# Supporting Information

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## SI Text

**SI Materials and Methods.** ***Growth of an SD culture started from a single cell descended colony.*** As shown in the results, cultures of extensively modified SD cells suffered a long lag phase when started at low cell density, probably because a significant percentage of the cells was damaged. A principal rule for culturing extensively modified SD strains is therefore not to start cultures below a density of $10^7$ cells/mL, because low cell densities will create a long lag phase prior to exponential growth. We used the following procedure to grow a 6803 culture from a colony descended from a single cell. A single SD colony is picked by a sterilized needle and used to inoculate 1 mL modified BG-11 medium buffered by 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) NaOH (pH 8.2) in a glass test tube. The tube is incubated with illumination and intermittent shaking for 2–4 days. These starter cultures can be scaled up by "1 into 10" inoculations after achieving an $OD_{730\,nm}$ of 0.6 ($10^8$ cells/mL) with 10 mL buffered BG-11 medium culture grown in a 50-mL flask with 50 rpm rotation, 100 mL buffered BG-11 medium culture grown in a 250-mL flask with 100 mL/min aeration with air and without shaking, and 1 L modified BG-11 medium grown without TES buffer with 300 mL/min air sparged with an air stone. Once a 1 L culture achieves $OD_{730\,nm} \sim 0.6$, aeration is switched from air to 3% $CO_2$-enriched air. This protocol uses TES buffer and air aeration to keep the pH around 8 at the beginning inoculation stages to minimize the lag phase. Free fatty acid (FFA)-producing strains need a sufficient $CO_2$ supply and a pH above 8 to maximize FFA-secretion yields. When the 6803 cell density achieves $10^8$ cells/mL, the culture is able to maintain its pH above 8 and can be supplied with $CO_2$-enriched air.

***Transformation procedures for 6803.*** We optimized the current genetic modification techniques for 6803 gene deletion, insertion, and substitution (1). Suicide vectors harboring a positive selection marker (e.g., $Km^R$) and a counterselection marker (e.g., *sacB*) are widely applied in a two-step gene deletion and insertion for 6803 without leaving any drug marker residuals (Fig. S5).

**Transformation of suicide vectors containing the sacB-$Km^R$ cassette.** About $10^6$ SD cells in 10 μl BG-11 medium are mixed with 400 ng suicide vector DNA (Table S1) containing the *sacB*-$Km^R$ cassette and incubated for 5 h. Then the mixtures were plated onto a filter membrane (Whatman PC MB 90 MM 0.4 μm) layered on a BG-11 agar plate. After segregation on the BG-11 plate for about 24 h, the membrane carrying the cyanobacteria was transferred onto a BG-11 plate containing 50 μg/mL of kanamycin. Generally, the colonies appear 4–5 days later. Individual colonies are restreaked with a sterile loop onto a kanamycin BG-11 agar plate and a 4.5% sucrose BG-11 agar plate. Those patches growing on a kanamycin BG-11 agar plate and not growing on a 4.5% sucrose BG-11 agar plate have the correct insertions with the *sacB*-$Km^R$ cassette.

**Transformation with markerless constructs.**
To replace the *sacB*-$Km^R$ selective marker with target gene segments, about $10^6$ *sacB*-$Km^R$ cells in 10 μL BG-11 medium are mixed with 400 ng suicide vector DNA containing the target genes and incubated for 5 h. The mixtures are inoculated into 2 mL buffered BG-11 medium and grown for 3–4 days. Then 1 mL inoculation is plated onto a 4.5% sucrose-containing BG-11 agar plate. Generally, the colonies appear 5–8 days later. Individual colonies are restreaked onto kanamycin BG-11 agar plates and 4.5% sucrose BG-11 agar plates. The patches growing on sucrose plates and not growing on kanamycin plates are positive candidates for further evaluation by PCR.

**Confirmation of replacement.**
Cells from a colony are resuspended in 2 μL water in a 200-μL PCR tube. The cell suspension is frozen at −80 °C for 2 min and then thawed in a 60 °C water bath. This freeze–thaw cycle needs to be performed three times. Then 1 μL frozen-thawed cell suspension is used as the PCR template for a 30 μL PCR reaction including the primers specific for the inserted gene segments or the deleted region.

***Genetic stability tests.*** When a foreign gene is introduced into 6803, it may cause an adverse effect on the growth and be subjected to gene loss or modification, because any cell losing the genetic alteration will likely have a higher growth rate to eventually take over the population. The genetic stability of foreign genes in 6803 is therefore tested by growing a culture of the strain with periodic dilution and subculturing for at least two months. After this time, the cells from the culture are plated onto BG-11 agar plates to obtain single isolated colonies. One hundred single colonies are picked and tested for all genetic attributes and confirmed for the presence of the foreign gene by PCR as described above. The percentage of positive colonies in the culture reflects the genetic stability of the foreign gene. Genes found to be unstable can be modified to eliminate nonfunctional hydrophobic domains that often are responsible for poor growth because of association with and impairment of cytoplasmic membrane function.

***Electrospray ionization mass spectrometry (ESI-MS) for the FFA produced by the SD strains.*** ESI-MS was performed to analyze the fatty acid profile of the secreted FFA and unsecreted FFAs (2). For the unsecreted intracellular FFAs, the cells are collected by centrifugation and extracted by the Folch method (3) for total lipids, which includes significant quantities of phospho-, galacto-, and sulfo-diglycerides (4).

Commercially available fatty acids, all with purities of at least 98%, used to determine the instrument response as a function of chemical properties (e.g., chain length and degree of unsaturation) were as follows. Myristic, palmitic, palmitoleic, heptadecanoic, linolenic, and oleic acids were from Sigma-Aldrich; lauric and linolenic acids were from Acros; stearic acid was from Alpha Aesar; and 3-hydroxymyristic acid was from TCI America.

Dried extracts were dissolved initially in chloroform:methanol (1:1) to give concentrated solutions. These samples were then diluted in methanol so that the most abundant fatty acids (palmitate and stearate) will have final concentrations of about 5 μM (typically between 200 and 1,000×). In cell extracts containing chlorophyll a, this can be accomplished by initially determining the chlorophyll a concentration from its absorbance at 665 nm after dilution with methanol and using a molar extinction coefficient of 70,000. Dilution of the sample to a chlorophyll concentration of 5–10 μM will yield a solution with about the right palmitate concentration. Samples were analyzed by using a Bruker MicrOTOF-Q mass spectrometer operated in the negative ion mode and tuned for good sensitivity in the low mass range. Instrumental parameters used were as follows: capillary voltage, 3,700 V; end plate offset, −500 V; dry gas flow and temperature, 4 L/min and 190 °C, respectively; funnel 1 and funnel 2

rf settings, 200 Vpp (peak to peak voltage); hexapole and collision cell RF settings, 100 Vpp; quadrupole isolation mass, 55.0 $m/z$; transfer time, 148 μsec; prepulse storage, 7 μsec. Mass calibration was done by using sodium iodide or sodium formate clusters sprayed from a 200-μM solution in acetone or in methanol, respectively.

Peak intensities for the acids relative to heptadecanoic acid, which was used as an internal standard, were obtained by spraying mixtures of the fatty acids dissolved in methanol containing 1 mM ammonium hydroxide at known concentrations. Fatty acid standards and samples were supplied to the ESI source of the MicrOTOF-Q from a 50-μL syringe at a flow rate of 3 μL/ min by using a KD Scientific model KDS-100-CE syringe pump, and the concentrations of fatty acids in the samples were determined from their peak intensities relative to the heptadecanoate internal standard, which was present at a concentration between 1 and 5 μM. Each sample was also analyzed in the absence of the standard to check and correct for the presence of trace amounts of heptadecanoate (typically less than 1% of the amount added as a standard) in the samples.

***Calculation for FFA field yield estimation.*** We theoretically scaled up the FFA yield of SD249 from lab conditions to field conditions by the following assumptions: (*i*) FFAs were transformed into fatty acid methyl ester for biodiesel calculation; (*ii*) cultures were grown in 20-cm-deep photobioreactors at a cell density of $1.5 \times 10^8$ cells/mL (0.23 g of dry weight/liter); (*iii*) by certain engineering means, the secretion efficiency of SD249 was kept at $133 \pm 12$ mg/liter of culture/day for one year; and (*iv*) the photobioreactors received 12 h light per day.

Starting with 133 mg FFA/liter of culture/day, we used C14 ($M_r = 228$) as the average FFA type and transformed C14 into myristic acyl methyl ester ($M_r = 242$). The production efficiency for biodiesel is

$$133 \times 242/228 = 141 \text{ mg biodiesel/liter of culture/day.}$$

Then the weight was converted by the average density of biodiesel (0.85 g/mL) into volume. The biodiesel yield in volume is

$$141/0.85 = 0.166 \text{ mL biodiesel/liter of culture/day.}$$

The production would persist 365 days per year but only 12 h each day. So the annual yield for biodiesel is

$$0.166 \times 365/2 = 30.3 \text{ mL biodiesel/liter of culture/year.}$$

We proposed growing the culture at 20-cm depth. The volume for 1 acre (4046.86 $m^2$) is

$$1 \text{ acre} \times 20 \text{ cm} = 4,046.86 \text{ m}^2 \times 0.2 \text{ m} = 809 \text{ m}^3$$
$$= 809,000 \text{ liters of culture.}$$

So, the annual yield per acre is

$$30.3 \times 809,000 = 2.45 \times 10^7 \text{ mL biodiesel/acre/year.}$$

Because 1 gallon equals 3,785 mL, the final biodiesel yield estimation in gallon is

$$24,500,000/3,785 = 6,473 \text{ gallons per acre per year.}$$


1. Liu X, Curtiss R III (2009) Nickel-inducible lysis system in *Synechocystis* sp. PCC 6803. *Proc Natl Acad Sci USA* 106:21550–21554.
2. Han X, Gross RW (2005) Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev* 24:367–412.
3. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509.
4. Wada H, Murata N (1990) Temperature-induced changes in the fatty acid composition of the cyanobacterium, *Synechocystis* PCC6803. *Plant Physiol* 92:1062–1069.


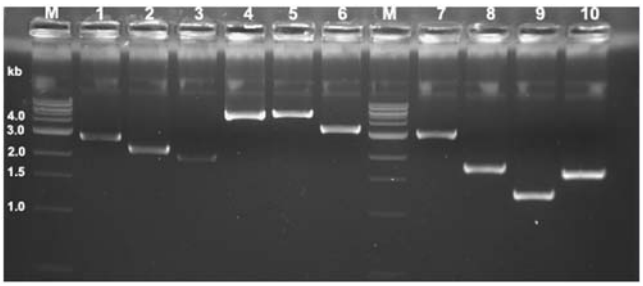


**Fig. S1.** PCR identification of deletions and insertions in SD249. The segregation-checking primers used in the PCRs are listed in Table S3. Wild-type DNA was used as the template for reactions loaded in the odd lanes. SD249 cell lysate prepared by freeze–thaw cycles was used as the template for reactions loaded in the even lanes. Lanes 1 and 2 used primers FadD-F1-Sequ and FadD-F2-A. Lane 1 indicated the wild-type *slr1609* region to be deleted in SD249 with an anticipated length of 2,596 bp. Lane 2 indicated the Δ*slr*1609::$P_{psbA2}$ *'tesA* cassette inserted in SD249 with an anticipated length of 2,171 bp. Lanes 3 and 4 used primers S4-seg100-S and S4-seg100-A. Lane 3 indicated the wild-type *slr1993-slr1994* region to be deleted in SD249 with an anticipated length of 1,878 bp. Lane 4 indicated the Δ(*slr*1993-*slr*1994)::$P_{cpc}$ *accBC* $P_{rbc}$ *accDA* cassette inserted in SD249 with an anticipated length of 4,727 bp. Lanes 5 and 6 used primers S5100S and S5100A. Lane 5 indicated the wild-type *sll1951* region to be deleted in SD249 with an anticipated length of 5,324 bp. Lane 6 indicated the Δ*sll*1951::$^*P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* cassette inserted into SD249 with an anticipated length of 3,109 bp. Lanes 7 and 8 used the primers S7 Seg 51S and S7 Seg 90A. Lane 7 indicated the wild-type *slr2001-slr2002* region to be deleted in SD249 with an anticipated length of 2,974 bp. Lane 8 indicated the Δ(*slr*2001-*slr*2002)::$^*P_{psbA2}$ Ch *fatB2* cassette inserted into SD249 with an anticipated length of 1,722 bp. Lanes 9 and 10 used the primers S9-S68 and S9-A71. Lane 9 indicated the wild-type *slr1710* region to be deleted in SD249 with an anticipated length of 1,256 bp. Lane 10 indicated the Δ*slr*1710::$^*P_{psbA2}$ Cc *fatB1* cassette inserted into SD249 with an anticipated length of 1,618 bp. Sequencing analysis of these PCR products proved that all the inserted sequences were correct in SD249 as expected.

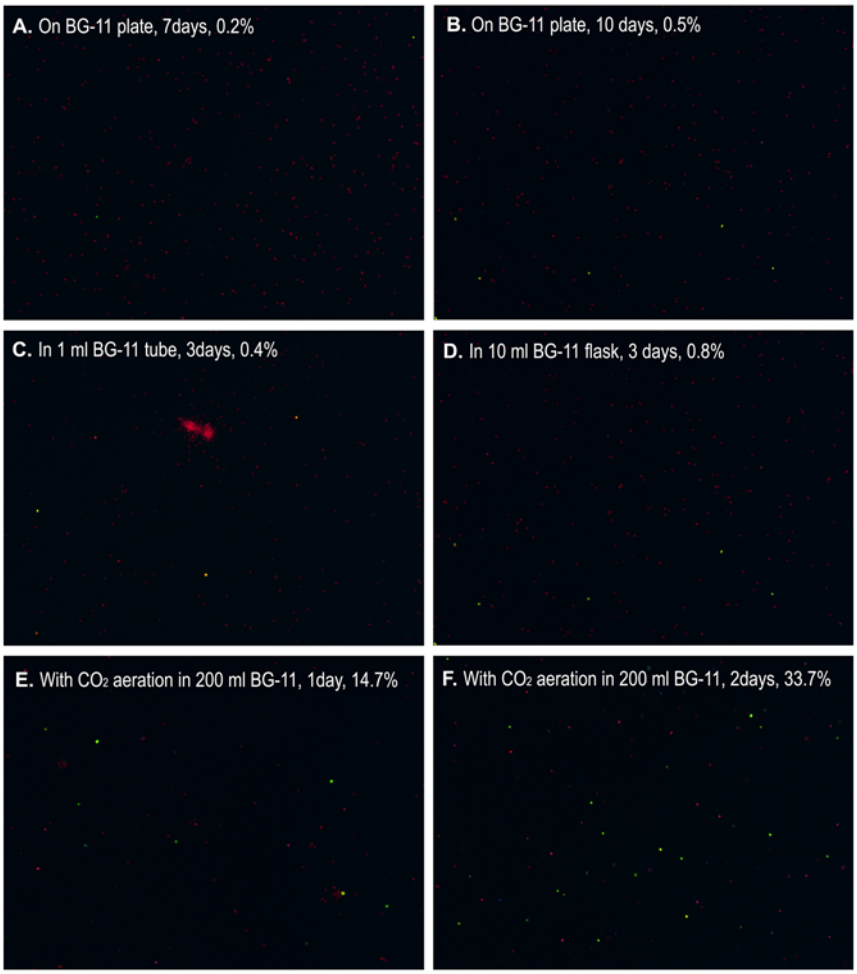


**Fig. S2.** The membrane damage of SD232 cells grown in different stages from a single cell. (*A* and *B*) A single cell derived colony had been growing on a BG-11 agar plate for 7 and 10 days (about $10^6$ cells/colony), respectively; (*C*), cells in a single cell derived colony were inoculated into 1 mL BG-11 medium in a glass tube and grown for 3 days with intermittent shaking (about $8 \times 10^6$ cells/mL); (*D*) the 1 mL SD232 culture was inoculated into 9 mL BG-11 medium in a flask and grown for 3 days with 60 rpm shaking (about $4 \times 10^7$ cells/mL); (*E* and *F*), the 10 mL SD232 was inoculated into 200 mL BG-11 medium and grown for 1 and 2 days, respectively, with 100 mL/ min aeration of 1% $CO_2$-enriched air. The high cell damage percentages in *E* and *F* indicated that the dilution into 200 mL for $4 \times 10^8$ cells was too much and too dilute. In the fluorescence dark optic field, membrane damaged cells are permeable to SYTOX green and fluorescence green, whereas healthy cells fluorescence red. Blue, purple, and yellow cells are all counted as damaged cells. The damaged cell percentages on the basis of counting of at least 400 cells are indicated.

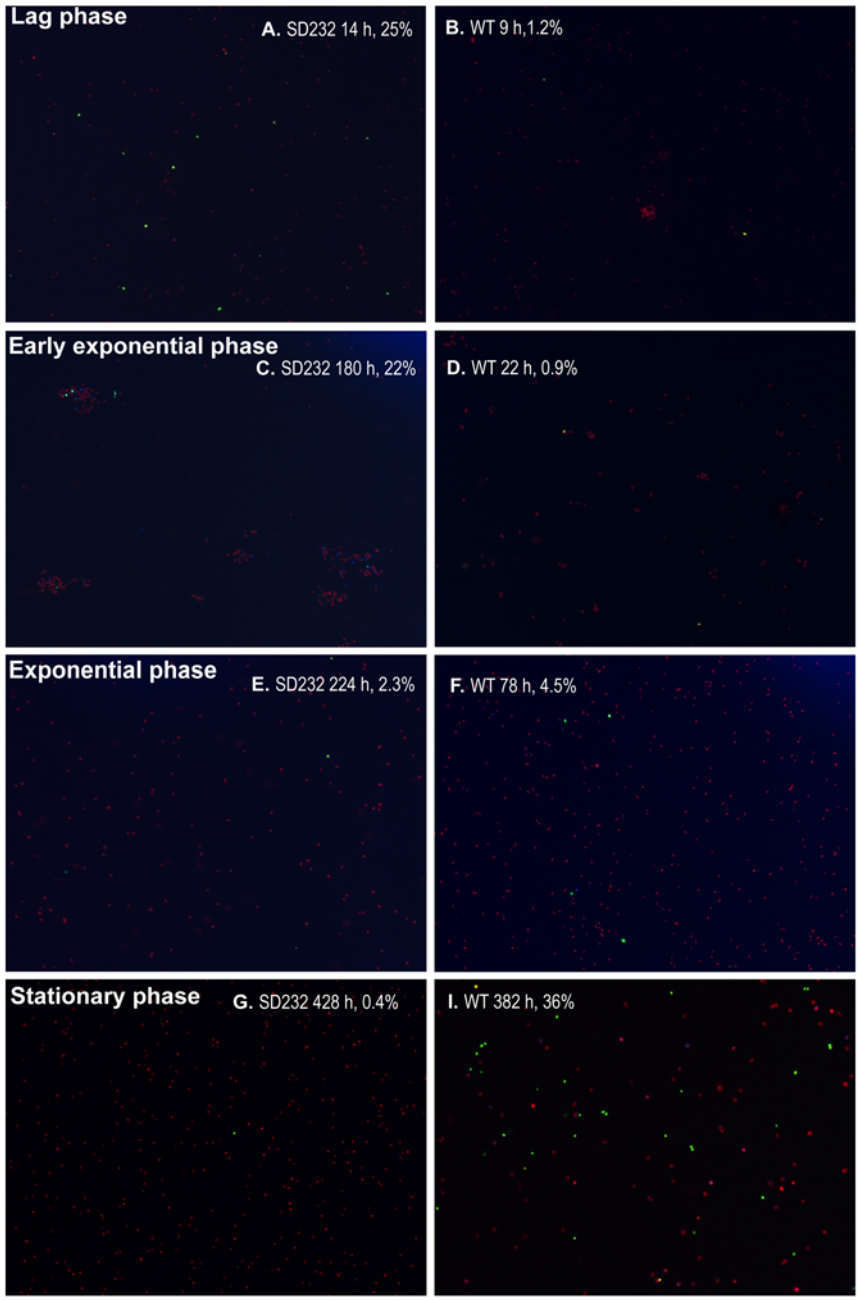


**Fig. S3.** The membrane damages during the growth of SD strains bubbled with 1% $CO_2$-enriched air. SD232 (*Left column*) and WT (*Right column*) cultures were started at $10^6$ cells/mL with 1% $CO_2$ aeration. Cell membrane damages were indicated by SYTOX green staining. The time for growing after inoculation and damaged cell percentages on the basis of counting at least 400 cells are indicated.

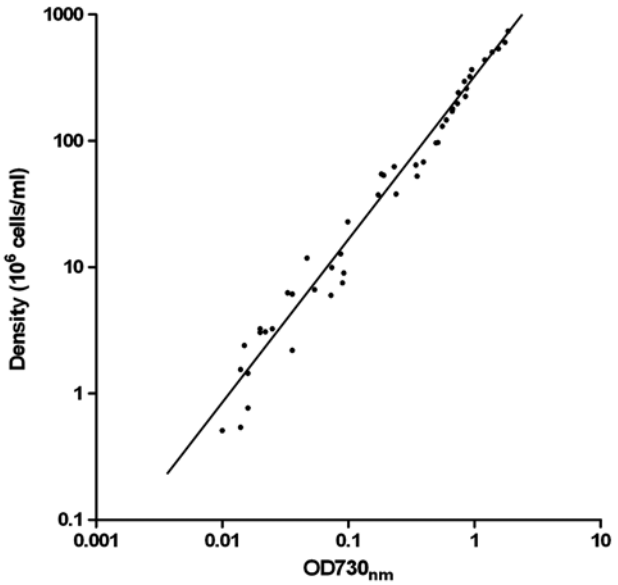


**Fig. S4.** The relationship between 6803 culture cell density and optical density. Forty-five samples from 6803 exponentially growing cultures were measured. Cell density was counted in a haemacytometer (Neubauer, 0.1 mm × 0.0025 $mm^2$). $OD_{730\,nm}$ was measured in a spectrometer (Genesys 10 VIS; Thermo Spectronic).

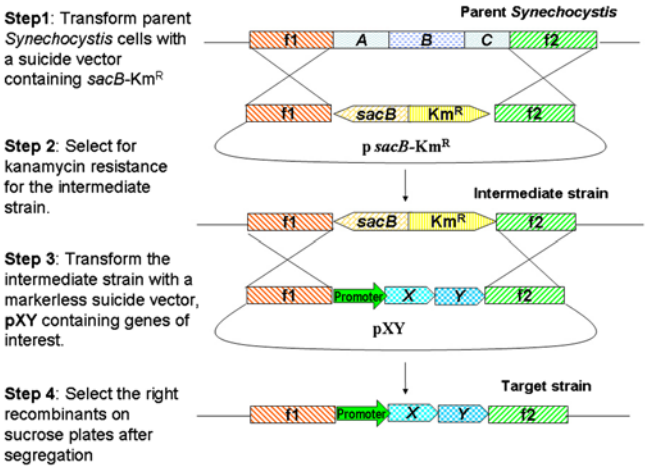


**Fig. S5.** The recombinant strategy used in this project for 6803 genetic engineering.

**Table S1. SD strains constructed for this study**

| SD no. | Genotype* | Construction | Remarks |
|---|---|---|---|
| SD100 | *Synechocystis* sp. PCC 6803 wild-type | | From Dr. Wim Vermaas' lab, School of Life Science, Arizona State University. |
| SD102 | Δ*nrsBAC*::$P_{nrsB}$ *13 sacB* $Km^R$ | Transform SD100 with pΨ102 (Δ*nrsBAC*::$P_{nrsB}$ *13 sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain with a *sacB* $Km^R$ cassette insertion replacing *nsrBAC*, which can be replaced by a further insertion. |
| SD214 | Δ*slr*1609::*sacB* $Km^R$ | Transform SD100 with pΨ214 (Δ*slr*1609::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for deleting fatty acid degrading pathway. The acyl-acyl carrier protein synthase gene (*slr1609*) in 6803 was deleted by *sacB* $Km^R$ insertion. |
| SD215 | Δ*nsrBAC*::$P_{nrsB}$ *'tesA* | Transform SD102 with pΨ215 (Δ*nsrBAC*::$P_{nrsB}$ *'tesA*) and select for sucrose survival | Inducible FFA-secreting strain. *E. coli* **tesA* gene is controlled by Ni inducible promoter. |
| SD216 | Δ*slr*1609::$P_{psbA2}$ *'tesA* | Transform SD214 with pΨ216 (Δ*slr*1609::$P_{psbA2}$ *'tesA*) and select for sucrose survival | Constitutive FFA-secreting strain. *E. coli* **tesA* gene is driven by $P_{psbA2}$, with the same orientation of $P_{fadD}$, and the *slr1609* gene is deleted. |
| SD219 | Δ*nsrBAC*::$P_{nrsB}$ *'tesA* Δ(*slr*1993-*slr*1994)::*sacB* $Km^R$ | Transform SD215 with pΨ207 (Δ(*slr*1993-*slr*1994)::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding genes into SD215. The poly-3-hydroxybutyrate (PHB) synthesis genes in SD215 were deleted by *sacB* $Km^R$ insertion. |
| SD220 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::*sacB* $Km^R$ | Transform SD216 with pΨ207 (Δ(*slr*1993-*slr*1994)::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding multiple genes onto SD216. The PHB synthesis genes in SD216 were deleted by *sacB* $Km^R$ insertion. |
| SD223 | Δ*nsrBAC*::$P_{nrsR}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* | Transform SD215 with pΨ223 (Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA*) and select for sucrose survival | 2nd generation inducible FFA-secreting strain. Acetyl-CoA carboxylase (ACC) overproduction and PHB deletion were incorporated into SD215. |
| SD225 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* | Transform SD216 with pΨ223 (Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA*) and select for sucrose survival | 2nd-generation constitutive FFA-secreting strain. ACC overproduction and PHB deletion were incorporated into SD216. |
| SD228 | Δ*nsrBAC*::$P_{nrsB}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*sacB* $Km^R$ | Transform SD223 with pΨ228 (Δ*sll*1951::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding genes into SD223. The S-layer gene (*sll1951*) in SD223 was deleted by *sacB* $Km^R$ insertion. |
| SD229 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*sacB* $Km^R$ | Transform SD225 with pΨ228 (Δ*sll*1951::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding genes into SD225. The S-layer gene (*sll1951*) in SD225 was deleted by *sacB* $Km^R$ insertion. |
| SD232 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* | Transform SD229 with pΨ231 (Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1*) and select for sucrose survival | 3rd-generation constitutive FFA-secreting strain. Plant C12:0, C8:0, and C10:0 thioesterases overproduction and S-layer deletion were incorporated into SD225. |
| SD240 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* Δ(*slr*2001-*slr*2002)::*sacB* $Km^R$ | Transform SD232 with pΨ240 (Δ(*slr*2001-*slr*2002)::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding genes into SD232. The cyanophycin genes (*slr2001 slr2002*) in SD232 were deleted by *sacB* $Km^R$ insertion. |
| SD243 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* Δ(*slr*2001-*slr*2002)::*$P_{psbA2}$ Ch *fatB2* | Transform SD240 with pΨ243 (Δ(*slr*2001-*slr*2002)::*$P_{psbA2}$ Ch *fatB2*) and select for sucrose survival | 4th-generation constitutive FFA-secreting strain. Plant C8:0, C10:0 thioesterase overproduction and cyanophycin deletion were incorporated into SD232. |
| SD248 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* Δ(*slr*2001-*slr*2002)::*$P_{psbA2}$ Ch *fatB2* Δ*slr*1710::*sacB* $Km^R$ | Transform SD243 with pΨ248 (Δ*slr*1710::*sacB* $Km^R$) and select for kanamycin resistance | Intermediate strain for adding genes into SD249. The penicillin binding protein 2 gene (*slr1710*) in SD243 was deleted by *sacB* $Km^R$ insertion. |
| SD249 | Δ*slr*1609::$P_{psbA2}$ *'tesA* Δ(*slr*1993-*slr*1994)::$P_{cpc}$*accBC* $P_{rbc}$*accDA* Δ*sll*1951::*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2* Δ(*slr*2001-*slr*2002)::*$P_{psbA2}$ Ch *fatB2* Δ*slr*1710::*$P_{psbA2}$ Cc *fatB1* | Transform SD248 with pΨ249 (Δ*slr*1710::*$P_{psbA2}$ Cc *fatB1*) and select for sucrose survival | 5th-generation constitutive FFA-secreting strain. Plant C14:0 thioesterase overproduction and penicillin binding protein 2 deletion were incorporated into SD243. |

*Genetic information: *13*, the holin gene from *Salmonella* phage P22; *sacB*, *sacB* gene, which is lethal for cyanobacteria in the presence of sucrose; $Km^R$, kanamycin resistance cassette; the other information was described in the Fig. 2 legend.

**Table S2. The FFA secretion of SD215 and SD228 after cell wall treatments**

| SD strain | $NiSO_4$ (μM) | Ampicillin (mg/L) | Secreted FFAs (mg/50 mL) | Secretion efficiency* (mg/L/day) | Observation remarks |
|---|---|---|---|---|---|
| SD215 | 0 | 0 | 0.118 | - | No lysis on the third day |
| | 7.0 | 0 | 0.292 | 3.48 | No lysis on the third day |
| | | 1 | 0.376 | 5.16 | No lysis on the third day |
| | | 3 | 0.459 | 6.82 | No lysis on the third day |
| | | 9 | 1.109 | 19.82 | Lysis on the second day |
| | | 25 | - | - | Lysis on the first day |
| SD228 | 0 | 0 | 0.245 | - | No lysis on the third day |
| | 7.0 | 0 | 2.708 | 49.3 | No lysis on the third day |
| | | 1 | 3.572 | 66.5 | No lysis on the third day |
| | | 3 | 3.886 | 72.8 | Lysis on the second day |
| | | 9 | - | - | Lysis on the first day |
| | | 25 | - | - | Lysis on the first day |

*Cultures of 200 mL (about $1.5 \times 10^8$ cells/mL) were induced by adding 7.0 μM $Ni^{2+}$ to the medium and treated with ampicillin of various concentrations.

**Table S3. Primers used in this study**

| Primer name | Sequence (5′ to 3′) |
|---|---|
| Construction of pΨ214 | |
| FadD-F1-A | TAA ACT CTG TAG GCC AGC GGC AA |
| FadD-F1-S | CGT CAA TGC CTA GAC CTA GCA GTA CC |
| FadD-F2-S | AAG GAT TTC CGT TTT ATC CCA GCA CCA |
| FadD-F2-A | GTA ATT GCC ACA GAC AAG CGT ATT CGG |
| KS-NdeI | ACC ATA TGC ATC CTA GGC CTA TTA ATA TTC CGG |
| KS-BamHI | GAA TTA GGA TCC GTC GAC CTG CAG G |
| Construction of pΨ215 | |
| NiF1-S-EcoRI | GAgAA TTc CAG ACG ACT ACG GGC AAA G |
| NiF1-A-toTesA | AAC GTG TCC GCC ATC ACA CCA CCT CAA ATT G |
| TesA-S-toNiF1 | GAG GTG GTG TGA TGG CGG ACA CGT TAT TGA T |
| TesA-A-toNiF2 | CAA AAG GAG CAA TGT GTT ATT TGT CAT CAT CGT CTT |
| NiF2-S-toTesA | GAT GAT GAC AAA TAA CAC ATT GCT CCT TTT GTG CG |
| NiF2-A-BamHI | ACG GAT CCG CAA GCA GTG AAA GAT AG |
| Construction of pΨ216 | |
| TesA-S | CAAATGGCGGACACGTTATTGATTCTG |
| TesA-A | CTT TGT AGT CTG AGT CAT GAT TTA CTA AAG GCT G |
| Test-S-to-pA2 | ATTATAACCAAATGGCGGACACGTTA |
| pA2-S | TCCCCATTGCCCCAAAATACATCC |
| pA2-A-to-TesA | CAA TAA CGT GTC CGC CAT TTG GTT ATA ATT CCT TA |
| Construction of pΨ207 | |
| S4F1-S-PstI | GActgcAGGTCATTGCCGATAAAGTTG |
| S4F1-A-XbaI | AGtctagATAATGTACAGGTCAAGCTGGTCT |
| S4F2-S-SacI | GAgagcTCATTGACACCGAAATGACTTTGG |
| S4F2-A-EcoRI | GAGAATTCTTTGCATTTCCGAAACCACCC |
| S4F2-S-XbaI | GATCTAGAATTGACACCGAAATGACTTTGG |
| S4F2-A-KpnI | GAGGTACCTTTGCATTTCCGAAACCACCC |
| Construction of pΨ223 | |
| Pcpc-S | TAG GCT GTG GTT CCC TAG GCA ACA GT |
| Pcpc-A-to-SynB | TCC GTA AAG TTA ATA GCC ATT GAA TTA ATC TCC TAC TTG AC |
| SynB-S-to-Pcpc | AGG AGA TTA ATT CAA TGG CTA TTA ACT TTA CGG AAC TGC G |
| SynB-A-to-SynC | CAT TGA ATT AAT CTC CTC TAG GGT TTA ATC CAC ATT AGG GTT |
| SynC-S | CCT AGA GGA GAT TAA TTC AAT GCA ATT CGC CAA AAT TTT AAT TGC |
| SynC-A | CTC TCC ATT GAC CTA GGG TGT TAA ATG CTC TTC G |
| SynC-S-to-SynB | GTG GAT TAA ACC CTA GAG GAG ATT AAT TCA ATG CAA TTC GC |
| SynC-A-to-Prbc | CTT TAC TTA TGG CAA TGC TCT CCA TTG ACC TAG GGT GTT |
| Prbc-S | AAC ACC CTA GGT CAA TGG AGA GCA TTG CCA T |
| Prbc-A-to-SynD | CAA TCA AAT AGA GAC ATC TAG GTC AGT CCT CCA TAA AC |
| SynD-S-to-Prbc | AGG ACT GAC CTA GAT GTC TCT ATT TGA TTG GTT TGC C |
| SynD-A-to-SynA | AGT CCT CCT TAA CCA TCT TGA TTG ACG GAA AT |
| SynA-S | GAC CTA GAT GAG TAA AAG TGA GCG TCG TGT TTT TCT |
| SynA-S-to-SynD | TCA AGA TGG TTA AGG AGG ACT GAC CTA GAT GAG TAA AAG TGA |
| SynA-A | TCA TTA CAC CGC CGT TTC TAA AAA TTG ACC CAA ATG |
| SynB-S-Seq | CCT TCG GCC ATC AAG AGA ATG CAG AG |
| SynA-A-Seq | TGA CGC AAC TGT TCA GCC CGA CT |
| Construction of pΨ228 | |
| S5F1S | CAC CAC TTT ACC CAT GAC GGA AGG TGG |
| S5F1A | TGT CTC GGA GTT GCT TAG GGT AAT CAT AGC A |
| S5F2S | TCG CGA ATT CCT GTT CAT CAA CAA CGG TG |
| S5F2A | AAA GCT AAA GCG ACT GAG GAA GTG CCA G |

| Primer name | Sequence (5′ to 3′) |
|---|---|
| Construction of pΨ231 | |
| Fats-S | AGA TAT CGC GTG CAA GGC CCA GTG |
| Fats-A | TGA TAT CAT TAA GAG ACC GAG TTT CCA TTG G |
| Construction of pΨ240 | |
| S7F1-S | GAC TTC CAA AAC GGC GAT CAA GCC AAC C |
| S7F1-A | GTC CAT TAG GGG AGT GTC CGC CAA CA |
| S7F2-S | GGT ACC ATG CAC TGG TGG ATT ACG CC |
| S7F2-A | GGG AAA TTG TTC CGT TAA CTG TTG ATA TTC CCG GT |
| Construction of pΨ243 | |
| ChFatB-s-to-Psba | CTG AAC GAA GGA ATT ATA ACC AAA TGG TGG CTG CTG CTG CTA GTT C |
| PsbA-a-to-ChFatB | GAA CTA GCA GCA GCA GCC ACC ATT TGG TTA TAA TTC CTT CGT TCA G |
| Construction of pΨ248 | |
| S9F1S | CAA TAG GAT TCG TAG AGA TTG AGA TAC TCC ATG GCG T |
| S9F1A | AGC CTT TTT TGA GGG CTA CCT TTT GGC TGT T |
| S9F2S | GGC TCC CTA CTT TTA CGG TTA CAT TTT TGG CGA AT |
| S9F2A | CTA CAA GGA AGC AAT TTG TCG CAT ATA TTG ACC CCA A |
| Segregation checking/sequencing | |
| FadD-F2-Seq | ATA AGT TTG GGT TAC CAC TGG TCG TTT GAG CTT C |
| FadD-F1-Sequ | CTTCCCTTCTTCCTTCCATCTGATTATGGT |
| S4-seg100-S | TGGCTCCCTGACCAATTTTTCGG |
| S4-seg100-A | CCA GGC AAT TTC CTC CGG TTT ACC |
| S5100S | TCA TCG TGT TAA CAG CGG TAT GCT TCT AGT CT |
| S5100A | CAA AGG TAC CGC TAA TAC CTG TAA GTT CTA CGA GG |
| S7 Seg 51S | GGG GAT CAA TTG CGT CTC TGT GGC |
| S7 Seg 90A | CAA AGC GTT GAC CGT GCC AGT TTT TGA C |
| S9-S68 | CCC TAA AAA AAG TCA AAC TAA CCT TTC CCA GGG TGG |
| S9-A71 | CTT CTT TGG CCA CAT CTT CGC CTA GTA AAT GGT T |

**Table S4. Synthesized DNA segments**

*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2*

AGatATcGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTCATCTCCATTGTCCCTGAAAATCAGTTGTGTCGCCCCT
CTACACAGCCCAGAACTATGGTAAAGGCGCACGAAAAACCGCCAGGTAAACTCTTCTCAACCCCCAAAACGCCCTCTGTTTACC
CATGGAAAAAACGACAATTACAAGAAAGTAAAACTTATGTCATCTATAAGCTTCGTGTATATTAACTTCCTGTTACAAAGCTTTACA
AAACTCTCATTAATCCTTTAGACTAAGTTTAGTCAGTTCCAATCTGAACTAAGGAATTATAACCAA
ATG GCt ACC ACC TCT TTA GCT TCC GCc TTt TGC TCG ATG AAA GCT GTA ATG TTa GCT CGT
GAT GGt CGG GGt ATG AAA CCt CGT AGt AGT GAT TTG CAA CTc CGT GCG GGA AAT GCG CCT
ACC TCT TTG AAA ATG ATC AAT GGG ACC AAA TTC AGT TAT ACG GAG AGC TTG AAA CGG TTG
CCT GAT TGG AGC ATG CTC TTT GCT GTT ATC ACC ACC ATC TTT TCG GCT GCT GAG AAA CAA
TGG ACt AAT CTA GAG TGG AAG CCG AAA CCG AAG CTA CCC CAG TTG CTT GAT GAT CAT TTT
GGA CTG CAT GGG TTA GTT TTC CGG CGC ACC TTT GCC ATC CGG TCT TAT GAa GTT GGA CCT
GAT CGC TCC ACC TCT ATT CTG GCT GTT ATG AAT CAT ATG CAG GAG GCT ACC CTT AAT CAT
GCG AAA AGT GTG GGA ATT CTA GGA GAT GGA TTC GGG ACG ACG CTA GAG ATG AGT AAG CGG
GAT CTG ATG TGG GTT GTT CGG CGC ACG CAT GTT GCT GTT GAA CGG TAC CCT ACT TGG GGT
GAT ACT GTA GAA GTA GAG TGC TGG ATT GGT GCT TCT GGA AAT AAT GGC ATG CGT CGT GAT
TTC CTT GTC CGG GAC TGC AAA ACC GGC GAA ATT CTT ACT CGC TGT ACC AGC CTT TCG GTG
CTG ATG AAT ACT CGC ACt CGt CGT TTG TCC ACC ATt CCT GAT GAA GTT CGT GGt GAa ATA
GGG CCT GCT TTC ATc GAT AAT GTT GCT GTg AAA GAC GAT GAA ATT AAG AAA CTA CAA AAA
CTC AAT GAT AGC ACT GCC GAT TAT ATt CAA GGA GGT TTG ACc CCT CGT TGG AAT GAT TTG
GAT GTC AAT CAA CAT GTT AAC AAC CTC AAA TAC GTT GCC TGG GTT TTT GAG ACC GTC CCc
GAt TCC ATC TTT GAG AGT CAT CAT ATT TCC AGC TTC ACT CTT GAA TAT CGT CGT GAG TGt
ACc CGT GAT AGC GTG CTG CGG TCC CTG ACC ACT GTC TCT GGT GGC TCG TCG GAG GCT GGG
TTA GTT TGC GAT CAT TTG CTC CAA CTT GAA GGT GGG TCT GAG GTA TTG CGT GCC AGA ACT
GAG TGG CGG CCT AAA CTT ACC GAT AGT TTC CGC GGc ATT AGT GTT ATT CCC GCC GAA CCG
CGC GTG TAA t gat at c a

*$P_{psbA2}$ Ch *fatB2*

AGatATcGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTCATCTCCATTGTCCCTGAAAATCAGTTGTGTCGCCCCTC
TACACAGCCCAGAACTATGGTAAAGGCGCACGAAAAACCGCCAGGTAAACTCTTCTCAACCCCCAAAACGCCCTCTGTTTAC
CCATGGAAAAAACGACAATTACAAGAAAGTAAAACTTATGTCATCTATAAGCTTCGTGTATATTAACTTCCTGTTACAAAGC
TTTACAAAACTCTCATTAATCCTTTAGACTAAGTTTAGTCAGTTCCAATCTGAACTAAGGAATTATAACCAA
ATG GTG GCT GCT GCT GCT AGT TCC GCT TTC TTC CCT GTT CCA GCC CCc GGA GCC TCC CCT
AAA CCC GGG AAG TTC GGA AAT TGG CCC AGT AGC TTG AGC CCT TCC TTC AAG CCC AAG TCA
ATC CCC AAT GGC GGA TTT CAG GTT AAG GCT AAT GAC AGC GCC CAT CCA AAa GCc AAt GGT
TCT GCc GTT AGT CTA AAG TCT GGC AGC CTC AAC ACT CAa GAa GAC ACT AGT TCC TCC CCT
CCT CCT CGG ACT TTC CTT CAt CAG TTG CCT GAT TGG AGT CGt CTT CTG ACT GCT ATt ACc
ACC GTG TTC GTG AAA TCT AAG CGT CCT GAC ATG CAT GAT CGG AAA TCC AAG CGT CCT GAC
ATG CTG GTG GAC TCC TTT GGG TTG GAG AGT ACT GTT CAG GAT GGc tTa GTG TTC CGA CAG
AGT TTT TCC ATT CGT TCT TAT GAA ATA GGC ACT GAT CGA ACG GCC TCT ATA GAG ACC CTT
ATG AAC CAC TTG CAG GAA ACC TCT CTC AAT CAT TGT AAG AGT ACC GGT ATT CTC CTT GAC
GGC TTC GGT CGT ACT CTT GAG ATG TGT AAA CGC GAC CTC ATT TGG GTG GTA ATT AAA ATG
CAG ATC AAG GTG AAT CGC TAT CCA GCT TGG GGC GAT ACT GTC GAG ATC AAT ACC CGt TTC
agC CGG TTG GGG AAA ATt GGT ATG GGT CGC GAT TGG CTA ATT AGT GAT TGC AAC ACC GGA
GAA ATT CTT GTA CGG GCT ACG AGC GCG TAT GCC ATG ATG AAT CAA AAG ACG CGG AGA CTC
TCC AAA CTT CCA TAC GAG GTT CAC CAG GAG ATT GTG CCT CTT TTT GTC GAC TCT CCT GTC
ATT GAA GAC AGT GAT CTG AAA GTG CAT AAG TTT AAA GTG AAG ACT GGT GAc agC ATT CAA
AAG GGT CTA ACT CCG GGG TGG AAT GAC TTG GAT GTC AAT CAG CAC GTA AGC AAC GTG AAG
TAC ATT GGG TGG ATT CTC GAG AGT ATG CCA ACA GAA GTT TTG GAG ACC CAG GAG CTA TGC
TCT CTC GCC CTT GAA TAT CGC CGG GAA TGC GGA CGC GAC AGT GTG CTG GAG TCC GTG ACC
GCT ATG GAT CCC TCC AAA GTT GGA GTC CGT TCT CAG TAC CAG CAC CTT CTG CGG CTT GAG
GAT GGG ACT GCT ATC GTG AAC GGT GCT ACT GAG TGG CGG CCG AAG AAT GCA GGA GCT AAC
GGG GCG ATc agC ACG GGA AAG ACT TCC AAT GGA AAC TCG GTC TCT TAA t gat at c a

*$P_{psbA2}$ Cc *fatB1*

AGatATcGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTCATCTCCATTGTCCCTGAAAATCAGTTGTGT
CGCCCCTCTACACAGCCCAGAACTATGGTAAAGGCGCACGAAAAACCGCCAGGTAAACTCTTCTCAACCCCCAAA
ACGCCCTCTGTTTACCCATGGAAAAAACGACAATTACAAGAAAGTAAAACTTATGTCATCTATAAGCTTCGTGTAT
ATTAACTTCCTGTTACAAAGCTTTACAAAACTCTCATTAATCCTTTAGACTAAGTTTAGTCAGTTCCAATCTGAA
CTAAGGAATTATAACCAA
ATG AAA ACT ACT TCT CTC GCC TCT GCC TTC TGT TCT ATG AAA GCT GTT ATG CTG GCg CGG
GAT GGT CGC GGT ATG AAA CCC CGT TCC AGT GAT CTG CAA TTA CGG GCT GGC AAC GCT CAG
ACC TCC TTG AAG ATG ATT AAC GGC ACT AAA TTC AGT TAT ACC GAA TCT TTG AAG AAA CTC
CCC GAT TGG AGC ATG TTG TTC GCC GTG ATT ACC ACC ATt TTt AGT GCT GCC GAA AAA CAA
TGG ACC AAT CTC GAA TGG AAA CCC AAA CCC AAC CCC CCG CAG CTG CTC GAT GAC CAT TTT
GGc CCC CAC GGC TTG GTG TTT CGG CGT ACC TTC GCT ATC CGG TCT TAT GAA GTC GGT CCC
GAT CGG AGC ACT TCC ATC GTC GCT GTT ATG AAT CAC TTG CAA GAA GCC GCT TTG AAC CAt
GCT AAA tct GTT GGG ATT CTG GGT GAT GGC TTC GGT ACC ACT CTG GAG ATG AGT AAG CGC
GAT CTG ATC TGG GTA GTA AAG CGT ACt CAT GTG GCC GTG GAA CGT TAt CCg GCC TGG GGT
GAT ACC GTA GAA GTG GAG TGT TGG GTA GGC GCC TCC GGT AAC AAC GGT CGG CGT CAC GAC
TTC TTG GTG CGT GAC TGT AAA ACt GGc GAG ATC CTG ACC CGC TGT ACT TCC CTG AGC GTT
ATG ATG AAC ACC CGG ACC CGT CGC TTA TCC AAG ATT CCC GAA GAA GTT CGC GGG GAA ATT
GGt CCt GCT TTC ATT GAT AAC GTT GCT GTT AAG GAT GAG GAG ATT AAA AAG CCG CAA AAG

*$P_{psbA2}$ Uc *fatB1* $P_{rbc}$ Ch *fatB2*

CTC AAT GAT TCT ACC GCC GAT TAC ATT CAA GGG GGT CTG ACT CCC CGT TGG AAT GAT CTG
GAT ATT AAT CAG CAT GTG AAT AAC ATC AAA TAT GTG GAT TGG ATT CTG GAG ACT GTG CCC
GAC TCT ATT TTC GAG TCC CAC CAC ATT agc AGT TTT ACC ATT GAA TAT CGT CGC GAA TGT
ACT ATG GAC AGT GTT TTG CAA TCC CTG ACC ACC GTC TCC GGC GGT TCC TCT GAA GCT GGC
CTG GTG TGC GAA CAC CTC TTG CAA CTC GAA GGC GGT AGT GAA GTg CTc CGt GCC AAG ACC
GAA TGG CGG CCC AAA TTG ACC GAC TCC TTT CGC GGG ATT TCT GTG ATT CCC GCC GAA TCC
TCC GTC TAA GATATCAT