


High-Light-Induced Stress Activates Lipid Deacylation at the *Sn*-2 Position in the Cyanobacterium *Synechocystis* Sp. PCC 6803

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Cyanobacterial mutants defective in acyl-acyl carrier protein synthetase (Aas) produce free fatty acids (FFAs) because the FFAs generated by deacylation of membrane lipids cannot be recycled. An engineered Aas-deficient mutant of *Synechocystis* sp. PCC 6803 grew normally under low-light (LL) conditions (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) but was unable to sustain growth under high-light (HL) conditions (400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), revealing a crucial role of Aas in survival under the HL conditions. Several-times larger amounts of FFAs were produced by HL-exposed cultures than LL-grown cultures. Palmitic acid accounted for $\sim 85\%$ of total FFAs in HL-exposed cultures, while C_{18} fatty acids (FAs) constituted $\sim 80\%$ of the FFAs in LL-grown cultures. Since C_{16} FAs are esterified to the *sn*-2 position of lipids in the *Synechocystis* species, it was deduced that HL irradiation activated deacylation of lipids at the *sn*-2 position. Heterologous expression of FarB, the FFA exporter protein of *Neisseria lactamica*, prevented intracellular FFA accumulation and rescued the growth defect of the mutant under HL, indicating that intracellular FFA was the cause of growth inhibition. FarB expression also decreased the 'per-cell' yield of FFA under HL by 90% and decreased the proportion of palmitic acid to $\sim 15\%$ of total FFA. These results indicated that the HL-induced lipid deacylation is triggered not by strong light per se but by HL-induced damage to the cells. It was deduced that there is a positive feedback loop between HL-induced damage and lipid deacylation, which is lethal unless FFA accumulation is prevented by Aas.

Keywords: Acyl-ACP synthetase • Lipid deacylation
• Photoinhibition • Toxicity to FFAs

Introduction

Free fatty acids (FFAs) are naturally produced by cyanobacteria via deacylation of membrane lipids, but the cells do not normally accumulate FFAs or secrete them out of the cells since the FFAs are readily esterified to acyl carrier protein (ACP) by acyl-ACP synthetase (Aas) and recycled to lipids (Kaczmarzyk and Fulda 2010). Initial physiological characterization of the Aas-null mutants of model cyanobacterial species *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 revealed secretion of FFAs out of the cells but there was no noticeable growth phenotype under illumination at 30–60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Kaczmarzyk and Fulda 2010, Ruffing 2014). Takatani et al. (2015) later showed that an Aas-deficient *S. elongatus* PCC 7942 grows slightly slower than the wild-type (WT) under low-light (LL) conditions (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and unlike WT, it cannot increase the growth rate in response to a shift to high-light (HL) conditions (400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). It was shown that lipid deacylation is enhanced under the HL condition and that the resulting FFAs destabilize photosystem II (PSII), making the cells more susceptible to photodamage (Takatani et al. 2015). These results showed the importance of Aas in acclimation of *S. elongatus* to the HL conditions. In this study, we characterized an Aas-deficient mutant (dAS11) constructed from *Synechocystis* sp. PCC 6803. HL-responsive lipid deacylation is shown to take place also in *Synechocystis* sp. PCC 6803, causing severe growth inhibition of the mutant. Involvement of an *sn*-2 specific lipase is deduced from the FFA composition of the HL-exposed cells. We show that heterologous expression of a bacterial FFA exporter prevents the

accumulation of intracellular FFAs, largely rescues the growth phenotype of the mutant, and at the same time attenuates the lipase activation under HL. Based on these findings, we propose that there is a positive feedback between photodamage to the cells, which is enhanced by intracellular FFA, and upregulation of an *sn*-2 specific lipase. Possible roles of lipid deacylation and FFA recycling in HL acclimation of cyanobacteria are discussed.

Results

Sensitivity of an *aas*-deficient mutant of *Synechocystis* sp. PCC 6803 to high-light conditions

Fig. 1 represents the appearance of the cultures of the WT strain and an *Aas*-deficient mutant (dAS11) of *Synechocystis* sp. PCC 6803 during cultivation under LL ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and HL ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions. The dAS11 culture became bluish after 3–4 days of cultivation under the HL conditions and completely lost pigmentation in 7 days, whereas no decrease in pigmentation was observed under the LL conditions (**Fig. 1**). In a previous study in *S. elongatus* PCC 7942, the *aas*-deficient mutant dAS1 was shown to suffer from enhanced photodamage to PSII under the HL conditions, but the cells could sustain growth under HL albeit at a lower rate as compared to the WT strain (Takatani et al. 2015). These results indicated that *Aas* plays a more crucial role in *Synechocystis* sp. PCC 6803 than in *S. elongatus* PCC 7942 in the acclimation of the cells to the HL conditions.

Functional expression of a *Neisseria lactamica* FFA exporter in *Escherichia coli*

Aas-deficient cyanobacterial mutants produce FFAs, which can be lethal if accumulated at high levels in the cell (Kato et al. 2015, 2016, 2017). To examine whether an increase in the FFA export activity could rescue the HL-sensitive phenotype of the

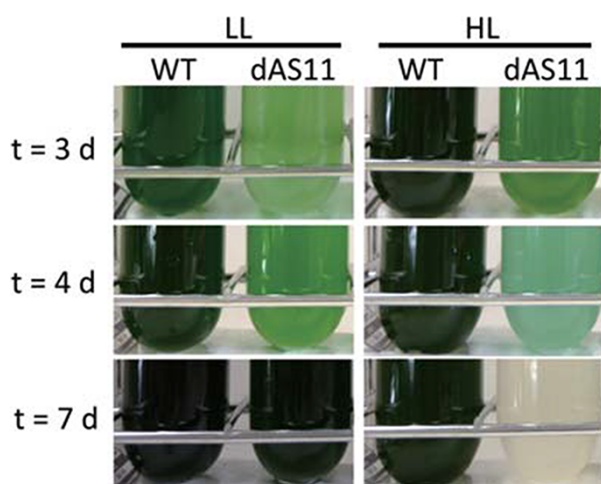


Fig. 1 Appearance of the WT and dAS11 cultures during growth under the HL and LL conditions. Cells grown under LL conditions ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were inoculated into new medium to give an OD_{730} value of 0.01 and incubated under the LL or HL ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions for the indicated period.

dAS11 mutant, we planned expression in dAS11 of the tripartite FFA efflux pump conserved among the bacteria of the *Neisseria* genus (Lee and Shafer 1999, see also **Supplementary Fig. S1**), which is composed of FarB (an MFS family transporter), MtrE (an outer membrane channel protein, OMP) and FarA (a membrane fusion protein, MFP). Since heterologous expression of the *Neisseria* FFA efflux pump had not been reported, we first tried it in *Escherichia coli*. Construction of a plasmid carrying the transcriptional fusion of the *farAB* operon and the *mtrE* gene under the control of the P_{trc} promoter was unsuccessful in *E. coli* even in the absence of isopropyl β -D-1-thiogalactopyranoside (IPTG) (data not shown), suggesting that expression of the whole set of the proteins comprising the FFA pump might be toxic to the *E. coli* host. We hence examined the effects of plasmid-based expression of the P_{trc} -*farAB*, P_{trc} -*farA* or P_{trc} -*farB* genes in an *E. coli* K12 Δ *acrAB* Δ *emrB* mutant, which lacks two (*AcrAB*-*TolC* and *EmrAB*-*TolC*) of the three efflux pumps known to mediate FFA efflux (Lennen et al. 2013). While the WT *E. coli* cells were resistant to lauric acid (12:0) and palmitic acid (16:0) added to the medium to a concentration of 1 mM, the Δ *acrAB* Δ *emrB* mutant was sensitive to 12:0 (**Fig. 2**). Plasmid-based expression of *farA* or *farAB* did not confer the cells the tolerance to exogenously added 12:0, but surprisingly, that of *farB* rendered the cells tolerant to 12:0 (**Fig. 2C**). These results indicated that expression of FarB supported export of 12:0 out of the cell, but co-expression of FarA interfered with it.

Negative effect of FarA on the tolerance of *Synechocystis* cells to extrinsic linolenic acid

Fig. 3 shows the effects of exogenously added linolenic acid (18:3) on growth of the WT *Synechocystis* sp. PCC 6803 strain, the dAS11 mutant and the dAS11 derivatives expressing either *farA* and *farB* or both *farA* and *farB*. *Aas* has been shown to facilitate passive entrance of extrinsic FFAs into the cell by consuming intracellular FFAs (Kaczmarzyk and Fulda 2010). Exogenously added 18:3 is generally toxic to cyanobacteria (Sakamoto et al. 1998, Maeda et al. 2005), but as reported previously (von Berlepsch et al. 2012, Kojima et al. 2016), the *Aas*-deficient mutant was tolerant to the fatty acid (FA) added to the medium to 100 μM . Expression of *farA* in dAS11 completely abolished the ability of the cells to grow in the presence of the FA, while that of *farB* did not affect it. Interestingly, the cells co-expressing *farA* and *farB* were tolerant to 18:3, showing that FarB could partially suppress the negative effect of FarA.

Positive effect of FarB on the tolerance of *Synechocystis* cells to extrinsic FFAs

Fig. 4 shows the growth curves of WT, dAS11 and the dAS11-*farB* strains in liquid media supplemented with various FFAs under the LL conditions. Growth of the WT strain was severely inhibited by 100 μM of 12:0 and 18:3 (**Fig. 4B, E**, black circles), but the inhibitory effects of these FFAs were partially attenuated by the deficiency of *Aas* as previously reported (**Fig. 4B, E**, white circles; von Berlepsch et al. 2012, Kojima et al. 2016; see also **Fig. 3**). Expression of *farB* was found

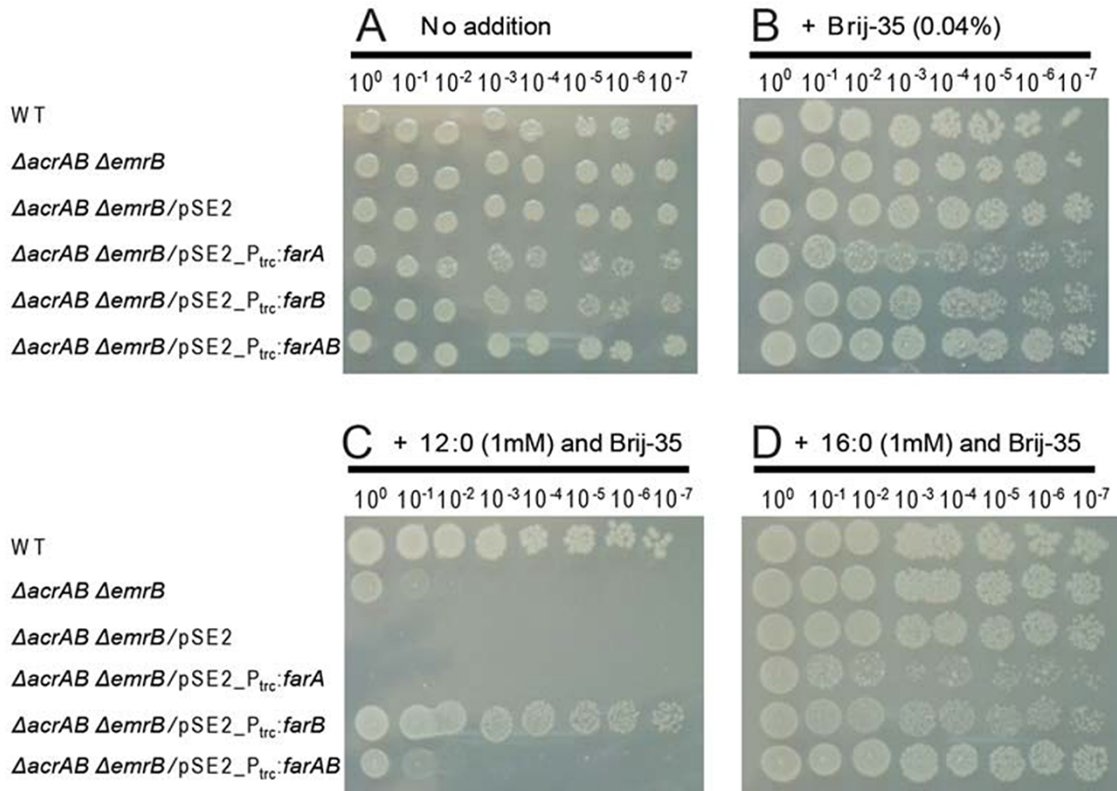


Fig. 2 Effects of expression of either or both of the *N. lactamica* *farA* and *farB* genes on growth of the *E. coli* K12 Δ acrAB Δ emrB mutant in the presence of exogenously added FFAs. Three microliters of the *E. coli* cell suspensions ($OD_{600} = 1.0$) and their 10-fold serial dilutions were spotted onto solid media and incubated for 1 day at 37°C. (A, B) Control experiments performed without (A) and with (B) 0.04% Brij-35. (C, D) Viability test performed in the presence of 0.04% Brij-35 and 1 mM 12:0 (C) and 16:0 (D). Numbers on the top indicate the dilution factor. The results from one of the three experiments, which yielded essentially the same results, are shown.

to further improve the growth of the *aas*-deficient strain in the presence of these FFAs (Fig. 4B, E, gray circles), suggesting the involvement of FarB in the export of the toxic FFAs. Unlike 12:0 and 18:3, 16:0 is normally non-toxic to cyanobacteria (Ruffing and Trahan 2014), but at a concentration of 500 μ M, it significantly inhibited the growth of dAS11 (Fig. 4C, white circles). Similar effects of exogenously added 16:0 were previously reported for a FFA-producing mutant of *S. elongatus* PCC 7942 and ascribed to an interference of the secretion of the endogenously produced FFA out of the cells (Kato et al. 2017). The inhibitory effect of 16:0 on dAS11 was rescued by expression of *farB* (Fig. 4C, gray circles), showing that FarB could mediate export of 16:0 as well as of 12:0 and 18:3.

Effects of *farB* expression on growth and photosynthetic activity of the *aas*-deficient *Synechocystis* mutant under high-light conditions

Fig. 5 shows the growth curves of the WT, dAS11 and dAS11_ *farB* strains under the LL and HL conditions. Under the LL conditions, the final optical density of the dAS11 culture was comparable to that of WT, although the growth of the mutant was slower than that of WT (Fig. 5A). Cultivation

under the HL conditions increased the growth rates of both WT and dAS11 as compared to those under the LL conditions, but in the case of the mutant, cell growth ceased after 3 days of exponential growth (Fig. 5B). The cells thereafter showed a

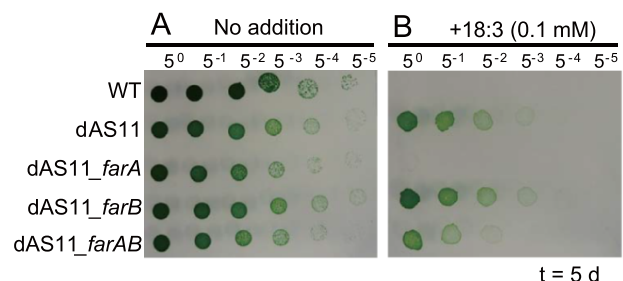


Fig. 3 Effects of expression of either or both of the *N. lactamica* *farA* and *farB* genes on growth of the Aas-deficient *Synechocystis* sp. PCC 6803 mutant in the presence of 18:3. Three microliters of the *Synechocystis* cell suspensions ($OD_{730} = 1.0$) and their 5-fold serial dilutions were spotted onto solid media and incubated for 5 d at 30°C under illumination at 25 μ mol photons $m^{-2} s^{-1}$. Growth of the cells in the absence (A) and the presence (B) of 0.1 mM 18:3 is compared. Numbers on the top indicate the dilution factor. A set of data from one of the three experiments, which yielded essentially the same results, is shown.

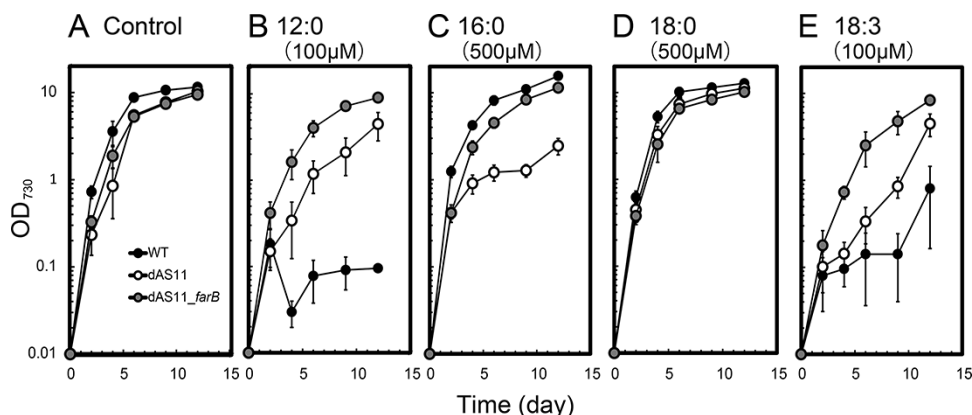


Fig. 4 Effects of Aas deficiency and *farB* expression on *Synechocystis* growth in the presence of various FAs. Growth curves of the WT (black circles), dAS11 (white circles) and dAS11_ *farB* (gray circles) strains were compared in the presence or absence of FAs (labeled on the top of the panel). Data shown are mean \pm SE from biological triplicates.

decrease in pigmentation as mentioned above (Fig. 1). Interestingly, growth of the mutant resumed several days after the growth arrest, showing a recovery of pigmentation. Growth of dAS11_ *farB* was essentially the same as that of dAS11 under the LL conditions (Fig. 5A). Even under the HL conditions, the *farB* expressing strain did not show the growth arrest or the bleaching phenotype (Fig. 5B). Thus, expression of *FarB* largely rescued the growth defect caused by the deficiency of Aas, although the final cell density of the dAS11_ *farB* strain was somewhat lower than that attained by WT (Fig. 5B).

The PSII activity, as determined by measuring the Fv Fm⁻¹ ratio, showed a sharp decline between the third and fourth days of incubation of the dAS11 cells under HL (Fig. 5C, D), suggesting that the decrease of PSII activity was the cause of the growth arrest. In contrast, no such decline was observed in the PSII yield of WT or the dAS11_ *farB* strain. In the *aas*-deficient cells of *S. elongatus* PCC 7942, intracellular FFA accumulation was shown to render PSII unstable and to make the cells sensitive to HL (Takatani et al. 2015). The tolerance of PSII of the dAS11_ *farB* strain to the HL conditions therefore suggested that *FarB* prevented FFA accumulation in the cells and thereby alleviated photoinhibition.

Effects of *farB* expression on the PSII activity and photodamage to PSII in the *aas*-deficient *Synechocystis* mutant

Fig. 6A compares the PSII activity of LL-grown cells of WT, dAS11 and dAS11_ *farB*. The PSII activity of dAS11 was lower than that of WT by about 40%, whereas that of the dAS11_ *farB* strain was essentially the same as that of WT. Expression of *farB* thus restored the activity of PSII of the *aas*-deficient strain to a level comparable to that observed in WT.

To further investigate the effects of *aas* deficiency and *farB* expression on the process of photoinhibition of PSII, the time courses of photoinhibition of WT and the mutant strains were compared in the absence and presence of lincomycin,

which inhibits the repair of PSII (Fig. 6B). In the absence of lincomycin (Fig. 6B), PSII activity of the LL-grown cells was decreased by about 40% after 20 min of exposure to illumination of 1,500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in all the strains. In WT and dAS11_ *farB*, there was only a small decrease in the PSII activity thereafter, whereas dAS11 appeared to suffer from further decrease in the PSII activity, which was decreased to \sim 40% of the initial level in 60 min of HL irradiation. However, the difference in PSII activity between dAS11 and the other strains was not statistically significant. When lincomycin was added to inhibit the repair of photodamaged PSII, on the other hand, the HL-induced decrease of PSII activity was significantly faster in dAS11 than in WT or dAS11_ *farB* (Fig. 6B), showing that PSII was more sensitive to photodamage in dAS11 than in the other two strains. Expression of *farB* thus stabilized the activity of PSII in the *aas*-deficient mutant, suggesting that intracellular accumulation of FFA was responsible for the hypersensitivity of PSII to photodamage in dAS11.

Effects of light intensity and *farB* expression on the intracellular and extracellular FFA levels

Fig. 7A compares the cellular FFA content of the WT and the *aas*-deficient mutants after 7 days of cultivation. The intracellular FFAs were determined by LC-MS analysis of the extracts of the cells collected by centrifugation. Under the HL conditions, dAS11 cells accumulated FFAs to a level as high as 250 $\mu\text{g per } 1 \times 10^8$ cells, which corresponded to 0.3 g of FFA ml^{-1} of cell volume. The cells of the *farB*-expressing *aas*-deficient mutant also accumulated larger amounts of FFAs under the HL conditions than under the LL conditions, but the FFA level in the HL-grown cells was only \sim 20 $\mu\text{g per } 1 \times 10^8$ cells (Fig. 7A). The amounts of total intracellular FFA were calculated for each of the cyanobacterial cultures (Fig. 7C), using the data in Fig. 7A and the cell density of each culture (Fig. 7B). In spite of the lower cellular FFA content of dAS11_ *farB* cells as compared to that of dAS11 (Fig. 7A), the total intracellular FFA in the dAS11_ *farB*

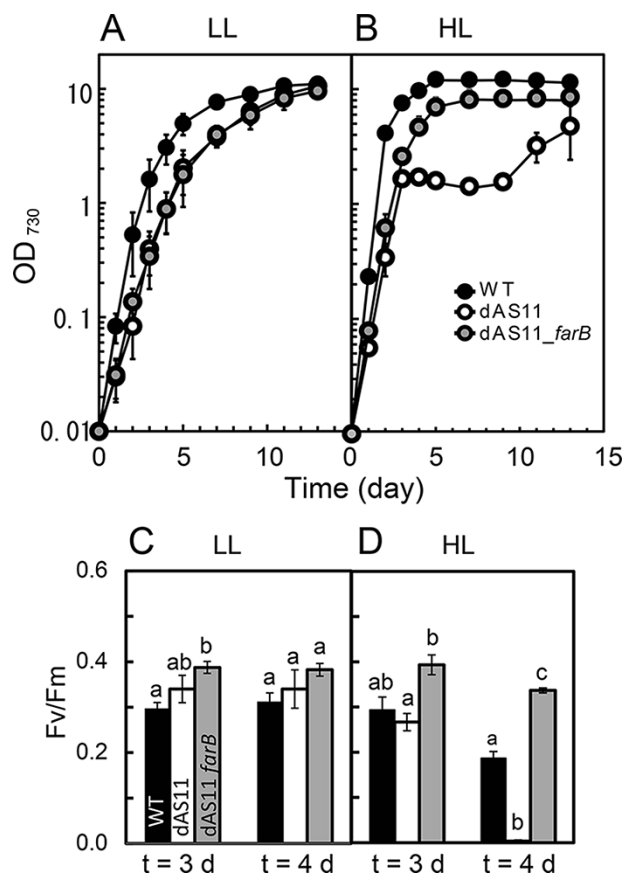


Fig. 5 Rescue by *farB* expression of the HL-sensitive phenotype of the *Synechocystis aas* mutant. Upper panels compare the growth curves of the WT (black circles), dAS11 (white circles) and dAS11_ *farB* (gray circles) strains under the LL (A) and HL (B) conditions. Lower panels show the photosynthetic yield of the three strains after 3 and 4 d of incubation under the LL (C) and HL (D) conditions. Data shown are mean \pm SE from biological triplicates. The letters denote significant differences ($P < 0.05$, Tukey's test).

culture was comparable to that in the dAS11 culture because of the high cell density of the dAS11_ *farB* culture (Fig. 7B, E).

The amounts of FFAs secreted from the cells, as determined enzymatically after removal of the cells from the cultures by centrifugation, are shown in Fig. 7D. Low but significant amounts of extracellular FFAs ($2\text{--}4\ \mu\text{g ml}^{-1}$ of culture) were detected in LL-grown cultures of all the strains including WT. When grown under the HL conditions, the extracellular FFA level was increased in all the strains, but the highest level was attained by dAS11_ *farB*; The HL-grown dAS11_ *farB* cultures had 3 and 5 times more extracellular FFAs than the dAS11 and the WT cultures, respectively. The higher extracellular FFA concentration and the lower cellular FFA content in HL-grown cultures of dAS11_ *farB* than in those of dAS11 (Fig. 7A, D) verified the contribution of FarB to FFA secretion out of the cells.

Comparison of the data in Fig. 7C, D indicated that in the HL-grown cultures of dAS11 and dAS11_ *farB*, over 80% of FFA

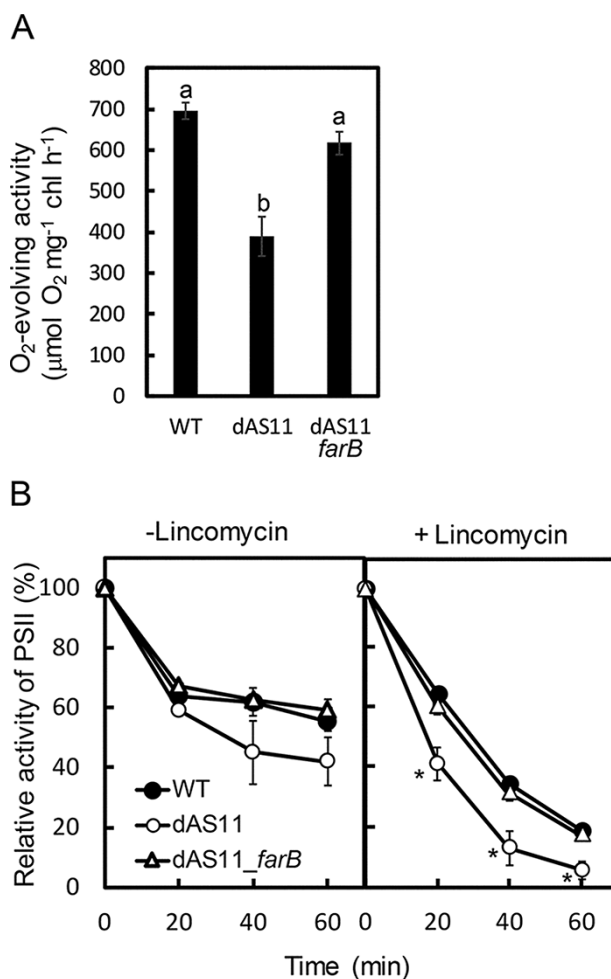


Fig. 6 Effects of *aas* deficiency and *farB* expression on the PSII activity (A) and the sensitivity of PSII to photoinhibition (B) in *Synechocystis* sp. PCC 6803. (A) PSII activity of the WT, dAS11 and dAS11_ *farB* cells grown under LL conditions ($50\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) for 24 h. The letters denote significant differences ($P < 0.01$, Tukey's test). (B) Susceptibility of the WT (filled circles), dAS11 (open circles) and dAS11_ *farB* (open triangle) cells to photoinhibition. The LL-grown cells were incubated under $1500\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ light conditions for 60 min in the absence or presence of $200\ \mu\text{g ml}^{-1}$ lincomycin and the PSII activity was measured at the indicated time points. One hundred percent activity for each of the strains was in the range shown in (A). Data shown are mean \pm SE (bars) from three independent experiments. Asterisks indicate significant differences compared with WT ($P < 0.01$, *t*-test).

was retained in the cells (Fig. 7C, D). The total amount of the FFA produced by the cells under HL, as calculated from the data in Fig. 7C, D, was essentially the same in the two strains, being about $190\ \text{mg l}^{-1}$ (Fig. 7E), from which the average rate of FFA production was calculated to be $\sim 1.1\ \text{mg l}^{-1}\ \text{h}^{-1}$. Due to the much higher cell density in the dAS11_ *farB* cultures (Fig. 7B), dAS11_ *farB* produced only 8.6% of FFA as compared to dAS11 on a 'per-cell' basis (Fig. 7F).

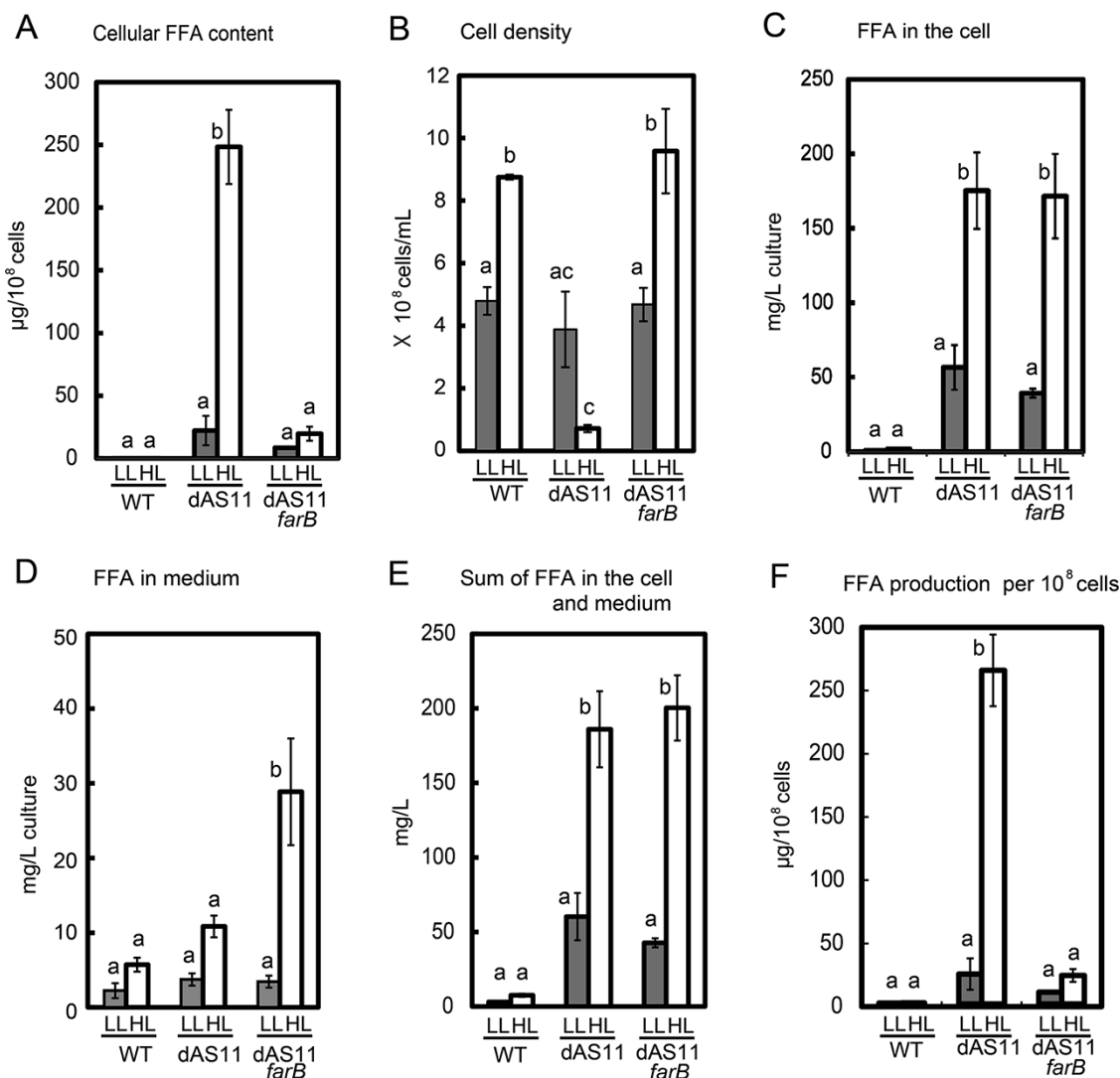


Fig. 7 Effects of light intensity and *farB* expression on extracellular and intracellular FFA levels of the *Synechocystis aas* mutant cultures. Cells of the WT, dAS11 and dAS11_ *farB* strains were grown in the liquid medium under the LL or HL conditions as in Fig. 5. Intracellular FFA content (A), the cell density of the culture (B) and the FFA concentration in the medium (D) were measured for each culture after 7 d of cultivation and used to calculate total intracellular FFA l^{-1} of culture (C), total FFA in the culture (E) and FFA production per cell (F). Data shown are mean \pm SE from biological triplicates. Different letters denote significant differences ($P < 0.05$, Tukey's multiple comparison test).

Effects of light intensity and *farB* expression on the composition of the FFAs

Table 1 shows the effects of light conditions on the composition of the FFA pool in the Aas-deficient mutants. In the LL-grown dAS11 cells, the C₁₈ FFAs including stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) and linolenic acid (18:3) constituted 86% of the FFAs. Since the C₁₈ FFAs are esterified to the *sn*-1 position of the membrane lipids in *Synechocystis* sp. PCC 6803 (Wada and Murata 1989), the results indicated preferential deacylation of the lipids at the *sn*-1 position under these conditions. In the HL-grown cultures, by contrast, 16:0 was the major constituent of the FFAs accounting for 70% of the total. Given that 16:0 is esterified to the *sn*-2 position of the membrane lipids of *Synechocystis* sp. PCC 6803 except in SQDG where some 16:0 is esterified also to the *sn*-1 position (Wada and Murata 1989),

it was deduced that HL irradiation activated deacylation of the lipids at the *sn*-2 position. In the case of the dAS11_ *farB* strain, on the other hand, the major constituents of the FFAs were the C₁₈ FFAs irrespective of the light conditions. The results indicated that the strong light per se was not responsible for the activation of lipid deacylation at the *sn*-2 position; it was deduced that HL-induced stresses triggered the lipid deacylation at *sn*-2.

Discussion

FFA production using cyanobacteria is considered to be one of the promising approaches to sustainable production of biofuels (Wang et al. 2020). Targeted inactivation of the *aas* gene is an essential step in the engineering of FFA-producing cyanobacterial mutants. In addition, a

Table 1 Relative amounts of the intra- and extracellular FFAs in HL- and LL-grown *Synechocystis* mutants

Strain	Light conditions	FFA (mol%)								
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	Total C ₁₆	Total C ₁₈
Intracellular FFA										
dAS11	LL	2 (1)	20 (12)	<0.5	60 (11)	5 (0)	7 (1)	5 (2)	20 (12)	77 (13)
	HL	3 (0)	80 (1)	1 (0)	10 (0)	1 (0)	5 (0)	1 (0)	80 (1)	17 (1)
dAS11_ <i>farB</i>	LL	<0.5	7 (1)	<0.5	68 (2)	6 (0)	8 (0)	9 (1)	7 (1)	91 (1)
	HL	1 (1)	20 (5)	1 (0)	50 (10)	1 (0)	15 (3)	11 (2)	21 (5)	77 (6)
Extracellular FFA										
dAS11	LL	2 (2)	5 (3)	2 (0)	14 (1)	3 (0)	17 (2)	52 (5)	7 (3)	87 (7)
	HL	3 (0)	62 (3)	1 (0)	16 (1)	1 (0)	10 (1)	5 (1)	63 (3)	31 (2)
dAS11_ <i>farB</i>	LL	<0.5	1 (0)	2 (0)	23 (10)	2 (0)	15 (2)	55 (9)	2 (1)	96 (1)
	HL	1 (0)	9 (2)	3 (1)	21 (8)	1 (0)	21 (3)	37 (4)	12 (3)	80 (5)
Total FFA										
dAS11	LL	2 (2)	8 (3)	1 (0)	36 (11)	4 (1)	13 (1)	33 (4)	10 (3)	85 (7)
	HL	3 (0)	69 (2)	1 (0)	13 (1)	1 (0)	8 (1)	3 (1)	70 (1)	25 (1)
dAS11_ <i>farB</i>	LL	<0.5	5 (1)	1 (0)	53 (6)	5 (1)	10 (1)	25 (7)	6 (1)	93 (1)
	HL	1 (0)	13 (3)	2 (1)	32 (8)	1 (0)	19 (2)	27 (3)	16 (4)	79 (5)

heterologous thioesterase gene(s) (e.g. the *tesA* gene of *E. coli*), which cleaves FFA from acyl-ACP, is introduced to increase FFA production. Although cyanobacteria have endogenous lipase activity (Kaczmarzyk and Fulda 2010), contribution of the lipase(s) in FFA production has not attracted much attention. The present results show that the cultures of the *Synechocystis* Δaas mutants (dAS11 and dAS11_ *farB*) produce large amounts of total FFA (i.e. the sum of intra- and extracellular FFA) under the HL conditions (Fig. 7E), with an average rate of 1.1 mg l⁻¹ h⁻¹ during 7 days of cultivation. Since the corresponding rates reported for the thioesterase-expressing Δaas mutant of *S. elongatus* PCC 7942 range from 2 to 3 mg of total FFAs h⁻¹ l⁻¹ (Kato et al. 2016, 2017), we conclude that the endogenous lipase(s) of cyanobacteria can make a significant contribution to FFA production under certain conditions.

Takatani et al. (2015) previously showed in *S. elongatus* PCC 7942 that PSII is more readily photodamaged in an *aas*-deficient mutant than in WT and speculated that FFA, which accumulated in the absence of Aas, may have destabilized PSII. The present results show that the deficiency of Aas results in destabilization of PSII also in *Synechocystis* sp. PCC 6803 (Fig. 6B). The rescue by the FFA exporter FarB of the adverse effect of Aas deficiency (Fig. 6B) verifies that FFA is the cause of PSII destabilization, excluding the possible involvement of lysolipid, the other product of lipid deacylation. However, the molecular mechanism of the PSII destabilization remains elusive, as FFA can act as a surfactant and have direct or indirect biochemical or biophysical effects on membranes and proteins.

In the case of *S. elongatus* PCC 7942, Δaas mutant dAS1 can sustain growth under illumination of 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Takatani et al. 2015), but the Δaas mutants expressing *tesA* suffer from severe growth inhibition caused by intracellular accumulation of FFA even at 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Kato et al. 2015, 2016). Overexpression of the endogenous RND-type efflux pump (Kato et al. 2015) and removal of FFA from the culture medium (Kato et al. 2016) have been shown to help growth and FFA production of the *Synechococcus* mutants by

decreasing intracellular FFA. The successful functional expression of FarB, an MFS family FFA exporter (Figs. 6, 7A, D), adds a new tool for further improving FFA production using engineered cyanobacterial mutants.

Functional expression of FarB not only rescued the growth deficiency and photodamage of dAS11 under HL (Figs. 5B, 6) but also drastically decreased FFA production 'per cell' (Fig. 7F) and the proportion of C₁₆ FA species in the FFA fraction (Table 1). It is thus clear that strong light itself is not the signal for induction of the *sn-2* specific lipase activity. From the trade-off between robust growth and lipase upregulation, we presume that HL-induced damage acts as a signal for activation of the lipase(s). For efficient production of biofuels, it is important to increase the 'per cell' production of FFA. To fully exploit the activity of endogenous lipase of cyanobacteria for production of FFA, it is important to prevent inactivation (or deactivation) of the lipase(s). Currently, there is little information about cyanobacterial lipases. The *Synechocystis* sp. PCC 6803 genome database shows two ORFs, slI0482 and slI1969, as putative lipase genes, but with no genetic or biochemical evidence. Identification of the gene responsible for the HL-induced, *sn-2*-specific lipase activity and elucidation of the molecular mechanism of the regulation of the lipase protein are needed.

Although Aas has been thought to play a physiological role in uptake and utilization of exogenous FFA (Kaczmarzyk and Fulda 2010), our results show that it plays a more important role in the survival of *Synechocystis* sp. PCC 6803 under HL (Fig. 1); Aas prevents the formation of a harmful, positive feedback loop between photodamage and lipase upregulation by lowering intracellular FFA levels. Importance of FFA recycling has thus become apparent, but the physiological significance of lipid deacylation remains enigmatic. In this respect, a recent report suggesting involvement of FFA in the repair of photodamaged PSII is interesting (Jimbo et al. 2020). Under illumination, PSII is photodamaged, and the damaged PSII is repaired by the PSII repair mechanism. The degree of photoinhibition is therefore determined by the balance between damage and

repair (Nishiyama and Murata 2014, Jimbo et al. 2019). By comparing the effects of exogenously added FFAs on PSII repair by *Synechocystis* sp. PCC 6803 cells, it was shown that saturated FFAs, i.e. palmitic and stearic acids, enhance the repair of PSII by accelerating de novo synthesis of the D1 protein, while linolenic acid is inhibitory to the repair process (Jimbo et al. 2020). In *Synechocystis* sp. PCC 6803, C₁₈ and C₁₆ FFAs are esterified to the *sn*-1 and *sn*-2 positions of membrane lipids, respectively (Wada and Murata 1989), and the fatty acyl moiety at the *sn*-1 position is unsaturated by the action of desaturases (Wada and Murata 1989, 1990). Since FFAs are not always available from the natural environment of cyanobacteria, the *sn*-2 specific HL-inducible lipase may provide the saturated C₁₆ FA to facilitate the repair of PSII.

FarB is an MFS-family transporter identified in *Neisseria gonorrhoeae* as a component of a tripartite efflux pump FarAB-MtrE, where MtrE is an OMP and FarA is a MFP (Lee and Shafer 1999). It was therefore unexpected that introduction of FarB alone could enhance FFA export of the *E. coli* K12 Δ acrAB Δ emrB mutant (Fig. 2) and the *Synechocystis* sp. PCC 6803 dAS11 mutant (Fig. 4). It should be noted that gram-negative bacteria have several efflux pumps associated with multidrug resistance, among which some are capable of FFA export. Most of the multidrug resistance-associated pumps are RND-type pumps consisting of an Rnd family transporter, an inner membrane protein and an MFP. *E. coli* has several of this type of exporters, at least two of which (i.e. AcrAB-TolC and MdtEF-TolC) have FFA export activity (Lennen et al. 2013). There also are tripartite efflux pumps involving an MFS family protein as a transporter component, which are exemplified by FarAB-MtrE of *Neisseria lactamica*. Since *E. coli* has a FarAB-MtrE homolog (i.e. EmrAB-TolC), there are at least three efflux pumps for FFA. The *E. coli* mutant used in this study is deficient in AcrAB-TolC and EmrAB-TolC, but retains the MdtEF-TolC pump. The cyanobacterium *S. elongatus* PCC 7942, on the other hand, has two sets of genes encoding MFP and Rnd proteins, each forming an operon, and one of these (i.e. *rndA1B1*) was shown to be involved in FFA export (Kato et al. 2015). The expression level of *rndA1B1* genes is low in the WT cells but inactivation of the operon results in loss of resistance to various exogenously added FFAs (Kato et al. 2015). *Synechocystis* sp. PCC 6803 have several genes for MFP and Rnd proteins, including the homologs of *rndA1* and *rndB1*, and shows similar levels of FFA resistance as observed in the WT *S. elongatus* PCC 7942 cells (Kojima et al. 2016). It is therefore reasonable to assume that at least one RND-type FFA pump is functioning in *Synechocystis* sp. PCC 6803. It is, however, unlikely that FarB formed a heterologous tripartite complex with the proteins of *E. coli* or *Synechocystis* sp. PCC 6803 to secrete FFA out of the cell. Given that RND-type pumps secrete the substrates out of the cell not only from the cytoplasm but also from the periplasm (Alvarez-Ortega et al. 2013), we presume that FarB enhanced FFA secretion by increasing the rate of FFA transfer from the cytoplasm to the periplasm. In contrast to that of FarB, expression of FarA reduced resistance of *E. coli* and *Synechocystis* sp. PCC 6803 cells to exogenously added FFAs (Figs. 2, 3). This is likely to be due to interference

by the heterologous MFP on the function of the endogenous efflux pumps.

Increased FFA production under HL, which is presumably due to activation of lipase activity, has also been reported in an *aas*-deficient mutant of *S. elongatus* PCC 7942 (Takatani et al. 2015), but as mentioned above, this mutant can sustain growth under the HL conditions (Takatani et al. 2015) and hence is more tolerant to high light conditions than the *Synechocystis* sp. PCC 6803 Δ *aas* mutant. The molecular basis of different light sensitivities of the two mutants is currently unclear. In the case of the *S. elongatus* PCC 7942 *aas* mutant, growth impairment due to accumulation of large amounts of intracellular FFA has been reported only when a foreign thioesterase is expressed to enhance FFA production (Kato et al. 2016, 2017). It is thus likely that the activity of the endogenous lipase is lower in *S. elongatus* PCC 7942 than in *Synechocystis* sp. PCC 6803. Another possible cause of the difference between the *aas*-deficient mutants of the two cyanobacterial strains may be the difference in FA compositions of membrane lipids. One of the notable differences between the two strains is that *S. elongatus* PCC 7942 does not contain polyunsaturated FFAs, whereas *Synechocystis* sp. PCC 6803 contain linoleic acid (18:2) and α -linolenic acid (18:3) (Wada and Murata 1989, 1990). Although palmitic acid is by far the major FFA in the dAS11 cells exposed to HL, the sum of 18:2 and 18:3 constitutes about 6% of total intracellular FFA (Table 1). Toxicity of extrinsically added 18:2 and 18:3 on cyanobacteria is well documented (Sakamoto et al. 1998, Maeda et al. 2005, von Berlepsch et al. 2012, Kojima et al. 2016). Given the high 'per-cell' FFA content of HL-grown dAS11 cells, the endogenously produced polyunsaturated FFAs may exert photooxidative damage to the cells under HL conditions. Further studies are needed to elucidate the hypersensitivity of the *Synechocystis* sp. PCC 6803 *aas* mutant to strong light.

Materials and Methods

Organisms and culture conditions

Synechocystis sp. PCC 6803 cells were grown at 30°C under continuous illumination using nitrate as the nitrogen source. HL illumination of the cultures was performed using white LED light at 400 μ mol photons m⁻² s⁻¹. For LL illumination of the cultures, the light intensity was 50 μ mol photons m⁻² s⁻¹ unless otherwise stated. The liquid medium used was a modification of BG-11 (Stanier et al. 1971) described previously (Suzuki et al. 1995). The solid medium was prepared by addition of 1.5% (w v⁻¹) agar and 0.3% (w v⁻¹) sodium thiosulfate to the liquid medium. Liquid cultures were bubbled with air supplemented with 2% (v v⁻¹) CO₂. Petri dishes containing the cells on the solid medium were incubated under the CO₂-enriched air. When appropriate, kanamycin and chloramphenicol were added to the medium at 15 μ g ml⁻¹ and 5 μ g ml⁻¹, respectively.

Construction of the *Neisseria farB*-expressing dAS11 mutant

The engineered *aas* insertion mutant dAS11 (Kojima et al. 2016) was the *aas*-deficient *Synechocystis* sp. PCC 6803 mutant used in this study. *N. lactamica* cells were obtained from ATCC and the genomic DNA was used for polymerase chain reaction (PCR) amplification of the DNA fragments carrying the *farAB* operon, the *farA* gene and the *farB* gene (Lee and Shafer 1999; GenBank

accession numbers AF132909 and AF132910), using the KOD plus DNA polymerase (Toyobo, Osaka, Japan) with the primer pairs a1/b3, a1/a2 and b1/b3, respectively (Supplementary Table S1). The amplified DNA fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and after verification of the nucleotide sequences excised from the plasmids by digestion with *EcoRI* and ligated into the *EcoRI* site of the *E. coli*-*S. elongatus* PCC 7942 shuttle vector pSE2 (Maeda et al. 1998) so that the *far* genes are transcriptionally fused to the *Ptrc* promoter on pSE2. The *cat* chloramphenicol resistance gene was amplified by PCR from the plasmid pHSG399 (Takeshita et al. 1987), using the primer pair d1/d2 (Supplementary Table S1) and ligated into the *XbaI* site of the pSE2 derivatives carrying the *Ptrc-far* fusions. Using the resultant plasmids as the template, DNA fragments carrying the *Ptrc-far* transcriptional fusions and the *cat* gene were amplified by PCR using the primer pair e1/e2 (Supplementary Table S1) and ligated into the *EcoRV* site of the plasmid pUM1, a cloning vector for gene introduction into the neutral site of the *Synechocystis* sp. PCC 6803 genome (Keta et al. unpublished results). The resultant plasmid DNAs, which were referred to as pUM1-*Ptrc-farAB_cat*, pUM1-*Ptrc-farA_cat* and pUM1-*Ptrc-farB_cat*, respectively, were used to transform the dAS11 cells through homologous recombination, generating kanamycin- and chloramphenicol-resistant transformants. After three rounds of streak purification of single colonies, selected colonies were analyzed by PCR to confirm the insertion of the *Ptrc-far* constructs into the neutral site of the chromosome with complete genome segregation (Supplementary Fig. S1).

FFA analysis

For the analysis of the intracellular and extracellular FFAs, 10-ml aliquots of the cultures were centrifuged at $1,700\times g$ for 15 min to separate the cells and the medium. The supernatant was transferred to a 15-ml plastic tube and the cells were resuspended in 1 ml of methanol. The samples were stored at -80°C until use. For determination of the total concentration of FFAs in the medium, the supernatant was analyzed using the Free Fatty Acid Quantification Kit (Biovision, California, USA) according to the manufacturer's instructions. For analysis of the extracellular and intracellular FFA profiles and the total FFA content in the cells, samples were extracted with a modified Folch method (Folch et al. 1957, Ikeda 2015) and analyzed by liquid chromatography-mass spectrometry (LC-MS) as described (Ikeda 2015, Takatani et al. 2015).

Effects of FFAs on *E. coli* growth

Construction of the *E. coli* K-12 $\Delta\text{acrAB}\Delta\text{emrB}$ strain was performed according to previously reported methods (Baba et al., Nakahigashi et al. 2009) using BW25113 as a background strain. For expression of the *Neisseria farAB*, *farA* or *farB* genes, pSE2-*Ptrc-farAB_cat*, pSE2-*Ptrc-farA_cat* or pSE2-*Ptrc-farB_cat* was introduced into the $\Delta\text{acrAB}\Delta\text{emrB}$ strain, respectively. A control strain was obtained by transformation of the $\Delta\text{acrAB}\Delta\text{emrB}$ strain with the pSE2 plasmid. For viability assays on the agar plates, cultures of *E. coli* in the late logarithmic phase of growth were incubated in the presence of 0.1 mM IPTG for 2 h and diluted to give an optical density (OD) of 1.0 at 600 nm, followed by serial dilution into fresh Luria-Bertani (LB) medium. About 3- μl aliquot from each dilution was spotted onto LB plates containing 1 mM lauric or palmitic acid and 0.04% (v v^{-1}) polyoxyethyleneglycol dodecyl ether (Brij-35). The plates were incubated for 1 day at 37°C in the dark.

Effects of FFAs on cyanobacteria growth

For viability assays on the agar plates, liquid cultures in the late logarithmic phase of growth were diluted to an optical density of 1.0 at 730 nm, followed by serial dilution into fresh liquid medium. About 3- μl aliquot from each dilution was spotted onto solid media containing 0.1 mM 18:3. The plates were incubated for 5 days at 30°C under illumination at a light intensity of $25\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$. For growth assays in liquid media, cells were inoculated into the media supplemented with 0.1 mM or 0.5 mM of various FAs to give an OD_{730} value of 0.01 and incubated under illumination at $50\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$.

Other methods

The cell number and volume were determined by using a particle counter analyzer (CDA-1000, Sysmex). The photosynthetic yield of PSII was determined by measuring the $F_v F_m^{-1}$ ratio using an AquaPen-C fluorometer (AP-C100, Photon Systems Instruments). The samples were dark-adapted for 5 min before measurement. For determination of the PSII activity of *Synechocystis* cells, the rate of oxygen evolution was measured by using an oxygen electrode (Oxygraph, Hansatech Instruments Ltd) in the presence of 1 mM *p*-benzoquinone.

Supplementary Data

Supplementary data are available at PCP online.

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

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