Identification of a Cyanobacterial RND-Type Efflux System Involved in Export of Free Fatty Acids

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An RND (resistance–nodulation–division)-type transporter having the capacity to export free fatty acids (FFAs) was identified in the cyanobacterium Synechococcus elongatus strain PCC 7942 during characterization of a mutant strain engineered to produce FFAs. The basic strategy for construction of the FFA-producing mutant was a commonly used one, involving inactivation of the endogenous acyl-acyl carrier protein synthetase gene (aas) and introduction of a foreign thioesterase gene (tesA), but a nitrate transport mutant NA3 was used as the parental strain to achieve slow, nitrate-limited growth in batch cultures. Also, a nitrogen-regulated promoter Pama was used to drive tesA to maximize thioesterase expression during the nitrate-limited growth. The resulting mutant (dAS2T) was, however, incapable of growth under the conditions of nitrate limitation, presumably due to toxicity associated with FFA overproduction. Incubation of the mutant culture under the non-permissive conditions allowed for isolation of a pseudorevertant (dAS2T-prl) capable of growth on nitrate. Genome sequence and gene expression analyses of this strain suggested that expression of an RND-type efflux system had rescued growth on nitrate. Targeted inactivation of the RND-type transporter genes in the wild-type strain resulted in loss of tolerance to exogenously added FFAs including capric, lauric, myristic, oleic and linolenic acids. Overexpression of the genes in dAS2T, on the other hand, enhanced FFA excretion and cell growth in nitrate-containing medium, verifying that the genes encode an efflux pump for FFAs. These results demonstrate the importance of the efflux system in efficient FFA production using genetically engineered cyanobacteria.

Keywords: Biofuel production • Cyanobacteria • Free fatty acid • RND-type efflux system.

Abbreviations: Aas, acyl-acyl carrier protein synthetase; ACP, acyl carrier protein; FFA, free fatty acid; IPTG, isopropyl-1-thio-D-galactopyranoside; Km, kanamycin resistance; MFP, membrane fusion protein; OMP, outer membrane protein; ORF, open reading frame; qRT–PCR, quantitative reverse transcription–PCR; RND, resistance–nodulation–division; SNP, single nucleotide polymorphism; Spc, small-plasmid-cured; WT, wild type.

Introduction

Photosynthetic microorganisms including cyanobacteria and eukaryotic algae are thought to provide a good platform for metabolic engineering of biofuel production pathways, because they have high photosynthetic activity and can be cultivated in non-arable land without competing with food production. However, the productivity of algal- or cyanobacterial biofuel production is currently too low to be commercially competitive with fossil fuel or to be environmentally sustainable (Clarens et al. 2010). One of the major causes of the low productivity of algal biofuel production is the limited capacity of the cells to store the photosynthetic products. Although green algae can accumulate triacylglyceride to a level as high as approximately 50% of cell dry mass (Hu et al. 2008), this means that the amount of the cell debris after lipid extraction equals the amount of the oil produced. If the annual demand for jet fuel is to be replaced with algal oil, approximately $2 \times 10^8$ t of cell debris would be produced. In other words, about half of the resource and energy consumed in production of algal oil is used for the production of cell debris. To avoid this problem, the prospect of ‘milking’ algal cells has been offered as an alternative strategy for biofuel production (Ramachandra et al. 2009); the fuel-related compounds are to be secreted or exported continuously from the cells, allowing for stable production of biofuel by the cells without continuing cell division.

Fatty acid production using genetically engineered cyanobacteria also aims at milking of cyanobacterial cells, having the cells to produce free fatty acids (FFAs) and to excrete the product into the medium. Although it is thought to be a promising
method of biofuel production, the product-to-cell debris ratio of the cyanobacteria-based FFA production is currently much lower than that of the algal triacylglyceride production system, being around 0.1 (Liu et al. 2011, Rufing and Jones 2012, Rufing 2014). This seems to be due to the fact that FFA excretion by the engineered mutants starts at a rather late stage of growth, i.e. in the cells approaching the stationary phase of growth, which is followed by rapid decline of the photosynthetic activity (Ruffing and Jones 2012, Rufing 2014). Thus, most of the light energy seems to be used for cell growth but not for FFA production.

To increase the efficiency of FFA production by engineered cyanobacteria, we attempted two things in this study; cells were grown under the constant stress of nitrogen limitation (but not nitrogen depletion) to reduce the rate of cell growth. To this end, the NA3 mutant of *Synechococcus elongatus*, which was constructed by deleting the *nrtABCD* genes coding for an ABC-type nitrate transporter from the genome (Maeda and Omata 1997), was used as a parental strain for the construction of FFA-producing mutants. *Synechococcus elongatus* mutants defective in the high-affinity nitrate transporter cannot take up low concentrations of nitrate, but can grow slowly in media containing a high concentration of nitrate, relying on passive influx of nitrate (Omata et al. 1989, Maeda et al. 1998, Aichi et al. 2004). Nitrate-limited growth was thus achieved in batch cultures in the presence of high concentrations of nitrate. Secondly, the thioesterase gene used to hydrolyze acyl-acyl carrier protein (ACP) was fused to the nitrogen-responsive promoter (P~nirA~) of the nitrate assimilation operon of *S. elongatus*, which is under the dual control of the NtcA and NtcB transcriptional activators and is most active during nitrate-limited growth (Ohashi et al. 2011). The attempt to force the cells to enhance FFA production during nitrate-limited growth, however, was unsuccessful; the cells were practically incapable of growth under the given conditions. We were, nevertheless, able fortuitously to isolate a pseudorevertant that could grow and excrete FFAs in nitrate-containing medium, which led to identification of a novel cyanobacterial efflux pump that can export FFAs. The usefulness of this transporter in improvement of FFA productivity in the engineered mutants and the physiological significance of this type of exporter in photosynthetic cells are discussed.

### Results

#### Growth defect of an FFA-producing mutant and isolation of a pseudorevertant

To examine the effects of nitrogen limitation on growth and FFA production, a nitrate transportless mutant of *S. elongatus* PCC 7942 (NA3; Maeda and Omata 1997) was used as a parental strain for construction of FFA-producing strains dAS2 and dAS2T. dAS2 was constructed by deleting a 0.66 kb internal fragment of the *aas* gene (Fig. 1A). For construction of dAS2T, the *tesA* coding sequence transcriptionally fused to the nitrogen-regulated promoter (P~nirA~) of the nitrate assimilation operon (nirA–nrtABCD–narB) of *S. elongatus* was introduced into the *aas* locus (Fig. 1A). P~nirA~ is inactive in the presence of ammonium in growth media and is most active under constantly nitrate-limited growth conditions, which can be achieved by forcing nitrate transportless mutants to grow on nitrate as the nitrogen source (Maeda et al. 1998). Both dAS2 and dAS2T grew normally in ammonium-containing medium under low light (50 μE m~2~ s~1~) and low CO2 conditions (0.04%) (hereafter referred to as N-replete conditions), showing the blue-green color typical of cyanobacteria (Fig. 1C). In nitrate (15 mM)-containing medium under high light (180 μE m~2~ s~1~) and high CO2 conditions (2%), NA3 and dAS2 grew with yellowish-green coloration indicative of nitrogen limitation, but dAS2T was practically unable to grow under the conditions of nitrogen limitation (Fig. 1C). These results suggested that enhanced production of FFA under the conditions of growth limitation was toxic. The cultures of dAS2T were therefore maintained by subculturing under the N-replete conditions. In a growth test carried out 1 year later under the non-permissive conditions, however, some cells were found to survive and grow while most of the cells in the culture died in 3 d. The cells that survived were purified and designated dAS2T-pr1 (Fig. 2A).

#### Molecular characterization of the pseudorevertant

To identify the mutation(s) responsible for the enhanced growth of dAS2T-pr1, genome re-sequencing analysis of SPc, NA3, dAS2T and dAS2T-pr1 was performed. The strain used as the wild type (WT) in this study was the *small-plasmid-cured (SpC)* derivative of *S. elongatus* strain PCC 7942 (Kuhlemeier et al. 1983), which was found to have a 20 bp deletion site and nine putative single nucleotide polymorphisms (SNPs) (Supplementary Table S1) compared with the reference genome sequence of *S. elongatus* PCC 7942 (NC_007604.1). Of the nine SNPs, three were ascribed to errors in the reference sequence (Supplementary Table S1). NA3 was constructed from the SpC strain by deleting the *nrtABCD* genes (Maeda and Omata 1997), but it carried four SNPs specific to this strain (Supplementary Table S1). The significance of these base substitutions and indels will be discussed elsewhere. The dAS2T mutant was constructed from NA3 in this study by replacing an internal 0.66 kb fragment of *aas* with a P~nirA~-*tesA* transcriptional fusion but was found to carry an additional mutation in synpcc7942_1620, resulting in a P141L amino acid substitution in an AAA+ ATPase of unknown function (Table 1). The synpcc7942_1620 gene is the ortholog of the *ycf46* gene found on the chloroplast genome of some eukaryotic algae. synpcc7942_1620 and its paralog synpcc7942_0417 are conserved in most cyanobacteria. In *Synechocystis* sp. PCC 6803, the ortholog of synpcc7942_0417 (slr0374) was shown to be defective in growth under low CO2 conditions, but no growth phenotype was found for the mutant of the ortholog of synpcc7942_1620 (slr0480) (Jiang et al. 2015). The physiological significance of the mutation of synpcc7942_1620 in dAS2T could not be inferred from its function. As expected, this mutation was also found in dAS2T-pr1. The other base substitutions specific to the genome of dAS2T-pr1 were found in the region upstream of synpcc7942_2368. Thus, 74%
Fig. 1 Characterization of the FFA-producing strains constructed from the nitrate transportless mutant NA3 of *S. elongatus*. (A) Map of the *aas* region of the genome of the parental strain (NA3) and the dAS2 and dAS2T mutants. dAS2 was constructed by deleting the central portion of the *aas* coding region. dAS2T was constructed by replacing the same genomic region with a transcriptional fusion of a truncated *tesA* coding sequence (‘tesA’, shown in red) to the nitrogen-regulated promoter of *S. elongatus* (*P_{nirA}*, shown in yellow). Arrows above the maps indicate the primers used for PCR analysis in (B). (B) PCR analysis of the *aas* region of the WT and the mutants, showing the absence of the WT chromosome copies in dAS2 and dAS2T. (C) Growth of the FFA-producing strains under nitrate-limited growth conditions. Cells grown under the N-replete conditions (see text) were inoculated into nitrate (15 mM)-containing medium to give an OD$_{730}$ value of 0.01 and incubated under high CO$_2$ (2%) and high light (90 μE m$^{-2}$ s$^{-1}$) conditions for 120 h.

Fig. 2 Characterization of the pseudorevertant obtained from dAS2T. (A) Growth of dAS2T and dAS2T-pr1 under the nitrate-limited growth conditions. (B) Map of the synpcc7942_2368 region of the genome of *S. elongatus*, showing the location of the base substitutions found in dAS2T-pr1. (C) qRT–PCR analysis of the transcripts of synpcc7942_2368 (*rndA1*), synpcc7942_2369 (*rndB1*) and synpcc7942_2370 in dAS2T and dAS2T-pr1. Cells grown under the N-replete conditions were inoculated into nitrate (15 mM)-containing medium to give an OD$_{730}$ value of 0.2 and incubated under high CO$_2$ conditions (2%) under illumination provided at 90 μE m$^{-2}$ s$^{-1}$. Total RNA was extracted at the indicated time points and subjected to analysis, using the *rnpB* transcript as a control. Data from a single experiment are shown.
of the total reads from this region had a C→T substitution at position –87 with respect to the initiation codon of synpcc7942_2368 and 14% of the total reads had an A→T substitution at position –84 (Table 1). Given the presence of several copies of chromosomal DNA in an S. elongatus cell (Mori et al. 1996, Griese et al. 2011), the results indicated incomplete segregation of the WT and mutant chromosomes. Since dAS2T-pr1 did not have any other mutations as compared with dAS2T, it was deduced that the base substitutions in this region were related to the improved growth of the pseudorevertant.

Table 1 List of mutated sites in the genome sequences from dAS2T and dAS2T-pr1 as compared with the parental NA3 strain

<table>
<thead>
<tr>
<th>Position</th>
<th>ORF ID</th>
<th>Databasea</th>
<th>dAS2Tb,c</th>
<th>dAS2T-pr1b,c</th>
<th>Amino acid change</th>
<th>Gene product in CyanoBase\d</th>
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<td>1686360</td>
<td>1620</td>
<td>C</td>
<td>T (100%)</td>
<td>T (99%)</td>
<td>P141L</td>
<td>ATPase</td>
</tr>
<tr>
<td>2433803</td>
<td>Upstream of 2368</td>
<td>C</td>
<td>C</td>
<td>T (74%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2433806</td>
<td>Upstream of 2368</td>
<td>A</td>
<td>A</td>
<td>T (15%)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\* This column shows the nucleotide in the reference genome sequence in the database (NC_007604.1).
\* These columns show the base substitutions and their frequency in dAS2T and dAS2T-pr1.
\* The engineered mutation (deletion of nrtABCD) in dAS2T and dAS2T-pr1 is not included in the list.

\d http://genome.microbedb.jp/cyanobase

Fig. 2B shows the gene arrangement in the synpcc7942_2368 region of the genome of S. elongatus. The synpcc7942_2368 gene is located 322 bases downstream from the synpcc7942_2370 gene for tRNAHis and encodes a protein of 376 amino acids. It is tightly clustered with the synpcc7942_2369 and synpcc7942_2370 genes encoding proteins of 1,056 and 69 amino acids, respectively, presumably forming a three-gene operon. The protein encoded by synpcc7942_2368 was identified as a periplasmic membrane fusion protein (MFP), which is widely distributed in Gram-negative bacteria. MFPs are known to interact with a transporter of various protein families in the inner membrane and an outer membrane protein (OMP) of the TolC family to form tripartite efflux systems. The deduced Synpcc7942_2369 protein is a membrane transporter component of a tripartite efflux system of the resistance–nodulation–division (RND) family, which is represented by AcrAB–TolC of Escherichia coli (Nikaido et al. 2011, Alvarez-Ortega et al. 2013). It seemed likely that the transporters encoded by rndA1 and rndB1 are involved in export of FFAs and their enhanced expression in dAS2T-pr1 rescued the growth defect of dAS2T.

Fatty acid-producing activity of the pseudorevertant

The FFA-producing activity of dAS2T-pr1 was evaluated by measuring the concentrations of FFAs in the medium and the cells after 192 h of cultivation under the N-limited ‘tesA’-driven conditions (60 mM nitrate, 2% CO2, 180 μM m–2 s–1). Since dAS2T could not grow under these conditions, dAS2 was used as a reference. The concentration of total extracellular FFAs in the culture of the pseudorevertant was 45 mg l–1 (Fig. 3A), which was similar to that attained previously by 480 h cultivation of an S. elongatus aas mutant carrying a Ptrc-driven ‘tesA’ (Ruffing and Jones 2012). As a consequence, the average FFA excretion rate attained in this study (0.23 mg l–1 h–1) was more than twice the previously reported rate (0.10 mg l–1 h–1; Ruffing and Jones 2012). The sum of the extracellular and intracellular FFA amounted to 380 and 100 mg l–1 in dAS2T-pr1 and dAS2, respectively, indicating a large contribution of the ‘TesA thioesterase in production of FFAs. The
enhanced FFA production was largely attributable to the enhanced production of palmitic acid (16:0) in dAS2T-pr1 (Fig. 3B, C). Comparison of the data in Fig. 3A and C, however, revealed that nearly 90% of the FFAs produced by dAS2T-pr1 cells remained in the cell. The FFA content of the dAS2 cells was about one-third of that in dAS2T-pr1, with approximately 70% of the produced FFA remaining in the cell. Because most of the FFA is retained in the cell, the composition of intracellular FFAs was essentially the same as that of the sum of the extracellular and intracellular FFAs in both dAS2 and dAS2T-pr1 (Fig. 3B, C). In accordance with a previous report by Kaczmarzyk and Fulda (2010), palmitoleic acid (16:1) was more effectively excreted than palmitic acid (16:0). The most prominent difference in the FFA composition other than that in the 16:0 content between dAS2 and dAS2T-pr1 was the presence of a small but significant amount of lauric acid (12:0) in the latter. Given that the membrane lipids of S. elongatus do not carry 12:0-acyl residues (Murata et al. 1992, Kaczmarzyk and Fulda 2010), it was deduced that 12:0 was produced by the action of TesA on lauroyl-ACP. Interestingly, 12:0 was detected only in the medium but not in the cells of dAS2T-pr1, which suggested operation of a mechanism for export of the fatty acid, although there was quantitatively no sign of enhanced FFA export activity in dAS2T-pr1 (Fig. 3A).

Functional characterization of the RND-type transporter

To determine whether the products of the rndA1 and rndB1 genes contribute to FFA export, a mutant defective in these genes (dRND1) was constructed from the WT strain (SPc) (Fig. 4A). PCR analysis of the genomic DNA preparation from the dRND1 mutant showed complete segregation of the mutant chromosomes, indicating that these genes are not essential for growth of S. elongatus under the standard growth conditions (Fig. 4B). The WT strain was also transformed with the pRND1 plasmid to examine the effects of expression of the plasmid-borne rndA1B1 genes. Fig. 4C compares growth of the WT and the mutant strains on agar plates supplemented with various FFAs. In accordance with the report by Ruffing and Trahan (2014), the WT cells could grow well in the presence of 500 μM 16:0 or stearic acid (18:0) (Fig. 4C). The WT cells also grew well in the presence of 500 μM myristic acid (14:0). In contrast to the long length saturated FFAs, polyunsaturated FFAs were inhibitory to cell growth; the cells could tolerate 10 μM linolenic acid (18:3, Fig. 4C) but not 25 μM of the FFA (not shown) as reported previously (Ruffing and Trahan 2014). Linoleic acid (18:2) was also found to be toxic to the cells, completely inhibiting cell growth at a concentration of 25 μM (Fig. 4C). Compared with the polyunsaturated FFAs, oleic acid (18:1) was less toxic, permitting cell growth at 200 μM. Of the two medium length saturated FFAs tested, 12:0 was more toxic than capric acid (10:0); The cells could tolerate 25 μM but not 100 μM 12:0, while they could tolerate 100 μM 10:0 (Fig. 4C). Unlike the WT cells, the dRND1 cells failed to grow on the plates containing 100, 120, 140, 181 and 183; they could grow only on the plates containing 160 or 180 (Fig. 4C). Thus, the RND-type transporter is responsible for the tolerance of the cells to the exogenously added FFAs. On the other hand, the WT strain transformed with pRND1 showed enhanced tolerance to 12:0 and 18:2 compared with the WT and the control cells carrying the pSE1 vector (Fig. 4C).
These results supported the notion that the RND-type transporter mediates export of FFAs. The toxicity of 12:0 and its absence in the cells of dAS2T-pr1 (Fig. 3) suggested that extrusion of the toxic fatty acid was a major cause of enhanced growth of the pseudorevertant.

Fig. 5 shows the effects of expression of plasmid-borne rndA1B1 genes on growth and FFA production of dAS2T. The experiment was carried out under the high CO2 (2%) and high light (180 μE m−2 s−1) conditions in a medium containing 60 mM nitrate. Although the high concentration (60 mM) of nitrate was used to attenuate the expression of ‘tesA and to reduce the inhibitory effect of FFA production, the vector control cells carrying pSE1 were unable to sustain growth under these conditions; Cell growth ceased in 72 h after the inoculation irrespective of the presence or absence of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Fig. 5A) and the cells excreted only small amounts of FFAs into the medium (Fig. 5B). The cells carrying the pRND1 plasmid, in contrast, could continue growth (Fig. 5A) and FFA excretion (Fig. 5B) in the nitrate-containing medium, provided that IPTG was added to the medium. The enhancement of FFA excretion and growth in nitrate-containing medium verified that the rndA1B1 genes encode an efflux pump for FFAs.

Discussion

The tripartite efflux pumps of the RND family, which is represented by AcrAB–TolC of E. coli, are widely distributed in Gram-negative bacteria and involved in export of various toxic substances, including hydrophobic and amphiphilic compounds and heavy metals (Nikaido 2011, Alvarez-Ortega et al. 2013). The substrates of the transporters include not only the substances of exogenous origin but also the metabolic...
intermediates whose accumulation is toxic to the cell (Nikaido 2011, Alvarez-Ortega et al. 2013). Cyanobacteria also have this type of transporter; *Synechocystis* sp. PCC 6803 has six genes coding for RND proteins, one of which (*slr6043*) has been implicated in copper tolerance (Giner-Lamia et al. 2012). Of the six RND-encoding genes of *Anabaena* sp. PCC7102, *all3143* and *alr1656* have been shown to be essential for expression of N₂ fixation activity (Hahn et al. 2013). Although the natural substrate(s) of All3143 are yet to be determined, it was shown to export antibiotics and ethidium as well. Unlike most other bacterial and cyanobacterial strains having several RND-encoding genes, *S. elongatus* has only two of these genes (*rndB1* and *rndB2*), each of which forms a gene cluster with an MFP-encoding gene located upstream. One of these gene clusters, i.e. *rndA1–rndB1*, has been shown in this study to be involved in export of FFAs. This is the first report of the occurrence of FFA efflux activity in cyanobacteria. RND family efflux systems possessing the capacity to export FFAs have been identified in *E. coli* (*AcrAB–ToIC* and *MdtEF–ToIC*) (Lennen et al. 2013) and *Neisseria gonorrhoeae* (*MtrCD–MtrE*) (Hagman et al. 1995). Since these efflux systems are known to export various other compounds including bile salts, antibiotics and detergents, RndA1B1 may mediate export of other compounds as well. Further studies are required to determine the substrate spectrum of the cyanobacterial efflux pump.

Since they act as surfactants and exert adverse effects on biological membranes, FFAs generally possess antibiotic activities (Desbois and Smith 2010). FFA efflux pumps are therefore thought to provide a protective role in the survival and colonization of bacteria in the FFA-rich environments in the gut or on mucosal surfaces in human and animal hosts. In contrast, cyanobacteria are photoautotrophic organisms and are unlikely to encounter toxic concentrations of exogenous FFAs in their natural habitats. It seems likely that the cyanobacterial FFA efflux pump has a role in export of FFAs generated in the cell. Production of FFAs via deacylation of membrane lipids was recently shown to be activated by illumination of *S. elongatus* cells with high intensity light (Takatani et al. 2015). Because FFAs are recycled via Aas-mediated esterification to ACP, there normally is no intracellular accumulation of FFAs in the WT cells, but in the absence of Aas and hence of the recycling of FFAs, cells become hypersensitive to photoinhibition (Takatani et al. 2015). Even in the presence of Aas, excessive accumulation of FFAs may take place under certain conditions, making it necessary for the cells to excrete FFAs via the efflux pump.

Further studies on the properties of the *dRND1* mutant are needed to elucidate the physiological significance of the FFA-exporting activity of the RND-type efflux pump of *S. elongatus*.

The orthologs of the *rndB1* gene, defined as the bidirectional best hits in other genomes, are found in 60 out of the 80 completely sequenced cyanobacterial genomes available in GenBank as of July 2015 (excluding those of the five variants of *Synechocystis* sp. PCC 6803). Similarly, the orthologs of *rndA1* are found in 61 of the 80 completed cyanobacterial genomes. In the presence of multiple MFP- and RND-encoding genes in most of the cyanobacterial strains, inference of orthology on the basis of sequence similarities may not always be accurate. Nevertheless, the *rndA1* and *rndB1* orthologs thus identified are found to form a gene cluster similar to *rndA1–rndB1* in 45 of the cyanobacterial genomes, suggesting wide distribution of the encoded efflux pump in cyanobacteria. These gene clusters include *alr3143–alr3144* of *Anabaena* sp. PCC7102, whose products were shown to make a major contribution to multidrug resistance of the cyanobacterium and hence are suggested to be the functional equivalent of *E. coli* AcrAB (Hahn et al. 2013). The phylogenetic tree of the RND proteins from *E. coli*, *N. gonorrhoeae* and five model cyanobacterial species including *S. elongatus* shows that RndB1 and All3143 are closely related to each other and are more closely related to the bacterial RND proteins known to mediate FFA export than to RndB2 (Fig. 6). It is thus likely that All3143 also has FFA export activity, a possibility that merits further investigation.

The orthologs of RndB2 are found in 49 of the 80 cyanobacterial genomes. Gene clusters similar to *rndA2–rndB2*, consisting of the bidirectional best hits of *rndA2* and *rndB2*, are found in 35 of the genomes, indicating conservation of the encoded
Although the function of RndB2 (and RndA2) remains to be determined, wide distribution of the gene cluster suggests that they play important roles other than FFA export in cyanobacteria.

Although dAS2T-pr1 shows enhanced expression of rndA1B1 (Fig. 2C) and excretes FFAs at a much higher rate than was reported previously for a similar mutant constructed from the same cyanobacterial species (Ruffing and Jones 2012) (Fig. 3), most of the FFA in the culture is retained in the cells (Fig. 3). Using the average cell volume measured on the same culture (1.3 fl cell−1), the FFA content of the cells is calculated to be as high as 77 mg ml−1, showing that *S. elongatus* has high activity of FFA production but the FFA export activity is not high enough to prevent intracellular accumulation of FFA.

In *S. elongatus*, C16 fatty acids comprise nearly 90% of the fatty acyl residues in the membrane lipids (Murata et al. 1992). It is also known that monounsaturated fatty acids are produced by desaturation of the fatty acid residues esterified to the sn-1 position of the lipids (Murata et al. 1992). Both 16:0 and 16:1 FFAs are thus produced by decylation of membrane lipids in Aas-deficient mutants, but in the absence of recycling of the FFAs via esterification to ACP, the cellular acyl-ACP population would be dominated by de novo synthesized 16:0-ACP. This would account for the observation that the ‘tesA-expressing dAS2T-pr1 cells produce 16:0 as the major FFA species (Fig. 3C). Here, it is important to note that 16:1 is the major FFA species secreted by dAS2T-pr1 (Fig. 3A), while 16:0 is the major FFA species retained in the cell (Fig. 3B). This indicates that 16:0 export is crucial to increase the FFA level in the medium in the FFA production system using *S. elongatus*.

The molecular basis of the inefficient secretion of 16:0 from *S. elongatus* cells needs to be elucidated by further studies, but if it is related to the substrate specificity of RndA1B1, efficient export of 16:0 would require function of an FFA efflux system(s) other than RndA1B1. It should be noted that the heterotrophic bacteria found in FFA-rich environments have multiple exporters for FFAs; *E. coli* has three tripartite efflux systems contributing to excretion of FFAs, including the two RND-type transporters (AcrAB–TolC and MdtE–TolC) and an additional tripartite efflux system involving an MFS family protein (EmrB) as a transporter component (EmrAB–TolC) (Lennen et al. 2013); *N. gonorrhoeae* has also an EmrAB–TolC-like FFA export system (FarAB–MtrE) in addition to an RND-type exporter MtrCD–MtrE (Lee and Shafer 1999). Since the dRND1 mutant is highly sensitive to FFAs (Fig. 4C), *S. elongatus* does not seem to have other effective efflux pumps for FFAs. Thus, introduction of exogenous FFA efflux systems may be required to improve the FFA productivity of dAS2T-pr1. Heterologous expression of a huge RND-type pump may not be easy, but Dunlop et al. (2011) recently reported enhancement of limonene excretion from an engineered *E. coli* strain by heterologous expression of an RND exporter from *Alcanivorax borkumensis*. It should be noted, however, that functional expression of exogenous as well as endogenous transporters is not simple. In this study, IPTG-induced expression of *rndA1B1* was shown to enhance growth and FFA excretion by the dAS2T cells (Fig. 5), but, unlike dAS2T-pr1, the IPTG-induced dAS2T/prRND1 cells died within 180 h of cultivation, with complete loss of pigmentation (not shown). Although the cause of the growth inhibition observed in IPTG-induced dAS2T/prRND1 cells remains to be determined, overexpression of RndA1B1 is clearly toxic to the cell. The other factor that could affect the activity of FFA transport systems of foreign origin in cyanobacteria is the presence of a large amount of the intracellular thylakoid membrane. The cytoplasmic (plasma) and thylakoid membranes of cyanobacteria have distinct compositions of membrane proteins (Omata and Murata 1983, Omata and Murata 1984), most of which are localized exclusively to one or the other membrane.
system, but their targeting mechanism is unknown. Basic research on the mechanism of targeting of the membrane proteins in cyanobacteria would be required for proper targeting and functioning of foreign FFA transporters in cyanobacteria.

Materials and Methods

Strains and growth conditions

The cyanobacterial strain used as the WT was a derivative of *S. elongatus* strain PCC7942 that is cured of the resident small plasmid pUH24 (strain SPc) (Kuhlemeier et al. 1983). SPc and a nitrate transportless mutant NA3 constructed therefrom (Maeda and Omata 1997) were the parental strains of other mutant strains used in this study (Table 2). Cells were grown photoautotrophically at 30°C under continuous illumination provided by fluorescent lamps under either low CO₂ (0.04%) or high CO₂ (2%) conditions. The basal medium at 15 g l⁻¹ containing media, respectively. Solid media were prepared by adding 1.5% Bacto agar to the liquid medium and, when appropriate, supplemented with 0.1% ethanol and FFAs. The media were buffered with 20 mM HEPES-KOH (pH 8.2). When appropriate, spectinomycin and kanamycin were added to the medium at 15 μg ml⁻¹.

Construction of the FFA-producing strains

Transformation of cyanobacteria and isolation of homologous mutants were performed as described by Williams and Szalay (1983). dAS2 was constructed from a nitrate transportless mutant NA3 by deleting a 0.66 kb portion of the nrtABCD genes (Kuhlemeier et al. 1983) to construct dAS2T. dAS2T harboring plasmid pRL250 (Cai and Wolk 1990) to construct dAS2sacB, from which the nptI–sacB cassette with the *tesA* fusion gene was moved to the *S. elongatus* genome, following the procedure described by Takatani et al. (2003). dAS2sacB was transformed with plasmid pUC19 carrying the *tesA* gene to yield the plasmid pAAAS. A 552 bp DNA fragment carrying the truncated *tesA* gene was amplified by PCR from *E. coli* genomic DNA using the primers tesAF and tesAR carrying added restriction sequences, respectively (Table S2). After digestion with *Sac*I and *Xho*I, the *tesA* gene was cloned into the pSE1 vector (Maeda et al. 1998) carrying the *nia* promoter from *S. elongatus* to yield the plasmid pTEAS. The resulting plasmid was used to amplify the *PnirA–tesA* expression cassette with the *tesA* gene by PCR, using the primers PnirA-tesAF and PnirA-tesAR carrying added restriction sequences, respectively (Table S2).

Successful genome segregation was confirmed by PCR using the three pairs of primers, i.e. p1/p4, p2/p4 and p3/p4 (Table S2). To confirm segregation of the alleles, genomic DNA from selected transformants was analyzed by PCR using the primers 0918sF and 0918sR carrying added restriction sequences, respectively (Table S2).

Table 2. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>SPc</td>
<td><em>Synechococcus elongatus</em> strain PCC7942 cured of the pUH24 plasmid, wild type</td>
<td>Kuhlemeier et al. (1983)</td>
</tr>
<tr>
<td>NA3</td>
<td>SPc ΔnrtABCD, lacking nitrate transporter genes</td>
<td>Maeda and Omata (1997)</td>
</tr>
<tr>
<td>dAS2</td>
<td>NA3 Δ<em>as</em></td>
<td>This study</td>
</tr>
<tr>
<td>dAS2T</td>
<td>NA3 Δ<em>as</em>::<em>PnirA–tesA</em></td>
<td>This study</td>
</tr>
<tr>
<td>dAS2T-pr1</td>
<td>Pseudorevertant of dAS2T</td>
<td>This study</td>
</tr>
<tr>
<td>dRN1</td>
<td>SPc Δ<em>rndA1B1</em></td>
<td>This study</td>
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<td>SPc harboring pSE1</td>
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<td>SPc/pRND1</td>
<td>SPc harboring pRND1</td>
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<tr>
<td>dAS2T/pRND1</td>
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<td>This study</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pSE1</td>
<td>Km*, <em>S. elongatus</em> shuttle expression vector</td>
<td>Maeda et al. (1998)</td>
</tr>
<tr>
<td>pRND1</td>
<td>pSE1 derivative carrying <em>rndA1B1</em></td>
<td>This study</td>
</tr>
</tbody>
</table>
Plasmid-based expression of rndA1 and rndB1 genes

A shuttle expression vector carrying the trc promoter (pSE1) (Maeda and Omata 1997) was used for overexpression of the rndA1 and rndB1 genes in the WT and dAS2T. The 4,322 bp DNA fragment carrying the coding regions of rndA1 and rndB1 genes was amplified by PCR using the primer pairs 2368OXf and 2369OXr carrying added EcoRI and Nhel sites, respectively (Supplementary Table S3). The amplified DNA fragment was ligated between the EcoRI and XhoI sites of the pSE1 vector to yield the plasmid pRND1. The resulting plasmid was introduced into cells of the WT and dAS2T.

Genomic DNA sequencing and SNP analysis

Genomic DNA was prepared from Synechococcus cells as described previously (Williams 1988, Hiraide et al. 2015) with minor modifications. Extracted DNA was quantified using the Qubit dsDNA HS Assay Kit (Life Technologies). Sequencing of the genomic DNA was carried out using two different sequencers. For sequencing of the genomes of WT and NA3, Life Technologies’ SOLiD platform was used. Library construction and SOLiD sequencing were performed according to the protocol of Aoki et al. (2012). For sequencing of the genomes of dAS2T and dAS2T-pr1, the MiSeq platform (Illumina) was used. The Nextera XT library preparation kit (Illumina) was used to generate a paired-end library with dual eight base indices according to the manufacturer’s protocol. Prepared libraries were sequenced by 2×300 bp sequencing on the MiSeq platform using the MiSeq v3 Reagent Kit (Illumina). DNA sequences thus obtained were mapped onto the reference sequence of S. elongatus strain PCC7942 (GenBank accession No. NC_007604.1). For SOLiD csfasta analyses, SNPs and indel data were gained using an SNP program and a small indel program (Bioscope 2.1, Life Technologies). For illumina fastq analyses, the Burrows–Wheeler Aligner (BWA, version 0.7.5a-r405) algorithm was used for mapping (Bioscope 2.1, Life Technologies). The cell number and volume were determined by using a particle counter analyzer (CDA-1000, symex).

Preparation of RNA and qRT–PCR

Total RNA was extracted from dAS2T and dAS2T-pr1 cells, using a combination of the Trizol® Reagent (Life Technologies) and the SV total RNA isolation system (Promega). Aliquots of the cultures were mixed with an equal volume of ice-cold ethanol containing 5% (w/v) phenol and then centrifuged at 8,300 × g for 3 min at 4°C to collect the cells. The supernatant was removed by decantation and the pellet was resuspended in the remaining liquid. The suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 17,900 × g for 15 s at 4°C. After carefully removing the supernatant, the pellet was resuspended in 1 ml of the hot Trizol® Reagent (Life Technologies) and incubated for 2 min at room temperature. The suspension was then mixed vigorously with 200 µl of chloroform and centrifuged at 17,900 × g for 10 min at 4°C to sediment the cell debris. A 400 µl aliquot of the resulting supernatant was collected in a 1.5 ml microcentrifuge tube, to which 250 µl of ice-cold ethanol was added, and mixed vigorously. Total RNA was subsequently extracted using the SV total RNA isolation system (Promega) according to the manufacturer’s instructions. The isolated total RNA was used for the synthesis of cDNA, using a SuperScript® III First-Strand Synthesis System (Life Technologies) and random primers according to the manufacturer’s instructions. The obtained cDNA was used as the template for qRT–PCR analysis using LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science) according to the manufacturer’s instructions with the primers specific for rndA1, rndB1, Synpcc7942_2370 and mhpB, respectively. (Supplementary Table S3).

FFA analysis

For analysis of intracellular and extracellular FFA, 5–10 ml aliquots of the cultures were centrifuged at 1,700 × g for 15 min to separate the cells and the medium. The supernatant was transferred to Cryo Tubes (Thermo Fisher Scientific) and the cells were resuspended in 1 ml of methanol. The samples were stored at −20°C until use. For determination of the total concentration of FFA in the medium, the supernatant was analyzed using the Free Fatty Acid Quantification Kit (Biovision) according to the manufacturer’s instructions. For analysis of the extra- 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).

Other methods

The cell number and volume were determined by using a particle counter analyzer (CDA-1000, symex).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


