reached about 10^9 cells/mL with 1% CO_2 aeration (2–3 d later), a 20 mL sample was extracted by hexane. To measure the FFA-secretion efficiencies of the nickel-inducible strains, the secretion efficiencies in 1 d were calculated from the difference of the FFA-secretion values between before induction and 24 h after induction. In the experiment, to evaluate the effect of attenuating cell walls on FFA secretion, five subcultures (about 1.5×10^8 cells/mL) of 200 mL were induced by adding 7.0 μM Ni^{2+} to the medium and treated with 0, 1, 3, 9, and 25 μg Amp/mL.

Statistical Analysis. Most data are expressed as means ± standard deviation. The means were evaluated with one-way ANOVA for multiple comparisons among groups. Student's *t* test was used for pairwise comparisons. $P < 0.05$ was considered statistically significant.

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