Effects of fatty acid activation on photosynthetic production of fatty acid-based biofuels in *Synechocystis* sp. PCC6803

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**Abstract**

**Background:** Direct conversion of solar energy and carbon dioxide to drop in fuel molecules in a single biological system can be achieved from fatty acid-based biofuels such as fatty alcohols and alkanes. These molecules have similar properties to fossil fuels but can be produced by photosynthetic cyanobacteria.

**Results:** *Synechocystis* sp. PCC6803 mutant strains containing either overexpression or deletion of the *slr1609* gene, which encodes an acyl-ACP synthetase (AAS), have been constructed. The complete segregation and deletion in all mutant strains was confirmed by PCR analysis. Blocking fatty acid activation by deleting *slr1609* gene in wild-type *Synechocystis* sp. PCC6803 led to a doubling of the amount of free fatty acids and a decrease of alkane production by up to 90 percent. Overexpression of *slr1609* gene in the wild-type *Synechocystis* sp. PCC6803 had no effect on the production of either free fatty acids or alkanes. Overexpression or deletion of *slr1609* gene in the *Synechocystis* sp. PCC6803 mutant strain with the capability of making fatty alcohols by genetically introducing fatty acyl-CoA reductase respectively enhanced or reduced fatty alcohol production by 60 percent.

**Conclusions:** Fatty acid activation functionalized by the *slr1609* gene is metabolically crucial for biosynthesis of fatty acid derivatives in *Synechocystis* sp. PCC6803. It is necessary but not sufficient for efficient production of alkanes. Fatty alcohol production can be significantly improved by the overexpression of *slr1609* gene.

**Keywords:** Biofuel, Fatty alcohol, Fatty alkane, Cyanobacteria, *Synechocystis* sp. PCC6803, Fatty acid activation

**Background**

Biofuel production from renewable sources is considered as a feasible solution to the energy and environmental problems we are facing. It is very important to explore and develop advanced biofuels alongside traditional biofuels such as bioethanol and biodiesel to ensure sufficient supply of renewable energy at a time when demand for energy is set to increase over the coming decades. Advanced biofuels possess higher energy density, hydrophobic properties and compatibility with existing liquid fuel infrastructure including fuel engines, refinery equipment and transportation/distribution pipelines, whilst serving as better alternatives to fuels produced from fossil fuels [1].

In terms of fuel properties the best replacement of petroleum fuels is “Petroleum Fuels”. This means ideal biofuels produced from biological systems should be chemically similar to petroleum, such as fatty acid-based molecules including fatty alcohols and fatty alkanes [2].

As a candidate for biofuel-producing microbial systems, cyanobacteria have become more and more attractive due to their specific characteristics as photosynthetic bacteria.

Compared to generally utilized biofuel-producing microbes such as *E. coli* and *S.cerevisiae*, cyanobacteria are photosynthetic microbes, which can convert solar energy and carbon dioxide more efficiently into biofuels in one biological system. In contrast to plants and eukaryotic algae, cyanobacteria are prokaryotic microbes with the ability to grow a lot faster. Genetic engineering platforms for cyanobacteria are well established and they are highly tolerant to heterogeneous genes. So far over 40 genomic sequences of cyanobacteria strains are available, therefore...
genetic information on cyanobacteria are relatively robust 
http://genome.kazusa.or.jp/cyanobase. This makes genetic 
engineering toward efficiently producing biofuels in cyano-
bacteria to be a more realistic and feasible option [3-5].

Recently, the alkane biosynthetic pathway was identified 
in cyanobacteria with two enzyme families including an 
acyl carrier protein (ACP) reductase (AAR) and an alde-
hyde decarbonylase (ADC) [6]. Genes associated with an 
alcohol-forming fatty acyl-CoA reductase (FAR) have not 
been reported in cyanobacteria, C16:0 and C18:0 alcohols 
can be produced by engineered cyanobacteria containing 
the FAR gene derived from jojoba [7] or Arabidopsis thali-
ana [4]. The overall pathway of the fatty acid, fatty alcohol 
and fatty alkane in wild-type or engineered Synechocystis 
strains were illustrated in Figure 1.

The fatty acid molecules must be activated to fatty 
acyl-thioesters by fatty acyl-CoA synthetase (ACS, EC 
6.2.1.3) or fatty acyl-ACP synthetase (AAS, EC 6.2.1.20) 
prior to the synthesis of fatty alcohols and alkanes. 
Based on sequence identity analysis, Synechocystis sp. 
PCC 6803 encodes only a single candidate gene for fatty 
acid activation, annotated as AAS and designated as 
slr1609 [8]. The slr1609-deletion cyanobacteria mutant 
was incapable of utilizing exogenous fatty acids and thus 
secreted endogenous fatty acids into the medium. The 
detected free fatty acids are released from membrane 
lipids. The data suggest a remarkable turnover of lipids 
and a role of AAS activity in recycling the released fatty 
acids [8].

The overall pathway of the fatty acid, fatty alcohol and 
alcohol in wild-type or engineered Synechocystis strains 
are illustrated in Figure 1. Synechocystis sp. PCC6803 
mutant strains with either overexpression or deletion of 
slr1609 gene have been constructed in this study. The 
results indicated that the AAS gene was metabolically 
crucial for production of free fatty acids and fatty acid 
derivatives in Synechocystis sp. PCC6803.

Results and discussion

Construction of Synechocystis sp. PCC6803 mutants 
with either overexpression or deletion of slr1609 gene

To investigate the impact of AAS on production of free 
fatty acids and fatty acid derivatives, we constructed two 
plasmids pGQ11 (Figure 2B) and pGQ49 (Figure 2D) for 
over-expressing slr1609 gene, driven by a strong constitut-
ive promoter Prbc or PpsbA2 and integrated into slr0168 
[9] or pshB2 site, respectively. Two plasmids pGQ53 (Figure 2A) and pGQ17 (Figure 2C) were constructed for 
disruption of slr1609 gene with erythromycin or kanamycin 
resistance cassettes, respectively. The plasmid pGQ11 or 
pGQ53 was transformed into Synechocystis sp. PCC6803 
generating GQ3 and GQ8 strains respectively for analysis of fatty acid and alkane production. The plas-
mid pGQ49 or pGQ17 was transformed into fatty-alco-
hol-producing strain Syn-XT14 generating GQ5 and GQ6 
respectively for analysis of fatty alcohol production. Over-
expressed AAS protein with C-terminal His-tag in GQ3 
and GQ5 mutant were detected by western blotting as 
shown in Additional file 1: Figure 1.

Due to cyanobacterial cells containing multiple copies 
of chromosomes [10], the complete replacement of wild 
type alleles must be established and confirmed by PCR 
(Figure 3 and 4). Primers used in this study were listed in 
Additional file 1: Table 1. Because the whole inserted 
fragment is too long to amplify from genomic DNA, the 
over-expressed genes were checked by two reactions for 
successful insertion and correct orientation of the 
slr1609 or FAR gene and complete replacement of wild-
type alleles (Figure 3B and 4). The first reaction with the 
primer (0168-2 or Pd1-3) of insert site and primer 
(1609NdeI, 1609R or far-1) for the inserted gene verified 
the genes were inserted in the correct orientation. The 
second reaction with primers (0168-1/0168-2 or pD1-1/p 
Pd1-2d-2) of inserted site verified the wild-type was

Figure 1. The overall pathway of the fatty acid, fatty alcohol and fatty alkane in Synechocystis sp. PCC 6803. Dash arrow represents non-
native and heterologously introduced pathway. ACP, acyl carrier protein; AAR, acyl-ACP reductase; ADC, aldehyde decarbonylase; ACC, acetyl-
CoA carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate-acyl transferase; ACAT, acyl-CoA-ACP transferase; ACR, acyl-CoA reductase.
replaced completely (Figure 3B and 4). The disrupted genes were checked with internal primers (Figure 3A and 4B) or primers that flanked the insertion site to prove the wild-type allele was replaced completely. The results of the PCR indicated the correct mutants were constructed.

**The amount of free fatty acids can be doubled in the *Synechocystis* mutant strain with *slr1609* knockout**

The total free fatty acids of the wild-type strain and the mutant strain GQ8 with *slr1609* deletion were extracted using the methods described in the Experimental Procedures. The wild-type and the mutant strain displayed similar growth behaviors (Figure 5A). However, the content of free fatty acids showed substantial differences between two strains (Figure 5B). In the *slr1609* deletion mutant, the concentration of total free fatty acids was 6.7 ± 0.2 μg/mL/OD, while that of the wild type was 3.5 ± 0.25 μg/mL/OD. The deletion of *slr1609* increased free fatty acid accumulation close to two folds. It indicates that the dysfunction of fatty acid activation caused by the deletion of *slr1609* results in an increase of free fatty acid accumulation.

As to the contents of the pool of free fatty acids, the amount of unsaturated fatty acids with carbon chain length of C16 and C18 was significantly higher in the *slr1609* knockout mutant strain compared to the wild-type strain. Double bonds can only be introduced into free fatty acid coupled to the glycerol backbone of membrane lipids by acyl-lipid-type desaturases. Indicating that unsaturated free fatty acids being released from membrane lipids of senescent or damaged cells, while unsaturated free fatty acids in AAS deletion mutant can not be recycled and incorporated to membrane lipids.

In the mutant strain GQ3 with *slr1609* over-expression, there is no significant change to the production of free fatty acids compared to the wild-type strain (data not
shown). It has been confirmed that free fatty acids are released from membrane lipids in *Synechocystis* sp. PCC6803 [10]. Indicating free fatty acid production is not only determined by the fatty acyl-ACP pool size, but also by the biosynthesis of membranes and hydrolysis of membrane lipids which are physiologically regulated.

**The production of alkanes was significantly reduced in the slr1609 deletion mutant strain**

Alkanes are the predominant constituents of gasoline, diesel, and jet fuels. Production of alkanes has been reported in a diversity of cyanobacteria, with heptadecane and heptadecene being the most abundant hydrocarbons found in *Synechocystis* sp. PCC6803. In this pathway fatty acyl-ACP is reduced to a fatty aldehyde by a fatty acyl-ACP reductase (AAR) and then the fatty aldehyde decarbonylase (ADC) is able to convert the aldehyde into an alkane. Besides the fatty acyl-ACP produced by *de novo* fatty acid synthesis from acetyl-CoA, acyl-ACP synthetase (AAS) is essential for recycling fatty acids into fatty acyl-ACP. The results showed that the production of hydrocarbons was significantly reduced by around 90% in the mutant strain GQ8 with an *slr1609* deletion (0.047 ± 0.01 μg/mL/OD) compared with the wild-type strain (0.38 ± 0.07 μg/mL/OD) (Figure 6B), and this indicates that AAS plays an essential role in alkane biosynthesis. AAS can enhance the total amount of fatty acyl-ACP available for alkane production, and the acyl-ACP formed by AAS activity may be more accessible to the acyl-ACP formed by *de novo* fatty acid synthesis.

The production of alkanes was not enhanced by the over-expressing *slr1609* gene alone in the GQ3 strain (0.39 ± 0.03 μg/mL/OD) (Figure 6B). Due to the activities of downstream enzymes of the alkane producing pathway, AAR and ADC, are rather low and fatty acyl-ACPs might not be efficiently converted to alkanes [11]. Fatty acyl-ACPs are also a supplier of fatty acyl groups for biosynthesis of lipid A [12], phospholipids [13], and membrane-derived lipo-polysaccharides [14].

**Synechocystis** AAS plays an important role in fatty alcohol production

Fatty alcohols possess carbon chain length which range from C8 to C22, and can be used as detergents, precursors for synthesis of other chemicals or fuels. We have constructed a fatty-alcohol-producing strain Syn-XT14, by the introduction of a jojoba acyl-CoA reductase gene into wide-type strain in previous work [4], and the effect of *Synechocystis* AAS on fatty alcohol production were examined by over-expressing (GQ5) or deleting the *slr1609* gene (GQ6) in Syn-XT14. The results showed that fatty alcohol production was enhanced by about 60% in GQ5 (19.8 ± 2.3 μg/L/OD) or decreased by about 60% in GQ6 (4.9±0.1 μg/L/OD) compared with Syn-XT14 (12.5±2.0 μg/L/OD), respectively (Figure 7). The data indicates that *Synechocystis* AAS plays an important role in fatty alcohol production.
Although the native jojoba FAR has a preference for very-long-chain acyl-CoA substrate (C20, C22 and C24), assays of jojoba extracts indicated that it is capable of reducing C16:0-ACP and C18:0-ACP [7]. It's a reductase with broad substrate specificity. It may be possible that the acyl-ACP produced by AAS can also be accepted as substrate in addition to acyl-CoA by jojoba FAR in engineered *Synechocystis* strains. It is also possible that the acyl-ACPs, which are synthesized by *Synechocystis* AAS, could be in turn transacylated to acyl-CoAs by a reverse catalysis of acetyl-CoA-ACP-transacylase (EC 2.3.1.38) type reaction.

**Conclusions**

In this study the effects of fatty acid activation functionalized by a fatty acyl-ACP synthase on the production of fatty acid-based biofuels including fatty alcohols and alkanes in a photosynthetic cyanobacterium were evaluated and analyzed. We found fatty acid activation to be essential for efficient production of alkanes and plays a key role in manipulating fatty alcohol production. The results here provide promising clues for metabolically engineering cyanobacteria to improve photosynthetic production of fatty acid-based biofuels.
Methods

Chemicals and reagents

Pentadecanol, eicosane and nonadecanoic acid were obtained from Sigma-Aldrich (USA). Other chemicals were from Merck (Germany) or Ameresco (USA). Oligo nucleotides and gene synthesis were carried out by Sangon (Shanghai, China). Taq DNA polymerases and all restriction endonucleases were from Fermentas (Canada) or Takara (Japan). The DNA ladders were from Takara (Japan). The kits used for molecular cloning were from Omega (USA) or Takara (Japan).

Construction of Synechocystis sp. PCC683 mutant strains

All primers used in this study are listed in the Additional file 1.

The slr1609 gene was amplified from the genomic DNA of Synechocystis sp. PCC6803 with the primers 1609NdeI/1609R and subcloned into NdeI/XhoI site of the plasmid pET21b (Novagen, USA) to generate the plasmid pGQ7. The gene was cloned from pGQ7 with the primers 1609XbaI/1609DraI and subcloned into XbaI/SmaI site of the plasmid pFQ20 [4] to generate the plasmid pGQ11.

The plasmid pXT68 was constructed based on the site of psbA2 gene. Both upstream and downstream fragments of psbA2 gene were cloned from the genomic DNA of Synechocystis sp PCC6803 with the primers Pd1-2-f/Pd1-2-r and pD1-2-d-1/pD1-2-d-2 respectively and inserted into the TA cloning site of pMD18-T, to generate the plasmids pXT25 and pXT59. The kanamycin resistance (kan‘) gene (ck2) cassette was excised with EcoRV and Xbal from pRL446 [15] and inserted into the PstI site of pXT25 with blunt ends, to generate the plasmid pXT25. The 4.5 kb fragment containing ck2 and upstream of psbA2 gene was excised with Xbal and SphI from pXT62 and inserted into Xbal site of pXT59 with blunt ends, to generate the plasmid pXT68. Then, the slr1609 gene was excised with NdeI and DraIII (blunted end) from pGQ7.
and inserted into the NdeI/SalI (blunted end) site of pXT68, to generate the plasmid pGQ49.

The plasmids pGQ17 and pGQ53 were constructed and used to disrupt the slr1609 gene via homologous recombination in Synechocystis [16]. Genomic DNA was used as the template to amplify the 500 bp N-terminal and C-terminal fragments of slr1609 ORF using the primers 1609 kuF/R and 1609 kdF/R, respectively. The N-terminal and C-terminal fragments were cloned into pMD18-T to generate the plasmids pGQ12 and pGQ13, respectively. The ck2 gene from pRL446 [15] was cloned into the BamHI site of pGQ12 to generate plasmid pGQ14. After digestion with Dral and EcoRI and blunting by T4 DNA polymerase, the ck2 gene together with N-terminal fragment of slr1609 from pGQ14 were cloned into the SmaI site of plasmid pGQ13 to generate plasmid pGQ17. The erythromycin resistance gene cce2 was digested with EcoRV from pRL271 [17] and cloned into the blunted BamHI site of pGQ17 to generate plasmid pGQ53.

Plasmids constructed and used in this study were listed in Table 1.

All of the constructs were checked by enzyme digestion and then transformed to Synechocystis sp. PCC6803 cells [18]. The plasmids pGQ11 and pGQ53 were transformed to Synechocystis sp. PCC6803 wild-type to generate the mutant strains GQ3 and GQ8 respectively. The plasmids pGQ17 and pGQ49 were transformed to Synechocystis sp. PCC6803 mutant strain Syn-XT14 [4] to generate two new mutant strains GQ6 and GQ5 respectively. For the initial selection of transformants, the DNA/cell mixture was applied to BG11 agar plates. After 18 h the membrane filters were applied to fresh BG11 agar plates containing antibiotics (10 μg mL⁻¹ spectinomycin, 10 μg mL⁻¹ erythromycin or 5 μg mL⁻¹/5 μg mL⁻¹ spectinomycin/kanamycin). Homogeneous mutants were obtained by successive streaking on BG11 plates containing antibiotics. Complete segregation of all mutants was verified by employing PCR. Strains constructed and used in this study were listed in Table 2.

**Cultivation of Synechocystis sp. PCC683 strains**

Liquid cultures of Synechocystis sp. PCC 6803 were grown photo-autotrophically in BG 11 media [19] at 30°C under constant illumination at a photosynthetic photon flux density of approximately 30 μmol photons m⁻² s⁻¹ and with aeration by sterile air or in a shaker. When necessary, the following antibiotics were added: erythromycin (20 μg mL⁻¹) and spectinomycin (20 μg mL⁻¹). Growth was monitored by following the OD at 730 nm. The Synechocystis sp. PCC6803 wild-type strain, the mutant strains GQ8 with deletion of the slr1609 gene and GQ3 with overexpression of the slr1609 gene were respectively grown in 100 mL Erlenmeyer flask containing 50 mL of BG11 medium in a shaker for free fatty acid analysis. The Synechocystis sp. PCC6803 wild-type strain, the mutant strains Syn-XT14 with overexpression of the FAR gene, GQ6 with deletion of the slr1609 gene and GQ5 with overexpression of the slr1609 gene were respectively grown in a 500 mL Erlenmeyer flask with aeration by sterile air for fatty alkane or fatty alcohol analysis. The Synechocystis sp. PCC6803 wild-type strain, the mutant strains GQ8 with deletion of the slr1609 gene and GQ3 with overexpression of the slr1609 gene were respectively grown in a 500 mL Erlenmeyer flask with aeration by sterile air for fatty alkane analysis.

**Extraction and analysis of free fatty acids, fatty alkanes and fatty alcohols**

For extraction of free fatty acids, 20 mL of the culture was lysed by sonication (total 30 min with 10 s on and 5 s off intervals) when the stationary phase (about 240
h) reached. To each 20 mL aliquot, 20 mL of 2:1 (v/v) CHCl₃:CH₃OH were added and the resulting mixture was mixed well [20]. For GC-MS analysis of free fatty acids, 10 µg of nonadecanoic acid was added as the internal standard. A two-phase system (top: aqueous, bottom: organic) was generated after shaking for 1 h and centrifugation at 3000 rpm at room temperature for 5 min. The bottom organic phase was collected and concentrated under a stream of nitrogen at 55°C giving a residue that was resuspended in 600 µL of hexane. Aliquots of this mixture were analyzed by GC-MS with an Agilent 7890A-5975 C system equipped with Agilent HP-INNOWax (30 m × 0.25 m). Helium (constant flow 1 mL/min) was used as the carrier gas. The temperature of the injector was 250°C and the following temperature program was applied: 100°C for 1 min, increase of 5°C min⁻¹ to 200°C then increase of 25°C min⁻¹ to 240°C for 15 min.

Previous work in our lab showed that fatty alcohol and alkane can not be detected in relative culture media (data not shown). For extraction of fatty alkanes, *Synechocystis* cells at stationary phase (about 240 h) were harvested from 200 mL of culture by centrifugation. The cells were resuspended in 10 mL of TE buffer (pH8.0) and then lysed by sonication. The lysate added with 30 µg of eicosane as internal standard was extracted for 1 h at room temperature with 10 mL of 2:1 (v/v) CHCl₃:CH₃OH. The same following sample preparation and GC-MS analysis methods described above were used for fatty alkane analysis.

The same extraction methods described above for fatty alkane analysis were used for fatty alcohol, except adding 20 µg of 1-pentadecanol as the internal standard. The following temperature program was applied here: 50°C for 1 min, increase of 20°C min⁻¹ to 180°C then increase of 10°C min⁻¹ to 240°C for 20 min.

Additional material

**Table 2 Strains constructed and used in this study**

| Strain     | Genotypea, b | Reference       |
|------------|--------------|-----------------|
| 6803yu     | Synechocystis sp. PCC6803 Wild-type, Glucose-tolerance | Prof. Xu X.     |
| GQ3        | slr0168:ΩP₆₅₆-str1609 | This study      |
| GQ8        | str1609:ΩCCEII | This study      |
| GQ5        | str1608:Ωpac far (jojoba), psbA2::CK2 | This study      |
| GQ6        | str1608:Ωpac far (jojoba), str1609::CK2 | This study      |
| SynXT14    | str0168:ΩP₆₅₆ far (jojoba) T₆₅₆ | [4]             |

* P₆₅₆: 0.4 kb DNA fragment containing the promoter of petE gene. P₆₅₆: 0.3 kb DNA fragment containing the promoter of rbc operon. T₆₅₆: 0.2 kb DNA fragment downstream of rbc operon. P₆₅₆: 0.3 kb DNA fragment downstream of rbc operon. P₆₅₆: 1.5 kb DNA fragment containing the promoter of psbA2 gene. All promoters and terminators mentioned here are from Synechocystis sp. PCC 6803.

**Abbreviations**

AEC: acyl carrier protein; AAR: acyl-ACP reductase; ADC: aldehyde decarboxylase; FAR: fatty acyl-CoA reductase; ACS: acyl-CoA synthetase; AAS: acyl-CoA synthetase; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; GPAT: glycerol-3-phosphate-acyl transferase; ACAT: acyl-CoA transferase; ACR: acyl-CoA reductase.

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Authors’ contributions
XL conceived of the study. XL and QG designed the experiments. QG carried out the construction and cultivation of Synechocystis sp. PCC6803 mutant strains. QG carried out extraction and analysis of free fatty acids, fatty alkanes and fatty alcohols. WW and HZ participated in GC-MS analysis. XL, QG and WW wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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