# Production and secretion of fatty acids in genetically engineered cyanobacteria

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**Our purpose is to apply a fatty acid secretion strategy in photosynthetic microbial biofuel production, which will avoid the costly biomass recovery processes currently applied in algal biofuel systems. Starting with introducing acyl-acyl carrier protein thioesterases, we made five successive generations of genetic modifications into cyanobacterium *Synechocystis* sp. PCC 6803. The mutant strains were able to overproduce fatty acids (C10–C18) and secrete them into the medium at an efficiency of up to 133 ± 12 mg/L of culture per day at a cell density of $1.5 \times 10^8$ cells/mL (0.23 g of dry weight/liter). Fatty acid secretion yields were increased by weakening the S layer and peptidoglycan layers. Although the fatty acid secreting strains had a long lag phase with many cells having damaged cell membranes when grown at low cell densities, these strains grew more rapidly in stationary phase and exhibited less cell damage than wild-type in a stationary culture. Our results suggest that fatty acid secreting cyanobacteria are a promising technology for renewable biofuel production.**

Development of clean and 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 (TAGs) 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 metabolism (4). Additionally, cyanobacteria are much more genetically manipulatable compared to algae (5).

In the field of algal or cyanobacterial fuels, biomass serves as a criterion for their potential biofuel productivity (6). Typically, the cells with lipids need to be harvested, dried, and then extracted by various solvents. Usually biomass extraction processes are energy-intensive steps (2). To skip these steps, we genetically engineered cyanobacteria to continuously secrete free fatty acids (FFAs hereafter), 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 (fatty acids). As Ramachandra 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" (7).

Our FFA overproduction and secretion approach is based on John Cronan's research on the bacterial fatty acid synthesis (FAS) pathways of *Escherichia coli* (8). *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 would redirect FAS to FFA secretion to the culture medium (8). This concept is being industrialized for biofuel production by the bioenergy company LS9 by using *E. coli* (9). When applying this concept to cyanobacteria, a big advantage arises because cyanobacteria do not need additional carbon feed. In cyanobacteria, the precursor for FAS, acetyl-CoA, directly comes from the Calvin–Benson–Bassham cycle (10) (Fig. 1). Compared with *E. coli* FFA overproduction by using sugar, cyanobacterial FFA overproduction avoids the energy used for sugar synthesis in plants and on glycolysis in bacteria, thus streamlining metabolic pathways and increasing solar energy efficacy.

Cyanobacterial FAS type II (11) provides the fatty acid substrates for membrane lipids (4). In FASII, long-chain acyl-ACP molecules are important feedback inhibitors of the activity of FAS enzymes (12), such as acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the conversion of acetyl-CoA into malonyl-CoA (13); FabH, which catalyzes the first step of FASII (14); and FabI, which catalyzes the completion of acyl-ACP elongation (15). Overproduction of TE reduces the cellular acyl-ACP concentrations, thus stimulating the FAS flow by decreasing feedback inhibition (12). Bacterial FFA secretion is the result of intracellular FFA overproduction but will increase FFA overproduction by channeling the products into the extracellular metabolic sink (16).

Besides bacterial TEs, plant TEs are also able to hydrolyze acyl-ACP thioester bonds. The known plant TEs can be divided into two main classes (17). 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) production (18), Uc FatB1 from *Umbellularia californica* accumulated laurate (12:0) (19), and Ch FatB2 from *Cuphea hookeriana* provided thioesterase activity specific for 8:0- and 10:0-ACP substrates (20).

In this study, we applied the fatty acid uncoupling strategy in cyanobacterium *Synechocystis* sp. PCC 6803 (6803 hereafter) to overproduce and secrete FFAs. We also tested multiple means to improve the quantity and quality of FFA produced by 6803.

## Results

**Construction of 6803 SD Strains.** We constructed 5 generations of 6803 SD strains for FFA secretion (Fig. 2 and Table S1). In all cases, insertions were introduced in place of genes with either negative or competing consequences for FFA production. All the strains are fully segregated and genotypically pure, as shown in Fig. S1 for the fifth-generation strain SD249 as an example. In the first-generation SD strains, the *E. coli* TE gene '*tesA* (8) was expressed in 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 the addition of 7 μM $Ni^{2+}$ in the medium (21). In strain SD216, '*tesA* was constitutively expressed at a high level by the promoter $P_{psbA2}$ (22). Also in SD216, the fatty acid activation gene *aas* (*slr1609*),

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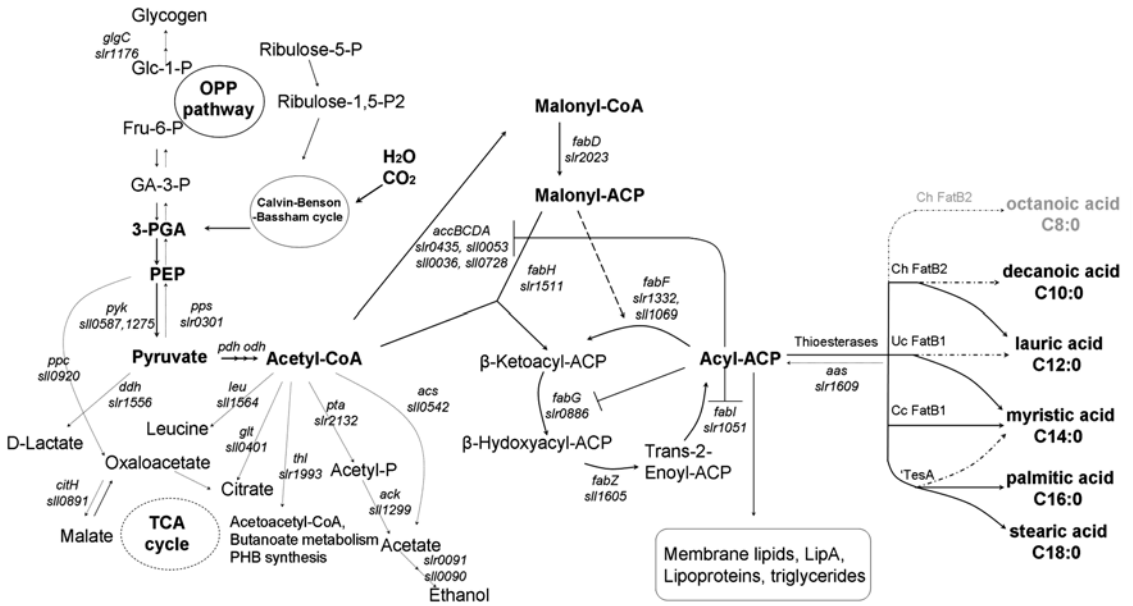


**Fig. 1.** The *Synechocystis* sp. PCC 6803 FAS pathways and modifications for FFA overproduction. The molecules and reactions in the primary pathways toward FFA overproduction are indicated as bold text, whereas those in the competing pathways that uncouple the carbon flux from FFA overproduction are indicated as regular unbolded text. Abbreviations: OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; GA-3-P, glyceraldehyde-3-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvic acid; Ch FatB2, thioesterase from *C. hookeriana*; Uc FatB2, thioesterase from *U. californica*; Cc FatB1, thioesterase from *C. camphorum*. The bold lines with arrow heads point to the major products of these TEs in 6803, whereas the dashed lines with arrow heads point to the minor products in 6803.

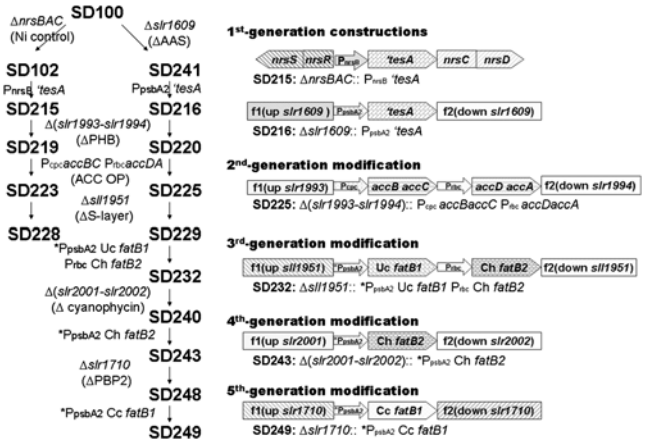


**Fig. 2.** The genetic modifications in the SD strains for FFA secretion. Five sequential genetic modifications were successively made to 6803 to increase FFA production and secretion. Their genealogy is shown on the left, and detailed modifications are shown on the right. As shown in SD215, $P_{nrsB}$ is the nickel-inducible promoter and also serves as the upstream flanking region for *'tesA* insertion, *nrsCD* is the downstream flanking region for the *'tesA* insertion, and *nrsBAC* are three deleted nickel resistance genes. In SD216, f1 and f2 are the upstream and downstream flanking regions, respectively (up *slr1609* and down *slr1609*) for deletion of *slr1609* (*aas*) and insertion of the $P_{psbA2}$ *'tesA* cassette, of which f1 contains the residual promoter of *slr1609* ($P_{aas}$), and $P_{psbA2}$ is the promoter of the 6803 *psbA2* gene. In SD225, f1 and f2 are the flanking regions (up *slr1993* and down *slr1994*) for deletion of *slr1993* and *slr1994*; $P_{cpc}$ is the promoter of the *cpc* operon; $P_{rbc}$ is the promoter of the *rbc* operon; *accB*, *accC*, *accD*, and *accA* are the genes coding for ACC subunits. In SD232, f1 and f2 are the flanking regions (up *sll1951* and down *sll1951*) for deletion of *sll1951* encoding the S-layer protein; $^{*}P_{psbA2}$ 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 SD 243, f1 and f2 are the flanking regions (up *slr2001* and down *slr2002*) for deletion of *slr2001* encoding cyanophycinase and *slr2002* cyanophycin synthetase. In SD 249, f1 and f2 are the flanking regions (up *slr1710* and down *slr1710*) for deletion of *slr1710* encoding penicillin binding protein 2; Cc *fatB1* is a TE gene from *C. camphorum*.

encoding an acyl-ACP synthetase (23), was knocked out by inserting the $P_{psbA2}$ *'tesA* cassette into the coding region of *slr1609*.

ACC has been postulated as the rate-controlling enzyme in fatty acid biosynthesis (24). For the second modification (SD223 and SD225), an artificial operon $P_{cpc}$ *accB accC* $P_{rbc}$ *accD accA* was introduced into SD215 and SD216 to overproduce ACC (Fig. 2). $P_{cpc}$ is the promoter of the *cpc* operon, which encodes the photosynthesis antenna protein phycocyanin (25); $P_{rbc}$ is the promoter of the *rbc* operon, which encodes ribulose 1,5-bisphosphate carboxylase (26). In SD223 and SD225, two poly-3-hydroxybutyrate (PHB) synthesis genes (*slr1993* and *slr1994*) were knocked out.

In the third-generation strain SD232, Uc *fatB1*, a 12:0 acyl-ACP TE encoding gene from *U. californica* (19), and Ch *fatB2*, an 8:0 and 10:0 acyl-ACP TE encoding gene from *C. hookeriana* (20) were synthesized in an artificial operon $^{*}P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* and inserted to knock out *sll1951* (Fig. 2), which encodes the monomer protein of the 6803 S layer (27). In the fourth-generation strain SD243, Ch *fatB2* was synthesized in an artificial operon $^{*}P_{psbA2}$ Ch *fatB2* and inserted to knock out *slr2001* and *slr2002* (Fig. 2), which encode cyanophycin synthetases (28). In the fifth-generation strain SD249, *Cc fatB1* from *C. camphorum* (18) was synthesized in an artificial operon $^{*}P_{psbA2}$ Cc *fatB1* and inserted to knock out *slr1710*, a 6803 penicillin-binding protein (PBP2) gene (29). The plant TE genes were synthesized after sequence optimization. $^{*}P_{psbA2}$ is a modified promoter sequence of *psbA2*, in which the AT box (9–18 bp upstream from the ATG start codon) was removed from $P_{psbA2}$ to enhance mRNA stability under dark conditions (22).

**Growth of the SD Strains.** During the growth of the FFA-secreting strains (SD232 for example) from a single cell to a 200-mL cell culture ($8 \times 10^8$ cells/mL, 1.2 g of dry weight/liter), cell permeability was revealed microscopically after staining cells with the vital dye SYTOX Green (30). 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 agar plates, suggesting that they were damaged.

SD232 colonies (containing $8 \times 10^5$ cells) descended from a single cell and grown on BG-11 agar plates for 10 days contained 0.5% cells permeable to the dye (Fig. S2*B*). When the entire colonies were inoculated into 1 mL BG-11 medium in a glass tube and grown for 3 days, 0.4% of the cells were permeable (Fig. S2*C*). However, when $4 \times 10^8$ SD232 cells 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, suggesting that $CO_2$ bubbling created significant cell damage at these low cell densities. A long lag phase was observed for the SD232 cultures when the cell density was below $10^7$ cells/mL and the permeable percentage was above 22.7% (Fig. 3). In the lag phase, SD232 cells aggregated as clusters of cells, which harbored a large number of viable growing cells (Fig. S3*C*). After the culture density achieved $10^7$ cells/mL, the cultures started exponential growth. The doubling times of wild-type ($7.4 \pm 0.8$ h), SD215 ($8.9 \pm 1.0$ h), SD216 ($11.9 \pm 1.1$ h), and SD232 ($12.4 \pm 1.3$ h) strains were calculated on the basis of the exponential growth period (Fig. 3). The damaged cell percentage for wild-type was below 2% before attainment of $6 \times 10^8$ cells/mL (Fig. 3). However, after completion of the exponential growth phase, the maintenance of cell viability for SD232 (0.39%, 428 h) was much longer than for the wild-type (35.7%, 382 h, Fig. S3 *G* and *I*).

**FFA Overproduction and Secretion.** FFA secretion was observed for the constitutively '*tesA* expressing strains, including SD216, SD220, SD225, SD229, SD232, SD243, and SD249 (Fig. 4). Each of 5 successive generations of genetic modifications resulted in increased FFA-secreting efficiency from the parent strains, but the intracellular FFA amount did not increase (Table 1). We noticed that ACC overproduction strains (SD225 and SD223) exhibited a 3-fold increase in FFA secretion over their parent strains (SD220 and SD219). This increase indicates that increasing the substrates for FAS will improve the FFA overproduction yields in 6803, which agrees with a previous study in *E. coli* (24). We

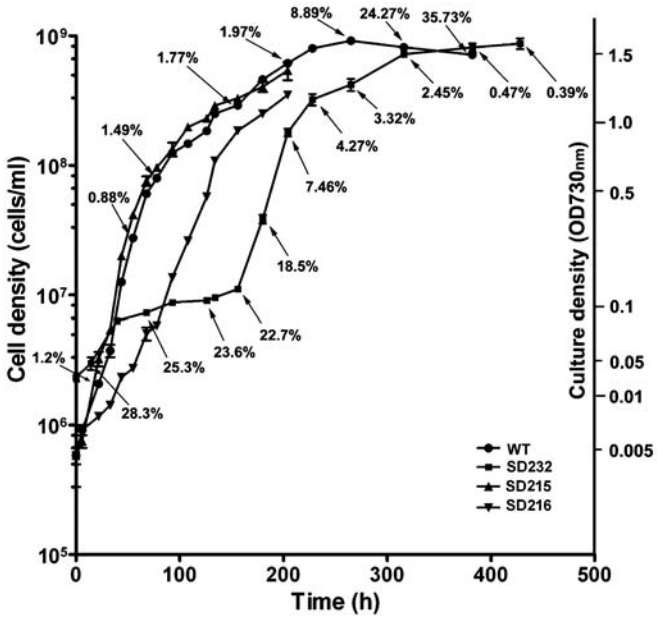


**Fig. 3.** Growth curves for SD strains. Cultures were grown at 30 °C in BG-11 medium and bubbled with 1% $CO_2$-enriched air. Cell density was transformed from culture optical density according to Fig. S4. The numbers pointed out by arrows are the damaged cell percentages in the SD232 and WT cultures at the specified times.

**Fig. 4.** The secreted FFAs (white deposit) from an SD232 culture. The picture on the left shows an 800-mL culture of SD232 grown in an aeration flask for 4 days. Notice the secreted FFAs precipitated out of the culture medium and formed a granular "ring" on the flask wall above the aqueous phase. The picture on the right shows the microscopy of FFA secretion. (Scale bar, 10 μm.)

noticed that deleting the S layer from the cell envelopes as done in SD229 caused a 3-fold increase in FFA secretion over the parents SD225 and also observed that weakening peptidoglycan layers by deleting PBP2 in SD249 caused a further significant increase. The intracellular FFA amount decreased with the S-layer deletion (SD229) and peptidoglycan weakening (SD249, Table 1), suggesting 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 FFA production. Starting with SD229, overexpression of extra plant TE genes further increased FFA-secretion efficiency up to $133 \pm 12$ mg/day per liter of culture (SD249), which counts for about 50% of the biomass ($10^8$ 6803 cells weigh 0.15 mg).

**Chemical Composition of the FFAs.** The chemical composition of the intracellular and secreted FFAs was analyzed by electrospray ionization mass spectrometry (ESI-MS hereafter) (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 17.6% (SD216 secretion) to 35.7% (SD249 secretion). Second, it was observed that the percentage of unsaturated fatty acids decreased in the FFA-overproducing strains. Third, we observed that the percentage of middle chain FFAs was higher in the secreted extracts than the intracellular unsecreted extracts (for example, the C12:0 and C14:0 FFA weight percent for SD232, SD243, and SD249 in Table 2), suggesting that the middle chain FFAs are easier to secrete than the longer chain FFAs.

**Weakening Cell Walls to Facilitate FFA Secretion.** S layers are regularly arrayed surface layers composed of a single protein species that provide a protective barrier for cyanobacterial cells (31) and a potential barrier to the efficient secretion of FFAs. The FFA-secretion efficiency of S-layer-deficient strain SD229 was much higher than that of its parent strain SD225 (Table 1). Weakening the peptidoglycan layers would also facilitate the secretion of FFAs. We used the controllable '*tesA* strains SD215 and SD228 (S-layer-deficient) to evaluate the FFA-secretion efficiency after attenuating the peptidoglycan layers by addition of ampicillin (Amp). After induction of '*tesA*, the secretion efficiency increased with Amp dose before cell lysis (Table S2). However, too much damage to the cell walls also killed the cells. On the basis of these observations, we further genetically weakened peptidoglycan layers by deleting a peptidoglycan assembly protein (PBP2) in SD249, which also increased FFA secretion (Table 1).

**Table 1. The secreted FFA and intracellular FFA of SD strain**

| Strain* | Secreted FFA† (mg/day/L) | Intracellular lipids/FFA‡ (mg/cell) | Modifications from parents§ |
|---|---|---|---|
| SD100 | 0.3 ± 0.02 | $4.4 \times 10^{-11}$ | Wild-type |
| SD216 | 6.2 ± 0.4 | $6.3 \times 10^{-11}$ | '*tesA* overexpression *aas* deletion |
| SD220 | 8.7 ± 0.5 | $7.5 \times 10^{-11}$ | PHB synthesis deletion |
| SD225 | 17.0 ± 2.5 | $12.5 \times 10^{-11}$ | ACC overproduction |
| SD229 | 47.6 ± 3.8 | $4.1 \times 10^{-11}$ | S-layer deletion |
| SD232 | 63.8 ± 5.2 | $4.5 \times 10^{-11}$ | C10:0 & C12:0 thioesterases |
| SD243 | 85.5 ± 6.4 | $6.8 \times 10^{-11}$ | C8-10:0 thioesterase cyanophycin deletion |
| SD249 | 133 ± 12 | $5.6 \times 10^{-11}$ | C14:0 thioesterase PBP2 deletion |
| SD215 | 4.5 ± 0.4 | $6.1 \times 10^{-11}$ | Controllable '*tesA* |
| SD219 | 6.5 ± 0.3 | $7.9 \times 10^{-11}$ | PHB synthesis deletion |
| SD223 | 23.3 ± 4.7 | $13.2 \times 10^{-11}$ | ACC overproduction |
| SD228 | 51.5 ± 6.3 | $4.4 \times 10^{-11}$ | S-layer deletion |

*The genotypes and constructions of the strains were elucidated in Table S1.

†For the constitutive strains (SD216–SD249), the late-log phase secreted FFA was measured for a 24-h interval with the culture density starting at about $1.5 \times 10^8$ cells/mL and reaching about $2 \times 10^8$ cells/mL. For the inducible strains (SD215–SD228), the induced FFA secretion was measured after addition of 7 μM $Ni^{2+}$ to the culture ($OD_{730\,nm} \sim 1.0$).

‡$2 \times 10^9$ cells in 10 mL hexane-treated culture were extracted by the Folch method.

§The parent for SD216 and SD215 is wild-type 6803; the parents for the other strains are the strains on the row above the new strain.

## Discussion

Our cyanobacterial FFA-secreting strategy resembles the FAS in plants. In plants, acyl-ACP TEs are nuclear-encoded, plastid-targeted proteins that terminate the FAS (17). 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 (32). By using genetic modifications, we proved that cyanobacteria have great potential to produce and export FFAs like plant plastids.

We found both disadvantages and advantages of FFA overproduction to the 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 (e.g., SD232). The permeability to the vital dye indicated that the cytoplasmic membranes of some cells were damaged when $CO_2$ aeration started. In addition, S-layer elimination and intracellular FFAs contributed to cell fragility. However, the high percentage of damaged cells (Fig. S2 *E* and *F*) of SD232 cultures was not observed for exponential or late-lag phase cultures (Fig. S3 *E* and *G*). Proper cell density is therefore important for SD cultures with multiple gene alterations to grow in a healthy manner with added $CO_2$ aeration. Therefore, we now always maintain cell densities above $10^7$ CFU/mL by stepwise scaling up the culture (*SI Text*).

FFA-overproduction strains exhibited less cell damage than wild-type cells at stationary phase (Fig. 3). This damage at stationary phase may be caused by excess electrons from photosynthesis when no significant NADPH consumption is required (3).

**Table 2. ESI-MS analysis of the FFA profile of the SD strains**

| FFA type* | Fatty acid weight percentage (%)† | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT | | | SD216 | | SD225 | | SD232 | | SD243 | | SD249 | |
| | Lipids | Secr | Cell | Secr | Cell | Secr | Cell | Secr | Cell | Secr | Cell | Secr | Cell |
| 8:0 | t‡ | ND§ | ND | 0.2 | ND | 0.3 | ND | 0.2 | ND | 0.3 | ND | 0.2 | ND |
| 10:0 | t | 1.7 | ND | 0.2 | ND | 1.1 | 0.4 | 0.3 | ND | 0.8 | 0.8 | 0.4 | 0.9 |
| 12:0 | t | 1.6 | 0.7 | 2.4 | 0.9 | 2.8 | 1.5 | 12.7 | 3.4 | 16.1 | 5.4 | 12.1 | 8.4 |
| 14:0 | t | 1.6 | 1.9 | 15.2 | 11.8 | 14.4 | 14.3 | 14.9 | 11.9 | 14.7 | 13.5 | 23.6 | 20.9 |
| 14:0-OH¶ | t | 6.4 | 7.6 | 3.9 | 4.2 | 5.2 | 3.9 | 3.1 | 6.6 | 2.3 | 2.5 | 2.8 | 6.9 |
| 16:1 | 3 | 10.8 | 5.1 | 0.5 | 1.6 | 0.5 | 0.9 | 0.3 | 0.6 | 0.4 | 1.0 | 0.3 | 1.2 |
| 16:0 | 52 | 14.7 | 26.9 | 63.4 | 52.4 | 61.7 | 59.6 | 55.6 | 55.7 | 52.7 | 55.4 | 48.4 | 40.7 |
| 18:3 | 29 | 28.7 | 9.4 | 1.4 | 3.8 | 1.1 | 2.6 | 1.0 | 1.6 | 1.3 | 3.0 | 1.1 | 3.4 |
| 18:2 | 11 | 17.9 | 20.5 | 1.2 | 2.9 | 1.0 | 1.9 | 1.0 | 1.4 | 1.1 | 1.9 | 1.1 | 2.3 |
| 18:1 | 5 | 5.8 | 16.1 | 0.8 | 1.7 | 0.9 | 1.1 | 0.9 | 1.1 | 0.9 | 1.4 | 0.8 | 1.4 |
| 18:0 | t | 10.8 | 11.8 | 10.8 | 20.7 | 11.0 | 13.8 | 10.0 | 17.7 | 9.4 | 15.1 | 9.2 | 13.9 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

*The number before the colon in the fatty acid name refers to the number of carbons, and the number after the colon refers to the number of double bonds.

†The weight percentage data are given as averages of triplicate experiments. All the variances are less than ±0.1% and are not shown in the table. The fatty acid percentages for wild-type 6803 membrane lipids (WT lipids) were obtained from Wada's report (4) as a baseline for the 6803 fatty acid profile. The fatty acid percentages for the other samples were based on the FFAs. Secreted samples (Secr) mean secreted FFAs in the culture medium extracted by hexane without disrupting the cells. Cell samples represent the unsecreted FFAs remaining inside the cells extracted from sedimented cells by the Folch method.

‡t, trace amount (less than 4%) (4).

§ND, not detected.

¶Fatty acid analysis of some strains revealed small but significant amounts of components with masses corresponding to 3-hydroxymyristic and 3-hydroxypalmitic acids, which are intermediates in chain elongation to produce the fully saturated acids. Possibly the 3-hydroxy acids are released from the acyl carrier protein by thioesterases together with the fully saturated fatty acids. The other intermediates in fatty acid biosynthesis, β-keto acids and α,β-unsaturated fatty acids, were not detected. This agrees with the finding of Kaczmarzyk and Fulda (23), who suggested that 3-hydroxymyristic could be a component of lipid.

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 culture compared to wild-type in the stationary phase of growth (Fig. S3 *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 the continuous FFA production using stationary-phase cyanobacterial cultures.

We showed that two factors significantly increased the FFA-secretion quality and quantity. One factor is increasing the cytosol TE activities. Our data have shown that introducing extra TEs into 6803 did increase the FFA-secretion efficiency (Table 1, from SD216 to SD249) and shorten the FFA chain length (Table 2, from SD216 to SD249). However, the product chain length of plant TEs in 6803 did not totally match their substrate preference in plants or *E. coli*. For example, Ch FatB2 produced a substantive amount of C12:0 in 6803 (Table 2), whereas it accumulated C10:0 and C8:0 in *C. hookeriana* and recombinant *E. coli* (20). Extra TEs also increased the fraction of fully saturated FFAs (Table 2). We hypothesize that the 6803 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 export. As Hamilton and colleagues have shown, long-chain fatty acids perform fast free diffusion by "flip-flopping" in the phospholipid bilayer (33). Because membranes are not substantial barriers to FFA secretion, we focused on compromising the layers made of polar molecules, i.e., S-layer and peptidoglycan layers. Removal of FFA will increase the intracellular FFA production, because removing the final products from a reaction system into a metabolic sink will push the equilibrium toward products (16) as well as relieve the consequences of feedback inhibition.

With our best constructs such as SD249 (Table 1), we can theoretically after scale up produce some 6,500 gal of biodiesel (transformed into fatty acid methyl ester; see *SI Text* for calculations) per acre of culture 20 cm deep at a cell density of $1.5\times 10^8$ cells/mL during a 1-yr period. 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 attenuate the competing pathway genes (Fig. 1). We will also make further improvements of growth conditions ($CO_2$ concentration, temperature, illumination, pH, and cell density) to enhance FFA yields. Because FFA production and secretion is healthy for a stationary-phase cyanobacterial culture, we thus expect that the 6803 FFA-secretion potential could be optimized for economic biofuel production.

## Materials and Methods

**Bacterial Strains, Media, and Growth Conditions.** All SD strains are derived from *Synechocystis* sp. PCC 6803. SD strains were grown at 30 °C in modified BG-11 medium (34) 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 *SI Text*. For plating and transformant selection, 50 μg/mL kanamycin or 4.5% (wt/vol) sucrose is added to 1.5% agar plates (wt/vol) and plates were grown under continuous illumination (50 μmol photons $m^{-2}\,s^{-1}$). All of our strains are 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 6803 culture optical density and cell density is used for conversion (Fig. S4). Staining with 5 μM SYTOX Green nucleic acid stain (Invitrogen Molecular Probes, Inc.) (30) for 5 min was used to detect damaged cells. Cells were observed under an Axioskop40 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 (35). The primers for constructions and genotype verifications are listed in Table S3. DNA sequences were analyzed in the DNA Sequence Laboratory at Arizona State University. Gene segments are synthesized at Genscript. The nucleic acid sequences of non-6803 genes were redesigned by codon optimization on the basis of the codon frequencies of highly expressed 6803 genes (Table S4). Also, the stem-loop hairpins in the predicted mRNA secondary structure were removed to smooth the transcription and to stabilize mRNA by prolonging its half-life (36).

**Gene Deletion and Introducing Genes into 6803.** Multiple gene modifications are applied into SD strains by using a *sacB*-$Km^R$ cassette (21) (Fig. S5). Detailed methods are described in *SI Text*.

**FFA Separation and Measurement.** The FFAs in the medium were quantitatively separated from the culture medium by hexane, which is unable to release FFAs and other lipids from intact 6803 cells. One hundred milliliters of culture was acidified by 2 mL $H_3PO_4$ (1 M) containing 1 g NaCl and extracted with 100 mL hexane. For the unsecreted intracellular FFAs and lipids, the cells were extracted by the Folch method (37) for total lipids. ESI-MS was done with a Bruker MicrOTOF-Q mass spectrometer by using an approach similar to the shotgun method for quantitative analysis of lipids described by Han and Gross (38).

To measure the FFA-secreting efficiencies of the constitutively producing strains, the accumulation of FFAs were measured for late-log phase cultures in a 24-h time interval. Briefly, during the continuous cultivation of a 50-mL culture, a 10-mL sample (about $1.5\times 10^8$ cells/mL) was extracted by hexane, and 24 h later, another 10-mL sample was also extracted. The secretion efficiencies were calculated by the difference in FFA amounts in the two samples. To measure the FFA-secretion efficiencies of the inducible strains, the secretion efficiencies per day 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, 5 subcultures (about $1.5\times 10^8$ 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/mL ampicillin.

**Statistical Analysis.** Most data were 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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# Retraction

**APPLIED BIOLOGICAL SCIENCES**

PNAS is retracting the following article: "Production and secretion of fatty acids in genetically engineered cyanobacteria," by Xinyao Liu, Daniel Brune, Wim Vermaas, and Roy Curtiss III, which appeared online in *Proc Natl Acad Sci USA* on March 29, 2010 (10.1073/pnas.1001946107).

The editors wish to note that it has come to our attention that not all authors approved the final version of the article and that there are ongoing disagreements between the authors over some of the results in the article. According to the Arizona State University Office of Research Integrity, "Drs. Vermaas and Brune, both authors on the original manuscript, have elected to remove their names and their data from the manuscript." The editors hereby wish to retract the article.

Randy Schekman
*Editor-in-Chief*

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