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Voshol, G.

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To *Natasha* and *Sabina*

Contents

List of abbreviations

'tesA	Multifunctional thioesterase I from *Escherichia coli* with the signal sequence, which normally directs it to the periplasm, removed.
2-OG	2-oxoglutarate
3PG	3-phosphoglycerate
aas	Acyl-ACP synthetase
ACCase	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
AD	anaerobic digestion
ADP-Glc	ADP- glucose
AGPase	ADP-glucose diphosphorylase
CCM	CO_2-concentrating mechanism
CoA	coenzyme A
DGAT	diacylglycerol acyltransferase
EPS	Extracellular polymeric substances
FAEE	fatty acid ethyl ester
FASII	dissociate fatty acid synthase
FFA	Free Fatty Acids
G1P	glucose-1- phosphate
G6P	Glucose-6-phosphate
GC-MS	Gas chromatography–mass spectrometry
GG	glucosylglycerol
LAHG	light-activated heterotrophic growth
LPSs	lipopolysaccharides
MUFA	Monounsaturated fatty acids
NADPH	The reduced form of Nicotinamide Adenine Dinucleotide Phosphate
OAA	oxaloacetate
OPP	oxidative pentose phosphate pathway
P-glycolate	2-phosphoglycolate
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PUFA	Polyunsaturated fatty acids
RuBP	ribulose-1,5-bisphosphate
SPS	sucrose-phosphate synthase
Suc6P	sucrose-6-phosphate
TCA	tricarboxylic acid cycle

Chapter 1

General introduction

The term petroleum is used to describe both crude oil and its refined products. Due to its high-energy density, transportability and relative abundance it has become an integral part of human society. Approximately 70-80% of the petroleum is used as a liquid fuel source, such as kerosene and gasoline. The remainder is used for other products including asphalt, tar, lubricants and many more (U.S. Energy Information Administration). However, due to recent concerns about global climate change, diminishing supplies of petroleum and political instabilities in oil producing countries there is a need to develop a clean sustainable alternative. Therefore the EU commission has established new climate and energy targets for coming decades. These targets include cutting the greenhouse gas emission by 40% by 2030 with an ultimate goal to reduce it by 85-90% by 2050 (European Commission). Additionally, the EU government aims to archive a 20% share of renewable energy in the total energy consumption by 2020 (Directive 2009/28/EC). In order to archive these targets, the efforts will be focused on using hydro, wind, solar power as well as biofuels. In the transport sector, which is currently almost entirely dependent on petroleum based fuels, the share of biofuel is set to become at least 10% by 2020 (Directive 2009/28/EC).

Bioethanol and biodiesel are the two dominant liquid biofuels with similar qualities as current petroleum based fuels. While the USA is mainly focused on bioethanol, Europe is the largest producer of biodiesel. The bulk of these biofuels are currently produced by using edible crops (i.e. first generation biofuels). Bioethanol is mainly produced from crops such as corn and sugar cane. Biodiesel is generally made from vegetable crops with high oil contents (e.g. rapeseed, sunflower), but can also be made from waste oil and animal fat. The first (from edible crops) and second-generation biofuels (from cellulosic biomass from non-food crops, agricultural waste, etc.) have successfully reduced the use of fossil fuels by 3%. However, despite this accomplishment, these biofuels have several disadvantages. First generation biofuels directly compete for arable land. Furthermore, both first and second-generation biofuels compete for fresh water, have the risk of reducing biodiversity, could cause land use changes (e.g. deforestation) and have limited potential for scaling up production (due to limitation in available land and yield

improvements). Research is currently focused on the development of third generation biofuels, which are produced using photosynthetic microorganisms.

Production of third generation biofuels using algae or cyanobacteria is a new fast growing technological development supported by the EU commission (European Commission). These microorganisms have simple nutrient requirements and an ability to produce a wide range of biofuel precursors. Moreover, algae and cyanobacteria do not compete for arable land and can be grown in either an open pond or a closed photo-bioreactor using fresh, brackish, saline or wastewater. After cultivation, the lipids can be extracted and converted into biodiesel, while the remaining fraction of protein and carbohydrates can be converted to bioethanol.

Currently, there is no commercial production of third generation biofuels. This is partially due to the high cost of harvesting the biomass, lack of efficient extraction methods and suboptimal strain productivity. To address these and other limitations it is necessary to enhance the biofuel production properties of these microorganisms. One approach to accomplish this is by genetic engineering. Cyanobacteria have several advantages, which make them good candidates for such optimisation strategies. Many are easily genetically transformable, have a high frequency of homologous recombination, have a sequenced genome and there are well-established genetic modification tools available (e.g. promoters, selection markers, etc.).

Aims of the thesis

The focus of this Ph.D. thesis is on optimizing the biodiesel production properties of strain *Synechococcus elongatus* PCC 7942. The aims were as follows:

1. To enhance the production and secretion of biofuel precursors
2. To isolate novel gene targets for further rational engineering

Outline of the thesis

Chapter 1 gives a general introduction to the main aims of this thesis. In **Chapter 2** an overview is given about the different types of bioenergy that can be produced using cyanobacteria including biodiesel and bioethanol. It also describes the biochemical pathways involved in the production of these biofuels and highlights several possibilities to improve the production of these compounds.

Chapter 3 describes the initial metabolic engineering of *S. elongatus* PCC 7942 for the production of biodiesel precursors. The engineered strains were subsequently characterized with regard to their intracellular and extracellular fatty acid profile, intracellular hydrocarbon profile and effect on growth. Out of all three engineered strains only the strain expressing a soluble thioesterase (responsible for the release of the fatty acid from their ACP) and lacking the acyl-ACP synthetase had a significant increase in the total amount of intracellular and extracellular free fatty acids. **Chapter 4** describes how this strain was further optimized for free fatty acid production by introducing all 4 subunits of the native ACCase. Fatty acid overproducing strains were also generated with transposon mutagenesis as described in **Chapter 5**. A transposon library was constructed and subsequently screened for fatty acid overproducing mutants. One of the higher producing transposon mutants was futher analysed. The genomic location of the transposon in this mutant was determined and the affected gene identified by DNA sequencing. This gene was subsequently expressed, partially deleted and the partial deletion was complemented with the introduction of the full gene construct. This resulted in identifying the GTP-binding protein Era as a promising novel target for the improvement of promising biofuel precursor production strains.

Chapter 6 provides a general discussion of the results obtained in this thesis including some additional unpublished results. In **Chapter 7**, a summary is provided in both English and Dutch describing the major findings of the thesis.

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Chapter 2

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Renewable energy from Cyanobacteria: energy production optimization by metabolic pathway engineering

Naira Quintana, Frank Van der Kooy, Miranda D. Van de Rhee, Gerben P. Voshol and Robert Verpoorte

Abstract

The need to develop and improve sustainable energy resources is of eminent importance due to the finite nature of our fossil fuels. This review paper deals with a third generation renewable energy resource, which does not compete with our food resources, cyanobacteria. We discuss the current state of the art in developing different types of bioenergy (ethanol, biodiesel, hydrogen, etc.) from cyanobacteria. The major important biochemical pathways in cyanobacteria are highlighted, and the possibility to influence these pathways to improve the production of specific types of energy forms the major part of this review.

Introduction

Fossil fuels, including oil, coal and natural gas, are providing about 85% of our energy need worldwide. The effective use of this energy resource in a productive and economic way still remains to be a major challenge. The main drawback of fossil fuels is that it is a finite resource and will be depleted in the near future. The term "peak oil" is commonly used to describe when peak oil production will be reached. Peak oil will be followed by a rapid decline in our oil reserves. Nashawi et al. (2010) predicted that peak oil will be reached as early as 2014. This finite nature of our fossil fuels and the dangers associated with nuclear energy, as evident by the recent nuclear disaster in Japan, emphasizes the importance of finding economically viable alternative energies. Alternative energy refers to renewable energy sources not derived from fossil fuels or nuclear power. Nowadays, there is a renewed interest in the development of sustainable energies promoted by the global concern that fossil fuels are finite, the rapid increase of energy consumption by industrialized countries, and the environmental problems caused by the burning of fossil fuels and from the management and storage of nuclear waste. Unlike fossil and nuclear fuels, alternative energy comes from natural resources (wind, sunlight, geothermal power and biomass), which are constantly replaced. Using these resources to supply our energy needs further supports sustainable development by lowering greenhouse gas emissions. The development and use of renewable energies provide a considerable number of benefits to nations around the world including an increment of the energy production, environmental protection, reduction in pollution and job creation. Solar (thermal or photovoltaic), wind, hydroelectric, biomass and geothermal energy currently constitute the most common sustainable sources of energy. Each one of these sources has particular properties that determine their usefulness and application in our society. The different characteristics of a specific energy resource can be evaluated in terms of sustainability indicators (Afgan and Carvalho 2002). In 2006, sustainable energies represented about 18% of the global total energy consumption (REN21 2007) and are able to substitute traditional fuels at different levels in our society including power generation, heating, transport fuel and rural energy. Because of its common use in developing countries for local energy supply, biomass represents the major source of renewable energy (constituting up to a 75% of the renewable energy sources) (Hall and Moss 1983). Bioenergy is fuel derived from biological sources (biomass) and is also referred to as biofuel. Biomass is defined as any

organic material coming from any form of life or its derived metabolic products. Biofuel (either biodiesel or bioethanol) is currently the only alternative energy source able to replace transport fuel in today's vehicles without involving major modifications to vehicle engines (Kaygusuz 2009). Biofuel is, however, not yet economically competitive with conventional energies. Additional input in order to collect, harvest and store the material is involved, resulting in higher manufacturing costs. Furthermore, biofuel possesses lower energy content than fossil fuels. Table 1 compares the calorific values for the different types of fuels. Biomass possesses important advantages if compared to other sustainable sources, for instance, it is available throughout the world, its processing is relatively simple without involving expensive equipment and it can be stored over long periods of time. In addition, bioenergy can be generated from organic waste material, which might otherwise be discarded thus contributing to the waste management. One of the main controversial issues related to the production of biofuel is the competition between energy crops and edible crops for arable land and water.

Table 1 Comparison of calorific values between conventional and alternative fuels and the corresponding references

Fuel type	Cal value	Reference
Gasoline	47.00 kJ/g	http://www.engineeringtoolbox.com
Diesel	45.00 kJ/g	(Hanumantha Rao 2009)
Biodiesel	37.27 kJ/g	http://www.berr.gov.uk
Methane	35.60 kJ/L	(Sialve et al. 2009)
Biogas	43.00 kJ/g	http://www.engineeringtoolbox.com
Hydrogen	150.00 kJ/g	http://www.engineeringtoolbox.com
Coal	27.00 kJ/g	(Matsunaga et al. 2009)
Ethanol	30.00 kJ/g	http://www.engineeringtoolbox.com
Bioethanol	26.72 kJ/g	http://bioenergy.ornl.gov
Rapeseed	39.70 kJ/g	http://www.biofuelsb2b.com
Sunflower	39.60 kJ/g	http://www.biofuelsb2b.com
Switchgrass	16.70 kJ/g	http://www.ecn.nl
Wheat	15.00 kJ/g	http://www.biofuelsb2b.com
Peanut	39.80 kJ/g	http://www.biofuelsb2b.com
Sesame	39.30 kJ/g	http://www.biofuelsb2b.com
Soybean	39.60 kJ/g	http://www.biofuelsb2b.com
Jatropha	39.07 kJ/g	(Hanumantha Rao 2009)
Chlorella	21.00–28.00 kJ/g	(Scragg et al. 2002)
Microalgae	25.80 kJ/g	(Matsunaga et al. 2009)

There is a scarcity of productive land available and areas occupied for bioenergy production may therefore serve for other more elemental uses, such as food production or conservation. Intensive cultivation of energy crops may also cause negative effects in the ecosystem biodiversity due to the substitution of local species and utilization of areas with some ecological value (RFA 2008).

Although biofuels are currently more expensive than fossil fuel, their production is exponentially increasing worldwide. Ethanol production experienced a twofold rise in the last 4 years reaching 67 billion litres in 2008. The increase in biodiesel production has even been more extraordinary, increasing six fold up to 12 billion litres, in the same period of time (REN21 2009). Biodiesel and bioethanol derived from edible crops, using today's technology, do not represent an effective alternative to substitute conventional fuel due to high costs of production and the land use competition with edible crops. Therefore, transition from the first (edible crops) and second generation (lignocellulosic biomass from dedicated non-edible crops like switchgrass and agricultural waste) to a third generation of biofuel, such as microalgae, is a promising option of sustainable biofuel production. For a description of all the different generations of biofuels, Gressel (2008) should be consulted. In addition to their higher yield per hectare, microalgae cultures do not compete with agriculture, requiring neither bio-productive lands nor freshwater (Chisti 2007, 2008; Griffiths and Harrison 2009; Mata et al. 2010, Rittmann 2008).

In this review, we will discuss the potential of a third generation of feedstock (focusing on cyanobacteria) as a viable biofuel source for energy production and compare it to first generation biofuel crops. We will also discuss the current state of the art for the production of H_2, ethanol, diesel, methane, electricity and photanol from these organisms. Additionally, we will focus on the carbohydrate, lipid and amino acid metabolism and discuss the possibilities of influencing these biochemical pathways in order to improve the production of a specific biofuel and to decrease the production costs.

Cyanobacteria as a producer of third generation biofuels

The most common feedstocks used in the first and second generations of biofuel include rapeseed, sunflower, switchgrass, wheat, peanuts, sesame seeds and soybean. These sources are used to generate different liquid forms of energy including alcohols (ethanol, propanol and butanol) and vegetable oil. As mentioned previously, the major constraint of these energy crops is based on the competition with our food sources for farmland and water. To overcome

this limitation, the third generation envisions a non-food biomass source for energy supply. Cyanobacteria possess certain properties, which have entitled them to be one of the most promising feedstocks for bioenergy generation:

- They can contain considerable amounts of lipids, which are mainly present in the thylakoid membranes.
- They possess higher photosynthetic levels and growth rates compared to other algae and higher plants.
- Cyanobacteria grow easily with basic nutritional requirements; they are able to survive if supplied with air [N_2 (nitrogen-fixing strains) and CO_2], water and mineral salts (especially phosphorous-containing salts) with light as the only energy source.
- Cultivation is therefore relatively simple and inexpensive.

The accumulation of lipids in algae occurs when the organism is under stress (e.g. nutrient deprivation) and in the stationary growth phase. Another secondary advantage is that cyanobacteria, being prokaryotes, can much more readily be genetically engineered in order to enhance the production of biofuels as opposed to eukaryotic algae. Cyanobacteria possess a relatively small genome and many of them have already been completely sequenced, thus it is also less complicated to perform system biology approaches in these organisms when compared to eukaryotic algae (Rittmann 2008). The genomes of 41 strains of cyanobacteria have already been sequenced including strains that are amenable to genetic manipulation (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Attempts to increase the biofuel content in cyanobacteria by genetic engineering has been mainly focused on *Synechocystis* sp. PCC 6803, which was the first cyanobacterium whose genome was sequenced (Kaneko et al. 1996) and *Synechococcus* sp. strain PCC 7942 whose genome has recently been sequenced (DOE Joint Genome Institute: http://genome.ornl.gov/microbiol/syn_PCC7942).

Therefore, cyanobacteria are a potential candidate for the production of biofuels and H_2 (Schütz et al. 2004, Rittmann 2008). They should, however, not only be viewed as a biocatalyst of sunlight, they also possess other additional properties which allow them to become ideal candidates for the development of bio-friendly systems for energy generation. Due to their ability to thrive in elevated CO_2 conditions, cyanobacteria have lately received considerable attention as a promising system for biological CO_2 mitigation, driving down CO_2 emissions from industrial activities. In addition, some

cyanobacterial species are also able to generate NH_4^+, relieving the dependency on chemical fertilizers. Moreover, cyanobacteria have been applied as bioremediation agents to remove heavy metals from aquatic ecosystems and reduce the excess of phosphate and nitrate in farmlands (Hall et al. 1995; Ono and Cuello 2007). In conclusion, it can be stated that the use of cyanobacteria to harness solar energy for the production of different types of bioenergy might represent a simpler and cleaner system for the production of sustainable energy.

Cyanobacteria

Cyanobacteria as a source of renewable energy

Cyanobacteria, being photosynthetic organisms, use the sun's energy, H_2O and CO_2 to synthesize their energy storage components, i.e. carbohydrates, lipids and proteins. These energy storage components form a potential feedstock which can be converted into bioenergy (Table 2) (SERI 1984). Of these three biochemical fractions, lipids have the highest energy content. To extract the energy from the lipid fraction, it has to be transesterified with a chemical process and the resulting hydrocarbons subsequently extracted. The hydrocarbons can then be used as transport fuel in the form of biodiesel. The carbohydrates may be transformed to ethanol by fermentation under dark, anoxic conditions (Stal and Moezelaar 1997) while alternatively, with the use of anaerobic digestion all three fractions can be converted to CH_4 gas (SERI 1984; Sialve et al. 2009). Cyanobacteria possess unique properties which make them a promising model to transform all these C sources into valuable fuels. The following sections discuss the wide range of fuels which can be potentially obtained from cyanobacterial biomass.

Table 2 Chemical composition of cyanobacteria (SERI 1984)

Fuel	Energy storage component		Fuel production	Total energy (MJ/kg)
Ethanol	Carbohydrate		0.329 L/kg	7.74
Oil	Lipids	Hydrocarbons	1.150 L/kg	44.96
		Fatty acids	1.250 L/kg	43.80
Biogas	Carbohydrates		0.370 (m^3/kg)	11.01
	Lipids		1.040 (m^3/kg)	30.95
	Proteins		0.490 (m^3/kg)	14.58

Hydrogen

Hydrogen can be produced by many strains of cyanobacteria by the reversible activity of hydrogenase. When cyanobacteria are grown under N2-limiting conditions, H_2 is formed as a by-product of N_2 fixation by nitrogenase (EC 1.7.99.2). It was also shown that non-heterocystous cyanobacteria are less efficient in H_2 production than the heterocystous organisms. Several reports have reviewed cyanobacterial species capable of producing H_2 (Abed et al. 2009; Das and Veziroglu 2001; Dutta et al. 2005) including at least 14 genera cultivated under different growth conditions. These genera include: *Anabaena*, *Oscillatoria*, *Calothrix*, *Cyanothece*, *Anabaenopsis*, *Nostoc*, *Synechococcus*, *Microcystis*, *Gloeobacter*, *Synechocystis*, *Aphanocapsa*, *Gloeocapsa*, *Microcoelus* and *Chroococcidiopsis* (Dutta et al. 2005). Among these genera it was shown that *Anabaena* spp. were able to produce the highest amount of H_2 (68 μmol mg^{-1} chl a h^{-1}). A comparison of the advantages which cyanobacteria have above other H_2-producing microorganisms has been described elsewhere (Hall et al. 1995). Research into the production of H_2 in cyanobacteria is at the moment focusing on the identification of new strains with specific H_2 metabolism, optimizing cultivation conditions in bioreactors and genetically modifying specific strains to enhance H_2 production (McKinlay and Harwood 2010; Schütz et al. 2004; McNeely et al. 2010). The main constraint for H_2 production in cyanobacteria is that hydrogenases are highly intolerant to the O_2 produced during photosynthesis. In addition, the availability of the reducing agents such as ferredoxin and NADPH is another bottleneck as these are also involved in other routes like respiration. In order to enhance H_2 production, it will be important to redirect part of the electron flow towards the H_2-producing enzymes and to engineer oxygen-tolerant hydrogenases (Angermayr et al. 2009; Weyman 2010). Recently, an attempt to eliminate pathways that consume reducing agents has been carried out by Dismukes' group. The mutants of *Synechococcus* sp. PCC 7002 lacking lactate dehydrogenase have resulted in a fivefold increment of the total H_2 production compared to the wild type (McNeely et al. 2010). Moreover, the emergence of synthetic biology approaches will facilitate the future development of specialised strains for biofuel production (Huang et al. 2010).

Biological H_2 production has been lately receiving considerably attention as a potential renewable energy source. Recently, the EC funded under the Framework Programme (FP7, 2008–2012) the SOLAR-H_2 project

with almost 4 million € (http://cordis.europa.eu) which aims to improve the photobiological H_2 production in Cyanobacteria.

Ethanol

Ethanol produced from renewable resources is an appealing energy source due to the fact that it can be mixed with existing diesel and used without any modification of existing diesel engines (Kaygusuz 2009). Currently, bioethanol is produced by fermentation of agricultural crops, mainly sugarcane in Brazil (Goldemberg 2007) and/or corn in the US (Hill et al. 2006). Due to its large-scale production from agricultural crops (sugarcane and corn) it remains to be a controversial alternative to fossil fuel due to its negative impact on food supply and food price sustainability (Rittmann 2008). The advantage that cyanobacteria have over the traditional energy crops in producing ethanol is that they ferment naturally without the need to add yeast cultures, as is the case with fermentation of traditional energy crops. This characteristic makes cyanobacteria a promising candidate for the production of ethanol. In order to study the fermentation ability of cyanobacteria, Heyer and collaborators (Heyer and Krumbein 1991) screened 37 strains and analyzed their ability of fermentation and the secretion of the fermentation products. Of the 37 strains studied, it was found that 16 strains were able to produce ethanol as one of the fermentation products while significant quantities of ethanol were produced in two *Oscillatoria* strains (>10 µmol/sample). Fermentation took place under dark conditions when no photosynthetical oxygen was produced, thus excluding respiration for energy production. Normally, fermentation does not represent a primary energy source for most algae and cyanobacteria. In these organisms, fermentation works at a minimum level, which allows them to survive. To overcome this problem and to increase the ethanol production, genetic modification might be a possible solution. The first cyanobacterial species to be genetically modified in order to produce ethanol was *Synechococcus* sp. PCC 7942. The strain was transformed by inclusion of coding sequences for pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis*, an obligately fermentative prokaryote. These genes were expressed under the control of the cyanobacterial *rbcLS* operon promoter, alone and in combination with the *Escherichia coli lac* promoter. The reported yields of ethanol produced by the transformed strain reached 54 nmol OD730 $unit^{-1}$. $liter^{-1}$. day^{-1} (Deng and Coleman 1999). The same genes have also been recently expressed in *Synechocystis* sp. PCC 6803 under the control of a

different promoter, the strong light driven *psbAII*. This strain showed an increase in ethanol production reaching 5.2 mmol OD730 unit^{-1}. liter^{-1}. day^{-1}) (Dexter and Fu 2009).

An alternative method for the production of ethanol is to produce it from cellulosic material. It has been observed that cyanobacteria deposits cellulose extracellulary at a yield of up to 25% of the cell dry weight (Dewinder et al. 1990). *Synechococcus leopoliensis* UTCC 100 (same strain as *Synechococcus elongatus* PCC 7942) was modified by Nobles and Brown with the cellulose synthase genes from *Gluconacetobacter xylinus* and this transformed strain was able to produce extracellular non-crystalline cellulose. The non-crystalline nature of cellulose makes it ideal as a feedstock for ethanol production facilitating its hydrolysis (Nobles and Brown 2008).

The focus of optimizing bioethanol-producing strains should start by screening several strains of cyanobacteria and using various promoters, afterwards, to establish the best combination. Studying different growth conditions as well as optimizing ethanol retrieval systems could lead an increase of ethanol production to levels where it will become economically feasible (Deng and Coleman 1999). The effect of salt stress conditions on the fermentation rate has been recently evaluated in Cyanobacteria. Ethanol production in a high salt concentration medium (1.24 M NaCl) was over 100-fold higher compared with the low salt conditions (0.24 M NaCl), resulting in a production of 0.75 mmol/g (Carrieri et al. 2010). In addition, Luo and co-workers have analysed the energy consumed and greenhouse gas emissions in different ethanol-producing systems employing cyanobacteria. This study showed that these two parameters are highly influenced by the concentrations of EtOH secreted by cyanobacteria. Their modelling results reveal that initial EtOH concentrations from 0.5 to 5 wt% would be enough to develop an environmentally friendly biofuel production system with reduced energy consumption and air pollution (Luo et al. 2010).

Different countries are currently funding projects in order to improve the bioethanol production efficiency in cyanobacteria. The US Department of Energy is sponsoring a project to the value of US $1.6 million for the DNA sequencing of six strains of *Cyanothece*, which shows promising ethanol production levels (http://news-info.wustl.edu/tips/page/normal/7719.html). The Federal Ministry of Education and Research in Germany will invest substantial funding for research on the production of bioethanol from cyanobacteria led by the Institute of Biology at Humboldt University

(http://www.drivehomesafe.com/news/the_bmbf_supports_research_on_producing_ethanol_cyanobacteria-6.html).

Ethanol is today the most common biofuel worldwide although longer chain alcohols have lately attracted some attention. The longer chain alcohols possess higher energy content and can be stored and transported easier than ethanol (Atsumi et al. 2008). Recently, Radakovits et al. (2010) pointed out that influencing the keto acid pathway and thereby producing isobutanol might be a promising source of biofuel in eukaryotic microalgae. The production of longer chain alcohols (C5–C8) in *E. coli* bacteria has been achieved by the overproduction of 2-keto acids, intermediates of the amino acid biosynthesis. These intermediates were later converted to butanol derivatives by the heterologous expression of 2-keto acid decarboxylase and alcohol dehydrogenase (Atsumi et al. 2008; Zhang et al. 2008). A very recent discovery that citramalate synthase occurs in cyanobacteria generates the possibility to produce propanol and butanol from 2-ketobutyrate, as this compound is an intermediate in the biosynthesis of citramalate (Wu et al. 2010). Engineering isobutanol biosynthetic pathway and overexpressing Rubisco have resulted in an enhanced production of isobutyraldehayde and isobutanol (6,230 and 3,000 μg L^{-1} h^{-1}, respectively) in *Synechococcus elongatus* PCC 7942 (Atsumi et al. 2009).

In addition, compared to isoprene, ethanol has lower energy content and is miscible in water which requires a time-consuming and expensive distillation process before it can be used. Thus, isoprene besides being useful in the industry as the basic unit of synthetic rubber could also be suitable as biofuel. In a recent study, *Synechocystis* PCC 6803 was genetically modified with the *Pueraria montana ispS* gene to enable the production of isoprene in this microorganism (Lindberg et al. 2010). Alkanes represent another appealing chemical feedstock fuel due the high energy content they possess. Recently, two-step alkane biosynthesis has been reported in cyanobacteria. This new finding opens new possibilities for alkane production by engineered mircroorganisms (Schirmer et al. 2010).

Photanol

In order to improve biofuel production, Hellingwerf and de Mattos have recently developed a new technology called the photanol approach (Hellingwerf and de Mattos 2009). Photanol has been one of the projects

funded by the Dutch Ministry of Agriculture under the Biorefinery Energy Innovation Agenda (http://www.senternovem.nl).

In this approach, the abilities of photosynthetic and fermenting bacteria are combined in a single organism (*Synechocystis* sp. PCC 6803). In photoautotrophic microorganisms, CO_2 is transformed into C_3 sugars like glyceraldehyde-3-phosphate (G3P), which are indispensable intermediates in the biosynthesis of complex molecules involved in the basic functions and structure of the organism. In chemotrophic organisms, however, different carbohydrates are first degraded to C_3 sugars to obtain energy (ATP) and converted afterwards into a variety of alcohols such as ethanol, butanol, propanediol and many others. In the photanol strategy, the properties of a chemotrophic organism have been included by means of genetic engineering into a photosynthetic organism (*Synechocystis* sp. PCC 6803). The C_3 sugar, G3P represents in this transformed organism the central linking compound between photosynthesis and fermentation.

Table 3 Bioenergy productivity of various energy sources using cyanobacteria as feedstocks

Fuel	Organism	Productivity	kJ/year[a]	References
Ethanol	*Synechoccocus* PCC 7942	54.0 nmol/L/day	<0.03	Deng and Coleman 1999
Ethanol	*Synechocystis* PCC 6803	5.2 mmol/L/day	2593.048	Dexter and Fu 2009
Ethanol by algenol biofuels	Cyanobacteria	56,000.0 L/ha/year	1.31 × 109/ha	Luo et al. 2010
Fatty acids	*E. coli*	4.5 g/L/day	67,671.00	Liu et al. 2010c
Fatty acids	*Synechocystis* PCC 6803	6.4 nmol/L/day	<0.03	Kaczmarzyk and Fulda, 2010
Fatty acids	*Synechoccocus* PCC 7942	8.4 nmol/L/day	0.03	Kaczmarzyk and Fulda, 2010
Isobutyraldehyde	*Synechoccocus* PCC 7942	6,230.0 μg/L/h	136.44	Atsumi et al. 2009
Isobutanol	*Synechoccocus* PCC 7942	3,000.0 μg/L/h	864.61	Atsumi et al. 2009
Methane	*S. maxima*	0.4 L/day	8,030.00	Varel et al. 1988
Hydrogen	*S. maxima*	400.0 μmol/L/h	994,435,200.00	Ananyev et al. 2008

[a]Calculated from previous published data

This modified organism uses solar energy to convert CO2 into biofuel with the advantage that the number of steps to do so has been minimized. This has lead to an increase in biofuel production efficiency compared to the current biofuel production processes reaching theoretical levels of 105 L ha^{-1} $year^{-1}$. Table 3 shows the energy productivity from the different bioenergy sources discussed above using mainly cyanobacteria as feedstock.

Diesel

For the production of lipid-based biofuels, cyanobacteria have received less attention than other feedstocks such as microalgae (Miao and Wu 2006; Rodolfi et al. 2009) or crops. As an energy source, cyanobacterial biomass has traditionally been associated with the production of ethanol (Deng and Coleman 1999; Dexter and Fu 2009) or H_2 (Hall et al. 1995). In 1998, 3,000 species of microalgae were screened in the Aquatic Species Program with the aim to identify species with high lipid content. In this program, little information regarding cyanobacteria was provided since they do not accumulate high amounts of lipids. It was, however, shown that cyanobacteria have the fastest growth rates and that the lipid productivity was amongst the highest in exponentially growing cultures (Sheehan et al. 1998). *Spirulina* also showed the highest overall utilization efficiencies in integrated liquid–gaseous fuel-processing options (SERI 1984). On the other hand, a recent comparison of different strains of microalgae revealed that although cyanobacteria possessed the highest biomass productivity, it showed a low lipid content reflecting the high metabolic cost of lipid synthesis (Francisco et al. 2010).

Around 2,000 species of cyanobacteria have been identified (Sheehan et al. 1998), but information regarding the production of biodiesel from these species or related parameters such as the biochemical profile, growth rate and energy content of the different species are scarce (Miao and Wu 2006). The implication of this is that the selection of adequate cyanobacteria strains for the production of biodiesel will not be an easy task. Table 4 summarizes the available information pertaining to the chemical composition of cyanobacteria. This information might assist in the evaluation of cyanobacteria species for industrial bioenergy production. To choose species for the large-scale production, a wide range of variables are important of which (Griffiths and Harrison 2009; Grobbelaar 2000) lipid content (percent dry weight), productivity (milligrams per litre per day) and growth rates (doubling time) are keys for the production of biodiesel. Griffiths and Harrison (2009) collected

Table 4 Different feedstock constituents from microalga (SERI 1984)

Species	Protein (% dw)	Lipid (% dw)	Carbohydrate (% dw)	Cal. Value (kJ/10 g dw)	References
Phormidium sp. (F)	62	11	16	d.n.a.	Cañizares-Villanueva et al. 1995
Calothrix crustacea (HF)	21	71a	8	25	Nagarkar et al. 2004
Calothrix contanerii (HF)	27	64a	8	29	Nagarkar et al. 2004
Gloeocapsa crepidinum (U)	56	36a	8	21	Nagarkar et al. 2004
Limicolaria martensiana (F)	19	76a	5	22	Nagarkar et al. 2004
Lyngbya semiplena (F)	27	64a	9	23	Nagarkar et al. 2004
Phormidium corium (F)	50	34a	16	33	Nagarkar et al. 2004
Phormidium tenue (F)	63	22a	15	31	Nagarkar et al. 2004
Spirulina subsalsa (F)	71	13a	17	35	Nagarkar et al. 2004
Spirulina labyrinthiformis (F)	68	17a	15	34	Nagarkar et al. 2004
Spirulina obliquus (F)	50–56	12–14	10–17	d.n.a.	Sialve et al. 2009
Spirulina platensis (F)	56–77	9–14	10–18	d.n.a.	Ciferri 1983
Spirulina maxima (F)	60–71	4	8–13	d.n.a.	Ciferri 1983
Oscillatoria formosa (F)	51	32a	9	15	Nagarkar et al. 2004
Oscillatoria salina (F)	42	47a	11	19	Nagarkar et al. 2004
Oscillatoria subbrevis (F)	45	57a	12	21	Nagarkar et al. 2004
Oscillatoria spp. (F)	d.n.a.	13	d.n.a.	d.n.a.	Griffiths and Harrison 2009
Synechocystis spp. (U)	d.n.a.	50	d.n.a.	d.n.a.	Rittmann 2008
Synechococcus spp. (U)	64	28	9	28	Nagarkar et al. 2004
Anabaena cylindrica (HF)	d.n.a.	5	d.n.a.	d.n.a.	Griffiths and Harrison 2009

Species	Protein (% dw)	Lipid (% dw)	Carbohydrate (% dw)	Cal. Value (kJ/10 g dw)	References
Anabaena sp. ATCC 33047 (HF)	45	10	28	d.n.a.	Vargas et al. 1998
Anabaena variabilis (HF)	47	11	22	d.n.a.	Vargas et al. 1998
Anabaenopsis sp. (HF)	52	11.4	16	d.n.a.	Vargas et al. 1998
Nodularia sp. (Chucula) (HF)	43	12.6	17	d.n.a.	Vargas et al. 1998
Nostoc commune (HF)	40	8.4	38	d.n.a.	Vargas et al. 1998
Nostoc paludosum (HF)	40	10.4	27	d.n.a.	Vargas et al. 1998
Nostoc spp.	37–47	7.9–11	16–32	d.n.a.	Vargas et al. 1998

Note that fuel production and energy are expressed in dry biomass of the class of compound (carbohydrate, lipid, protein)

HF heterocystous filamentous cyanobacteria, *F* non-heterocystous filamentous cyanobacteria, *U* unicellular cyanobacteria, *d.n.a.* data not available

[a]The lipid content was calculated as difference between 100 and the sum of proteins and carbohydrates

data on the biodiesel production of 55 microalgae species. *Synechococcus* with a production of 75 mg/L of lipids per day was among the highest yielding strains. Liu et al. (2010a) reported high secretion levels (133 mg L^{-1} day^{-1}) of FFA by an engineered *Synechocystis* sp.. However, this paper was retracted last July after some of its coauthors decided to remove their name and data from it (Liu et al. 2010b).

Vargas and co-workers (Vargas et al. 1998) analyzed the biochemical composition of 12 N_2-fixing cyanobacteria. The content of the lipids in these strains ranged between 8–12% dw, of which *Nodularia* and *Nostoc* contained the highest amount of lipids. Nagarkar et al. (2004) reported on the chemical composition and their respective calorific values of 13 cyanobacteria species (they did not report on the lipid content). They found that the calorific values varied between 15–33 kJ 10 g^{-1} dw with *Spirulina* and *Phormidium* responsible for the higher values. They could also correlate the high calorific values with high protein content. Vermaas and colleagues have genetically engineered a single gene mutant of *Synechocystis* able to accumulate up to 50% of dry weight in lipids (Rittmann 2008). A bioscience firm in the USA, Targeted Growth, has

recently claimed to have developed a new technology to increase the lipid content of cyanobacteria by approximately 400% (Timmerman 2009). It should be pointed out here that absolute values rather than percentages should be provided when discussing the improvement of lipid production. Percentages offer no possibility to have an idea about the lipid content reached and therefore to appreciate the real success of the new technique developed. Another company, Synthetic Genomics Inc., announced an agreement with ExxonMobil in 2009, to develop the next generation biofuels using photosynthetic algae including microalgae and cyanobacteria. ExxonMobil will invest US $600 million to develop more efficient means to harvest the oils which the photosynthetic algae produce (Howell 2009).

Biodiesel quality is also an important factor as it should meet various specifications before commercialization according to the European or American standards (UNE-EN 14214 and US ASTM D6751, respectively). Important parameters including oxidation stability, cetane number, iodine value and cold-flow properties are closely correlated to the fatty acid composition and are determined by the degree of saturation and the chain length of the fatty acids. Low cetane numbers are associated with shorter chain lengths and an increase in the level of unsaturation in the fatty acid. Moreover, a high content in unsaturated fatty acids is responsible for decreasing the oil oxidation stability while biodiesel consisting of saturated long chain fatty acids shows poor cold-flow properties (Knothe 2008; Pinzi et al. 2009). The fatty acids C10:0, C16:1 and C18:1, have the best combination of properties to produce high quality biodiesel. Investigation of the fatty acid profile of the raw material is thus important when selecting cyanobacteria species for biodiesel production. Several reports of the fatty acid composition of different cyanobacteria species grown under different conditions are available in the scientific literature. The fatty acid composition is often governed by the growth temperature where the degree of unsaturation increases at lower temperatures and the biosynthesis of shorter acyl chains occur (Liu et al. 2005). An increase of cyanobacterial biomass and lipid content is observed in strains grown in wastewater from the swine industry (Cañizares-Villanueva et al. 1995). The amount of polyunsaturated fatty acids (PUFA) was also decreased and those of monounsaturated fatty acids (MUFA) were increased at high light intensities (Walsh et al. 1997). The N_2 level is also known to influence the biochemical composition in cyanobacteria. The presence of a combined N_2 source drove up the protein content and drove down the amounts of lipids and carbohydrates in

cyanobacteria, although biomass productivity was only slightly affected. Finally, biochemical composition can also vary according to the growth phase, with the highest lipids occurring as the culture entered in the stationary phase (Vargas et al. 1998).

Generally, unicellular types of cyanobacteria lack PUFA, while most of the filamentous species contain high levels of di- and trienoic fatty acids (Kenyon 1972; Kenyon et al. 1972). Thus, unicellular strains represent the most suitable choice for high quality biodiesel production because they have a larger MUFA amount.

Methane

Cellular biomass can be transformed to CH_4, under anoxic conditions, through a process known as anaerobic digestion (AD). After lipid extraction from cyanobacterial biomass, the remaining material can be converted into CH_4 by this process raising the total energy recovery. This will lead to a more favourable or positive energetic balance of the overall biofuel production by cyanobacteria, which could also decrease the total costs of the process for bioenergy production. Furthermore, when the algae accumulate less than 40% of lipids, the oil recovery results in a 21% of the energetic value but the energetic costs of lipid recovery involved are higher than 30% (Sialve et al. 2009). In this case, AD represents an ideal choice for the total energetic recovery of biomass.

The production of CH_4 by an organism is highly correlated with its biochemical composition (C, N_2, phosphorous and oligonutrients). The methanogenic activity of cyanobacterial biomass in general is less desirable (0.31–0.47 L CH_4 L^{-1} day^{-1}) compared to cattle/swine waste or from mixed slurry (in the range of 3–6 L CH_4 L^{-1} day^{-1}) (Chellapandim et al. 2010; Varel et al. 1988). Therefore, not much research has been performed on the CH_4 production by cyanobacteria biomass leading to insufficient information in literature in order to compare the yield of different cyanobacteria strains. However, there are some examples that deserve to be mentioned. *Spirulina maxima* is one of the species studied in terms of the CH_4 production by AD (Samson and Leduy 1982, 1986; Varel et al. 1988). Mesophilic temperatures at 35°C seem to be optimum for a maximum yield (around 0.40 L CH_4/g VS fed) with an energy conversion efficiency of 59%. Compared to other microalgae, the CH_4 production of *S. maxima* is comparable to the yields obtained from *Scenedesmus* spp. and *Chlorella* spp. (0.4–0.8 L CH_4 L^{-1} day^{-1}). Further studies

should analyze the effect of different parameters on the production efficiency such as lack of nutrients, poor C/N ratio and accumulation of toxic compounds in the organisms which could reduce the gas productivity. The main constraints in the CH_4 production and the key factors influencing the AD yield in microalgae have recently been comprehensively analyzed by Sialve (Sialve et al. 2009).

Several studies have illustrated that the CH_4 production from cyanobacteria can be successfully combined with the natural ability of certain species to mitigate contaminants from the environment. The N_2-fixing cyanobacterium, *Anabaena* sp., was shown to be able to biodegrade cyanides and thereby producing CH_4 in batch reactors. This study carried out by Gantzer (Gantzer and Maier 1990) showed that *Anabaena* reduced cyanides by nitrogenase to CH_4 and NH_3, an enzyme normally responsible for the reduction of N_2. It was found that the rate for CH_4 production was ten times faster than expected based on literature values. Other examples in the literature describe cyanobacteria participating in a two-step system for the production of CH_4 either by producing nutrients (used by methanogenic bacteria) or by removing CO_2 from biogas and thereby improving its quality. Sustainable CH_4 production has been achieved in *Synechococcus elongatus* PCC 7942 from atmospheric CO_2 and solar energy, where the photosynthetic products including glucose or acetic acid were used as nutrients by a methanogenic bacterium in the CH_4 generation (Koshland 2009). Another species, *Arthrospira platensis* has also been involved in biogas production by removing CO_2 from the biogas formed by AD of sewage sludge (Converti et al. 2009).

Currently, "The Baltic EcoEnergy Cluster" is starting a project to produce biogas with high CH_4 and H_2 contents from algae and cyanobacterial biomass. The algae will be harvested from the Gulf of Gdansk and the Vistula submersion. The project is expected to be completed in 2013 (BiofuelDigest 2009).

From an economic point of view, the cost of CH_4 from cyanobacteria is still far more expensive when compared to the CH_4 derived from fossil fuels. In the coming years, this situation might change with efforts to improve the current technology resulting in economically reasonable prices of the gas. The process of CH_4 production alone or coupled to other bioenergy producing processes, therefore needs further investment in research (Rittmann 2008).

Electricity and fuel cells

It has recently been reported that microorganisms can convert light into electric energy with the use of photoelectrochemical cells. In these cells, high-energy electrons produced by the light excitation in the photosystems are transferred to an electron mediator, which in turn transfers them to an electrode and thereby producing electricity. Examples in the literature have studied strains like *Anabaena* (Yagishita et al. 1996) and *Synechococcus* or *Synechocystis* (Moriuchi et al. 2008; Tsujimura et al. 2001; Yagishita et al. 1996) for this purpose. Dr. Ilia Baskakov's research at the University of Maryland (Baltimore, USA) focused on the study of the electricity production in cyanobacteria has been funded by the prestigious Elkins Professorship Award (http://www.umbi.umd.edu/).

Cyanobacterial species can also act as natural resources of H_2 in fuel cells (Dawar et al. 1998). Behera and co-workers employed *Spirulina* in fuel cells for H_2 production. This species possess a known high nutritional value that could reduce the production costs of the energy production by this mean (Behera et al. 2007).

Central carbon metabolism in cyanobacteria

Cyanobacteria are aerobic photototrophic organisms generating ATP and NADPH during the light phase of photosynthesis. In the dark phase of photosynthesis, commonly known as the Calvin cycle, these molecules are used to produce sugars and other organic compounds from CO_2 and water. In short, during the first step in the Calvin cycle, CO_2 is assimilated by Rubisco (*rbcL*, EC 4.1.1.39) through the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form 3-phosphoglycerate (3PG). Glucose-6-phosphate (G6P) is in turn formed from 3PG via gluconeogenesis. Finally, RuBP is recovered from fructose 6-phosphate, G3P and dihydroxyacetone phosphate in a sequence of reactions similar to the non-oxidative branch of the pentose phosphate pathway.

Enzymes involved in the Calvin cycle are encoded by genes known as *cbb* genes. Gibson and Tabita showed that these genes are regulated by a common promoter activated by a LysR-type transcription factor, CbbR. Knockout strains with mutations affecting CbbR were impaired in the expression of *cbb* genes (Gibson and Tabita 1996).

It is well known that cyanobacterial Rubisco possesses a relative low affinity for CO_2 when compared to other algae or higher plants. In order to overcome this problem, they have developed a CO_2-concentrating mechanism

(CCM). The CCM contains two carbon-fixing enzymes, Rubisco and carbonic anhydrases, stored in carboxysomes. These storage microcompartments are thought to increase the CO_2 level surrounding Rubisco away from the competing O_2. A detailed understanding about the regulation of the main CCM constituents may enable the manipulation of this system to optimize the CO_2 fixation. A recent study has shown that in cyanobacteria, carboxysomes possess a specific organization through the cell, not found in other prokaryotes, and this distribution is closely linked to the CO_2 fixation efficiency. It seems that two cytoskeletal proteins, *par A* (involved in chromosome and plasmid segregation) and *merB* (involved in cell morphology), are involved in the organization of this specific carboxysomal pattern (Savage et al. 2010).

Inorganic carbon (Ci) transporters are also important CCM components and they are responsible for the intracellular delivery of CO_2 and HCO_3 (Badger et al. 2006). The overexpression of carbonic anhydrase, insertion of a more efficient Rubisco or multiple copies of Ci transporters could in principle increase CO_2 fixation levels in cyanobacteria. Although Rubisco is the main enzyme responsible for the C fixation, cyanobacteria possess an additional assimilation mechanism operated by phosphoenolpyruvate carboxylase (PEPC) and malic acid to assist them in the fixation of large amounts of CO_2, which is similar to the C4 pathway in plants (Yang et al. 2002).

The 2-phosphoglycolate (P-glycolate) is a noxious by-product of the Rubisco oxygenase activity, which inhibits important enzymes in the Calvin cycle (phosphofructokinase and triosephosphate isomerase). Besides photorespiration, in cyanobacteria, this molecule is degraded via the glycerate and decarboxylation pathway. Certain compounds derived from these pathways, including glycolate and glycine, seem to be potential candidates to control the Ci level in cyanobacteria (Eisenhut et al. 2008).

Some cyanobacteria strains are capable of assimilating some sugars and growth in dark conditions as facultative heterotrophs. Nevertheless, cyanobacteria grown in the dark have shown lower growth rates than when grown under light conditions (Stanier and Cohen-Bazire 1977).

Unlike most other phototrophs, in cyanobacteria, photosynthesis and respiration co-occur in a single compartment within the cell, the thylakoid membrane. In addition, constituents from both electron transfer chains such as the redox carriers cytochrome *bf* complex, plastoquinone, cytochrome *c*6 and plastocyanin are shared as well. Although they have common elements, some of them are still specifically associated to one of the pathways. Photosystem I

(PSI) and PSII are photosynthesis specific, whereas NADP dehydrogenase (*ndh*, EC 1.6.99.3), succinate dehydrogenase (*sdh*, EC 1.3.99.1) and terminal oxidases occur only in the respiratory chain. In contrast to higher plants, cyanobacteria often possess PSI/PSII ratios larger than 1. Under light conditions, PSI competes with terminal oxidase for electrons to maximize the amount of NADPH required for CO_2 fixation (Vermaas 2001). Recently, Nelson (2010) has shown how this PSI could be used as a small battery charger (Nelson 2010).

Carbohydrate metabolism

Sugars are the main and most common source of metabolic energy among living organisms. Sugar catabolic pathways [glycolysis, the oxidative pentose phosphate pathway (OPP) and tricarboxylic acid cycle (TCA)] are active mainly during the dark phase of the light–dark cycle. These pathways are responsible for producing NAD(P)H and other biosynthetic metabolites involved in the normal cellular functions. The major route of glc degradation is the OPP cycle and is considered as the main CO_2 fixation mechanism in cyanobacteria. The key enzymes in the oxidation of G6P through the OPP cycle are glucose-6-phosphate dehydrogenase (*zwf*, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase. Glucose-6-phosphate dehydrogenase controlled at the level of gene expression is especially interesting from a regulation point of view. In addition, low RuBP levels significatively reduce this enzyme activity (Kaplan et al. 2008; Stanier and Cohen-Bazire 1977; Vanderoost et al. 1989).

Modulation of sugar catabolic pathways in cyanobacteria during the light–dark transition has been reviewed by Osanai et al. (2007). In *Synechocystis* sp. PCC 6803, enzymes that participate in the sugar catabolism are stimulated by the light–dark shift and by the circadian rhythm. Regulatory proteins including histidine kinase, Hik8 and the RNA polymerase sigma factor, Sig E are involved in this activation. In addition, reduced N_2 concentrations also trigger the transcription of sugar catabolic genes via NtcA, the major N2 mediator. A detailed analysis of the transcriptional network in central metabolism during light periods has been provided by microarray data from *Cyanothece* ATCC 51142 (Stockel et al. 2008). This study revealed that the glycogen accumulated in diurnal periods is later degraded via glycolysis, OPP and the TCA cycle during dark or C depletion conditions. However, cyanobacteria possess an incomplete TCA cycle unable to work properly as a respiratory chain (discussed in the next section) (Stanier and Cohen-Bazire 1977). *Synechocystis* sp. PCC 6803 was shown to be able to grow under dark

conditions with periodic light pulses at glc expenses, a phenomenon known as light-activated heterotrophic growth (LAHG). The genes *hik8* and *sigE* seem to be involved in LAHG (Osanai et al. 2005; Singh and Sherman 2005). During LAHG conditions, the glycolytic enzyme fructose 1,6-bisphosphate aldolase (*fbaA*, EC 4.1.2.13) is induced by *sll1330* (a putative helix-turn-helix DNA-binding protein) in *Synechocystis* sp. PCC 6803 (Tabei et al. 2009). Central sugar metabolism differs among photoautotrophic, heterotrophic and photomixotrophic growth conditions (reviewed by Kaplan et al. 2008). Previous studies revealed that genomic and metabolomic data provide enough information to model the central metabolism and estimate the flux balance during different conditions in *Synechocystis* sp. PCC 6803 (Hong and Lee 2007). Modelling results were correlated with experimental data and may be extrapolatable to the whole cell metabolism in this organism.

Sugars and other organic compounds from the central metabolism participate in the biosynthesis of diverse cellular metabolites. Here, only the biosynthesis of major classes of carbohydrates will be discussed: glycogen, sucrose and the carbohydrates involved in salt stress response, trehalose and glucosylglycerol and cell wall polysaccharides.

Glycogen

Glycogen is the main carbon and energy storage polysaccharide in cyanobacteria. In the cell, glycogen is synthesised during light periods from assimilated CO_2 (Ball and Morell 2003). The enzyme ADP-glucose diphosphorylase (AGPase; EC 2.7.7.27, encoded by the *agp* gene, also known as *glgC*) controls glycogen synthesis in bacteria in an ATP-dependent reaction and seems to be regulated by 3PG (activator) and Pi (inhibitor) (Ballicora et al. 2003; Gomez-Casati et al. 2003).

$$\mathrm{G1P+ATP \leftrightarrow ADP-Glc+PPi}$$

As discussed below, sucrose synthase can also contribute to ADP-glucose (ADP-Glc) synthesis in filamentous cyanobacteria. The formation of glycogen involves the elongation of α-1,4-linked glucan by glycogen synthase (*glgA*, EC 2.4.1.21) which transfers ADP-Glc to the growing chain.

$$\mathrm{ADP-Glc+(glucosyl)}_n \rightarrow \mathrm{ADP+(glucosyl)}_{n+1}$$

The last step of glycogen synthesis is the α-1,6-glycosidic bond formation catalysed by a glycogen-branching enzyme (*glgB*, EC 2.4.1.18). The enzyme glycogen phosphorylase (*glgP*, EC 2.4.1.1) is responsible for the glycogen degradation, removing glucose units to form monomers of glucose-1-phosphate (G1P). The enzyme phosphoglucomutase (*pgm*, EC 5.4.2.2) catalyses the conversion of G1P to G6P, which can then be used in glycolysis or in the OPP pathway. Different conditions such as N2 depletion (Yoo et al. 2007) and salt stress (Page-Sharp et al. 1998) are known to influence the glycogen accumulation in cells.

Sucrose

Sucrose (α-D-glucopyranosyl β-D-fructofuranoside) accumulation in cyanobacteria during conditions of salt stress or desiccation plays a fundamental role as an osmotic protective agent (Billi et al. 2000; Miao et al. 2003a). In addition, a role as storage and signalling molecule has also been associated to this disaccharide (Desplats et al. 2005).

A schematic representation of sucrose metabolism is shown in Fig. 1 [adapted from (Curatti et al. 2008)]. The main enzymes involved in the sucrose synthesis are sucrose-phosphate synthase (*sps*, EC 2.4.1.14) and sucrose-phosphate phosphatase (*spp*, EC 3.1.3.24) (Salerno and Curatti 2003). The synthesis of sucrose-6-phosphate (Suc6P) is catalysed by SPS and followed by a dephosphorylation reaction by SPP leading to the sucrose formation. Sucrose is later hydrolyzed into glc and fru in an irreversible reaction catalyzed by invertases (EC 3.2.1.26). Genomic analysis revealed the importance of these enzymes under N2-fixing conditions in filamentous cyanobacteria (Vargas et al. 2003).

Sucrose may be produced as well from activated glucose (UDP/ADP-glucose) and fructose in a reversible reaction controlled by the enzyme sucrose synthase (*sus*, EC 2.4.1.13). This enzyme seems to be present only in filamentous N2-fixing cyanobacteria (Curatti et al. 2000; Salerno and Curatti 2003) where it is involved in the accumulation of storage (glycogen and sucrose) and structural (such as cellulose, see below) polysaccharides (Curatti et al. 2008).

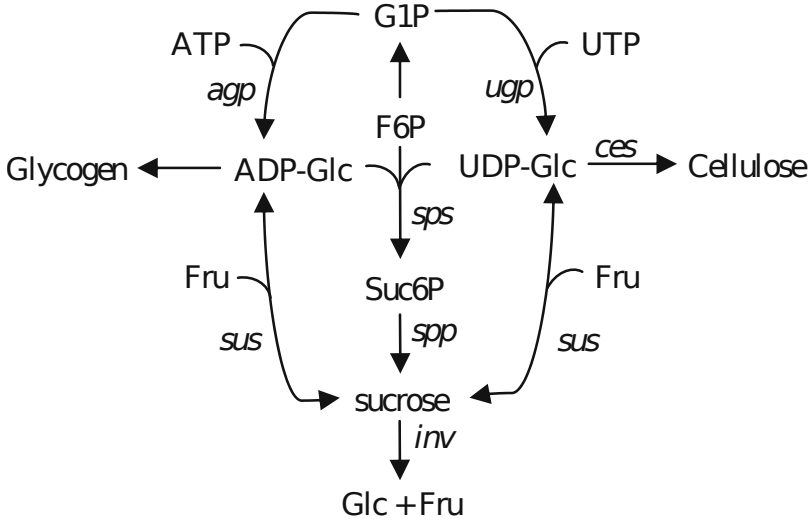

Fig. 1 Sucrose metabolic pathway in cyanobacteria (adapted from Curatti et al. 2008). Sucrose synthase has only been found in heterocyst-forming cyanobacteria. Genes encoding metabolic enzymes: *agp*, *ugp*, *sps*, *spp*, *sus*, *inv*, *ces*. Metabolites: ADP-Glc, G1P, UDP-Glc, F6Pm, Fru, Suc6P, Glc

Glucosylglycerol and trehalose

Other osmoprotectants like glucosylglycerol (GG, composed of a sugar and a polyol) and trehalose also occur in cyanobacteria and they are related to the salt tolerance of the strains. GG biosynthesis involves two successive reactions similar to sucrose synthesis catalysed by the enzymes GPP-S and GPP-P, respectively. In *Synechocystis* sp. PCC 6803, the role in salt resistance of *ggpS* and *ggpP* (*stpA*) has been supported by previous studies (Hagemann et al. 1997; Marin et al. 1998).

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is known to be produced through several biosynthetic routes in the different organisms (Avonce et al. 2006). TreY-TreZ pathway is involved in the trehalose formation in *Nostoc* and *Anabaena*. *TreY* encodes maltooligosyltrehalose synthase (*mts*, EC 5.4.99.15) and *treZ* encodes maltooligosyl trehalose trehalohydrolase (*mth*, EC 3.2.1.141). Gene disruption experiments indicated that trehalose, like sucrose, plays a crucial role in dehydration (Asthana et al. 2008; Higo et al. 2006) and salt stress tolerance (Salerno et al. 2004) in different organisms.

Cell wall polysaccharides

The cyanobacterial cell wall combines features from gram-positive and gram-negative bacteria. From inside to outside a cytoplasmic membrane, a highly cross-linked peptidoglycan layer and an outer membrane with lipopolysaccharides (LPSs) are the main constituents of this cell wall (Hoiczyk and Hansel 2000). Peptidoglycan strands in cyanobacteria consist of repeating subunits of the aminosugars *N*-acetylmuramic acid and *N*-acetylglucosamine. In addition, this strand contains cross-linked peptides and it is complexed with specific polysaccharides in its structure. Peptidoglycan biosynthesis, in bacteria, is mediated by the genes *murA-murG*, *mraY* and *pbp* and a schematic representation can be found in Garcia et al. 2008.

The LPSs in cyanobacteria have not been extensively documented. Previous studies indicated the different composition of the LPS in marine *Synechococcus* sp. and proteobacteria (Snyder et al. 2009). In addition, it has been suggested that a gene homologue of *lpxC*, *alr2270* participates in the LPS lipid A biosynthesis (Nicolaisen et al. 2009). Many cyanobacteria are also able to secrete diverse extracellular polymeric substances (EPS) into their immediate surroundings of the cell (reviewed by Pereira et al. 2009). Cellulose has been found as the main constituent of the EPS in several cyanobacteria (Nobles et al. 2001) and the genes involved in the synthesis of this polysaccharide (*cesA*, EC 2.4.1.12) possess sequence resembling the plant *cesA* genes. As mentioned above, the *sus* gene seems to be involved in the cellulose biosynthesis (Curatti et al. 2000). Recently, *Synechococcus* sp. PCC 7942 was genetically modified to secrete non-crystalline cellulose into the growth media, which is a promising candidate for ethanol production (Nobles and Brown 2008), as discussed in "Ethanol" section.

A possible strategy to enhance the lipid content for biofuel purposes could include knocking out the genes involved in the biosynthesis of storage or osmotic protectant substances. In addition, the overexpression of genes involved in the degradation of these compounds could also increase the lipid production in the cell. Previous studies on eukaryotic algae indicate that certain starch-impaired strains accumulate higher amounts of PUFA or TAG under N2 starvation (for a review see Radakovits et al. 2010).

Redirecting the C flux to the cellulose synthesis would be another approach in order to increase the fuel content in cyanobacteria. Currently, ethanol production is derived mainly from the fermentation of cellulose. The

overexpression of *cesA* plus a knockout of the *agp*, *spp* and *sps* genes could hypothetically cause an increase of the cellulose content in the cell.

Besides respiration, other polysaccharides could also in principle be catabolised by fermentation. However, as mentioned before ("Ethanol" section) in wild type cyanobacteria, fermentation does not seem to supply a significant amount of energy to the cell. Previous studies have already attempted to enhance the ethanol levels by developing genetically engineered organisms (Deng and Coleman 1999; Dexter and Fu 2009).

Lipid metabolism

Fatty acid and protein biosynthetic pathways possess phosphoenolpyruvate (PEP) as common substrate (Fig. 2). Thus, when PEP is converted to oxaloacetate (OAA) by phosphoenolpyruvate carboxylase (*ppc*, EC 4.1.1.31), it enters into protein synthesis and is directed to fatty acid synthesis when transformed to malonyl-CoA. PEP is converted to pyruvate by pyruvate kinase (*pyk*, EC 2.7.1.40) and then by pyruvate dehydrogenase (*pdhB*, EC 1.2.4.1) in a second reaction to form acetyl-coenzyme A (acetyl-CoA). In addition, pyruvic acid can be converted to alanine and thus participates in protein metabolism. On the other hand, acetyl-CoA can be converted to malonyl-CoA in a rate-limiting reaction catalysed by acetyl-CoA carboxylase (*accC*, EC 6.4.1.2), which is the first step towards the fatty acid synthesis. Whereas high concentrations of acetyl-CoA or free fatty acids stimulated PEPC activity in *E. coli*, (Izui et al. 1970) in certain cyanobacterial strains, the increased levels of acetyl-CoA did not influence PEPC activity (Chen et al. 2002; Luinenburg and Coleman 1993; Owttrim and Colman 1986). PEPC from *Synechococcus vulcanus* was strongly activated by fructose-1,6-diphosphate while aspartate acted as a strong suppressor. This compound has been reported to reduce the PEPC activity from *Coccochloris peniocystis*. PEPC seems to divert the carbon flux away from fatty acid biosynthesis. Thus, the antisense expression of PEPC-coding gene (*ppc*), in *Synechococcus* sp. PCC 7002, has led to a lipid content increase in this organism (Song et al. 2008).

As pointed out before, the first committed reaction in the fatty acid biosynthesis is an enzymatic reaction catalysed by ACCase. Whereas in eukaryotes, this enzyme is constituted by a single multifunctional polypeptide, bacterial ACCase contains four proteins, a biotin carboxyl carrier protein, biotin carboxylase and the α and β subunits of carboxyltransferase (Cronan and Waldrop 2002). Previous studies have already supported the important role

that ACCase possesses directing the C flow towards fatty acid synthesis (Lykidis and Ivanova 2008; Song et al. 2008). In plants, fatty acyl-ACP synthesised in the plastids is transformed to free fatty acids by acyl-ACP thioesterases and then transported from the chloroplast to the cytoplasm, thus the possibilities of influencing the control of fatty acid biosynthesis are remote.

Acyl-ACP synthesis from malonyl-CoA involves five different reactions catalysed in most bacteria by the type II or dissociate fatty acid synthase (FAS II). In the FAS II system, each reaction is catalyzed by an individual enzyme, while its eukaryotic counterpart is composed of a single multifunctional enzymatic entity (FAS I). First of all, the malonyl subunit from malonyl-CoA is transferred to ACP by the malonyl-CoA:ACP transacylase (*fabD*, EC 2.3.1.39). The resulting malonyl-ACP is then condensed to acetyl-CoA with the help of 3-ketoacyl-ACP synthase (*fabH*, EC 2.3.1.41). In *E. coli*, *fabH* is involved in the initial condensation reaction while further malonyl-ACP additions to the growing fatty acyl-ACP are carried out by *fabB* and *fabF*. Then, 3-ketoacyl-ACP reductase (*fabG*, EC 1.1.1.100) catalyses a reduction step yielding 3-hydroxyacyl-ACP which is dehydrated by 3-hydroxyacyl-ACP dehydrase (*fabZ*, EC 4.2.1.-) to produce trans-2-enoyl-ACP. The final reaction of the pathway is a reduction of the trans-2-enoyl-ACP by enoyl-ACP reductase (*fabI*, EC 1.3.1.9) resulting in fatty acyl-ACP used afterward in successive condensation steps. As mentioned earlier, ACCase activity is inhibited by this acyl-ACP and thereby this end product plays a fundamental role in the fatty acid synthesis control. In addition, Heath and coworkers (Heath and Rock 1995, 1996a, b) found that acyl-ACPs are also able to inhibit the 3-ketoacyl-ACP synthase and enoyl-ACP reductase activities. These findings might offer a possible explanation for the fact that the overexpression of ACCase causes a ~100-fold rise in malonyl-CoA levels, but only a sixfold rise of fatty acid synthesis (Davis et al. 2000).

The formed fatty acyl-ACPs are later directed to the synthesis of the membrane glycerolipids including monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidyl glycerol (PG) and sulfoquinovosyldiacylglycerol (Weier et al. 2005). The first step in lipid biosynthesis is the formation of a 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatic acid). In microorganisms, this compound is known to be produced by two different mechanisms. Either the fatty acyl-ACP is directly added to a 3PG (backbone for the glycerolipid synthesis) by a *sn*-glycerol-3-phosphate acyltransferase (GPAT or PlsB; EC 2.3.1.15) or by a newly discovered two-reaction system catalyzed by the enzymes, PlsX and PlsY

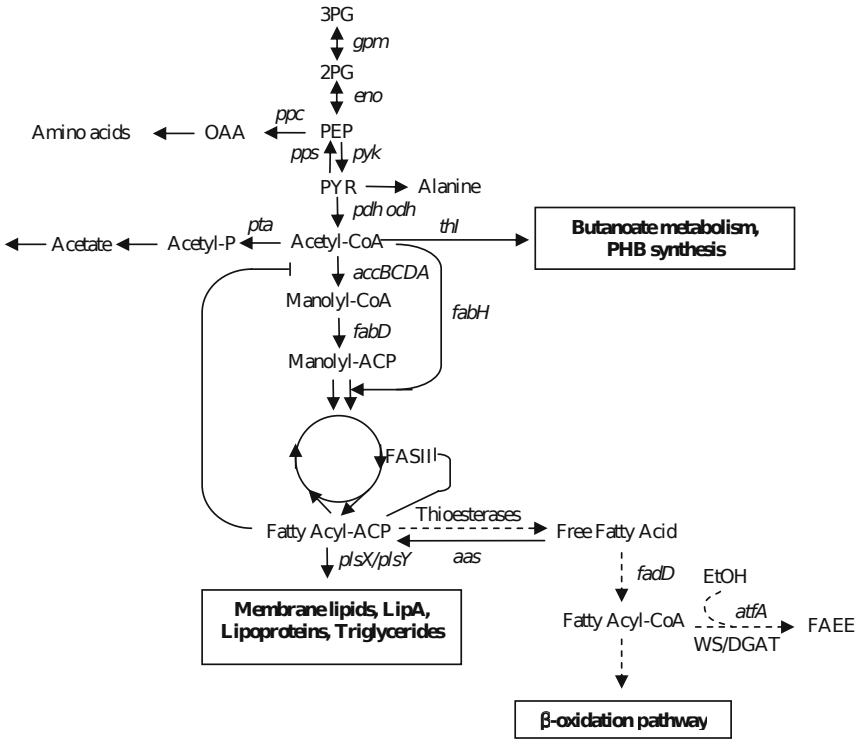

Fig. 2 Simplified overview of the fatty acid biosynthesis and some of the competing pathways in cyanobacteria (adapted from Liu et al. 2010a). Pathways not present in cyanobacteria or those which are unknown are indicated with *dashed lines*. Genes encoding metabolic enzymes: *aas*, *accBCDA*, *atfA*, *eno*, *fabD*, *fabH*, *fadD*, FASII, *gpm*, *odh*, *pdh*, *plsX/plsY*, *ppc*, *pps*, *pta*, *pyk*, *thI*. Metabolites: 2PG, 3PG, FAEE, OAA, PEP, PYR

(Zhang and Rock 2008). In this system, a molecule of phosphate is added to the fatty acyl group derived from a fatty acyl-ACP chain by PlsX and then transferred to G-3-P in a reaction catalyzed by PlsY (Lu et al. 2006). Further acylation by 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (AGPAT or PlsC; EC 2.3.1.51) forms 1,2-diacyl-*sn*-glycerol-3-phosphate (phosphatidic acid). The overexpression of GPAT or AGPAT led to higher lipid levels in *Arabidopsis thaliana* seeds (Ranalli 2007). It may be of interest to develop a similar strategy in cyanobacteria-overexpressing *pls XYC* genes.

Nutrient-limited conditions in *E. coli* trigger a series of reactions known as the stringent response. This response is modulated by guanosine tetra- (ppGpp) and pentaphosphate (pppGpp) which are included in a group of compounds called alarmones. These compounds mediate a wide spectrum of metabolic reactions. Previous results indicated that GPAT is negatively

regulated by ppGpp leading to a decrease in the lipid synthesis and consequently a decrease in the fatty acid production through the accumulation of fatty acyl-ACPs, responsible of the inhibition of several steps (previously mentioned) in fatty acid synthesis. It appears that an increase in the activity levels of GPAT, PlsX and a fatty acyl-ACP thioesterase (*tesA*, EC 3.1.2.14) overcomes this inhibition by detaching the fatty acid from the lipid biosynthesis (Davis et al. 2000; Heath et al. 1994; Paoletti et al. 2007).

TAGs are neutral storage lipids generally synthesised from diacylglycerol in an acyl-CoA-dependent reaction catalysed by diacylglycerol acyltransferase (DGAT) (Yen et al. 2008). Additionally, in plants and yeasts an acyl-CoA independent reaction catalysed by a phospholipid-diacylglycerol acyltransferase leads also to the TAG synthesis (Dahlqvist et al. 2000). In *E. coli*, the heterologous expression of bifunctional DGAT from *Acinetobacter baylii* resulted in fatty acid ethyl ester (FAEE) synthesis in this organism. This enzyme possesses both DGAT and wax ester synthase activities being able to use acyl-CoA molecules and esterify them to ethanol to create FAEEs *in vivo* (Kalscheuer et al. 2006). This *in vivo* production of fuel molecules circumvents the necessity of industrial transesterification of TAG. The accumulation of FAEEs by the transgenic organism accounted only for a 26% of the cellular dry weight, which needs further optimization to be profitable. These levels were lower than initially expected. Steen and co-workers have also used a similar approach to produce FAEE in *E. coli*. Fatty acid production was improved by overexpressing a native thioesterase and *fadD*, together with a heterologous acyl-CoA ligase and an ester synthase (*atfA*). The same authors used genetic engineering to over produce fatty alcohols in *E. coli* with the *acr1* from *Acinetobacter calcoaceticus* (Steen et al. 2010). Recently, similar strategies have been followed by Lu in *Synechocystis* sp. PCC 6803 (Lu 2010), although no yields have been reported. Radakovits engineered eukaryotic algae with specific thioesterases for the production of C12–C14 fatty acids. In this study, a high amount (around 80%) of the fatty acid produced was incorporated into triacylglycerols (Radakovits et al. 2011). In cyanobacteria, thioesterases overexpression results on the enhancement of fatty acid secretion (Roessler et al. 2009).

As previously mentioned, fatty acyl-ACP thioesterase has the capacity to uncouple fatty acid from lipid synthesis. This enzyme hydrolyses the ACP moieties from fatty acyl-ACP molecules to produce free fatty acids. In bacteria, CoA units are added to free fatty acids through an acyl-CoA synthetase (*fadD*,

EC 6.2.1.3) and then the resulting acyl-CoA enters into the *β*-oxidation pathway serving as a C and energy source (Fujita et al. 2007; Zhang and Rock 2008). As pointed out before, an increase in fatty acid production has been achieved already in *E. coli* by deleting *fadD* in combination with the overexpression of other key genes of the fatty acid pathway (Lu et al. 2008). In addition, long-chain acyl-CoA seems also to control the expression of genes encoding enzymes of fatty acid catabolism by interacting with the FadR transcriptional regulator. Previous results indicated that FadR is able to bind to specific DNA sequences inhibiting transcription of genes involved in fatty acid catabolism. If however, long-chain acyl-CoA is attached to FadR it is released from the DNA, allowing gene expression (Schujman and de Mendoza 2005; Zhang and Rock 2008). Another strategy to optimise the lipid content in the cell is to reduce lipid degradation through *β*-oxidation. These approaches have already been studied in plants and yeasts. However, *β*-oxidation is one of the major energy sources for the cells and mutation of genes involved in this pathway could lead to a decrease of the organism performance. In addition, many enzymes involved in the lipid metabolism possess common activities constraining the possibilities of eliminating single steps. In yeast, deletion of the fatty acid catabolism-encoding genes shows a fatty acids rise in the cells. The fatty acid secretion seems to be also stimulated in these mutants (Radakovits et al. 2010). Thus, similar strategies could be developed in cyanobacteria.

Any extra amount of energy or C produced during the cell growth is accumulated in storage products in the organisms. Although, as mentioned earlier, glycogen is the main carbohydrate reserve in cyanobacteria, these organisms also produced polyhydroxyalkanoates to store their excess of energy and C. There are three main enzymes participating in the cyanobacterial glycogen synthesis, an ADP-glucose pyrophosphorylase (*agp* (*glgC*), EC 2.7.7.27), a synthetase (*glgA*, EC 2.4.1.21) and a branching enzyme (*glgB*, EC 2.4.1.18). In *Synechocystis* sp. PCC 6803, it seems that mutation of the *agp* gene is linked to a higher accumulation of PHB compared to the wild type during photoautotrophic growth (Wu et al. 2002). Mixotrophic growth in presence of either glc or acetate resulted in an enhancement of the glycogen (in the wild type and increased growth in both wild type and mutant) and PHB (in mutant and wild type) contents, respectively. Since acetate provides the acetyl subunits required to form acetyl-CoA, the levels of PHB are higher when compared to photoautotrophic conditions. Nevertheless, acetate unlike glc did not seem to stimulate the cellular growth in a significant way. It has been suggested, that

the produced acetyl-CoA is not incorporated into pathways that contributes to the basic functions of the cells. The participation of acetyl-CoA in the rate-limiting step of the lipid biosynthesis catalysed by ACCase might provide a partial explanation to these findings. Another study on *agp*-deficient mutants suggested that low light intensities (45 μmol photon m^{-2} s^{-1}) increase moderately the cell growth, whereas higher intensities (82 μmol photon m^{-2} s^{-1}) reduce the growth (Miao et al. 2003b). These results indicate that *agp* mutants are able to harness the chemical energy from photosynthesis more efficiently at low light conditions; at higher intensities impaired glycogen synthesis would cause photosynthesis inhibition (feedback inhibition). The same authors later found that during salt stress, these modified organisms produce sucrose rather than GG as osmoprotectant (Miao et al. 2003a). This example further illustrates the complexity of metabolic networks and their modulation.

In *S. elongatus* PCC 7942, deletions in the *glgC* and *glgA* genes strongly repressed glycogen accumulation (Suzuki et al. 2010). Under constant conditions of light, the growth, photosynthesis and respiration rates were particularly low in these mutants. Although during salt and oxidative stress they were able to produce sucrose (PCC 7942 does not accumulate GG), these modified organisms presented an additional growth reduction compared to wild type. These results indicate that glycogen is indirectly linked to the strains ability to successfully deal with stress conditions, probably due to the correlation between glycogen and ATP availability. Previous studies in eukaryotic algae have shown that the inactivation of genes involved in the biosynthesis of starch, another storage compound, causes an enhanced lipid production (Ramazanov and Ramazanov 2006). Unfortunately, the lipid and protein contents were not examined in the glycogen-deficient mutants of *Synechococcus*.

Amino acids synthesis via the TCA cycle

The TCA cycle is in most organisms an essential aerobic pathway for the final oxidation of carbohydrates and fatty acids. Its major role is to supply reducing power (NADH and FADH2) to produce ATP and intermediates required by other biosynthetic reactions. In contrast, cyanobacteria possess a non-complete cycle mainly orientated to the 2-oxoglutarate (2-OG) synthesis, which is involved in amino acid biosynthesis and N2 fixation. The 2-OG is derived from citrate in a reaction catalyzed by isocitrate dehydrogenase (*icd*, EC 1.1.1.42).

Thus, in cyanobacteria, the reducing power is mainly generated during photosynthesis (Muro-Pastor and Florencio 1992).

The first enzymatic step of the cycle is mediated by a citrate synthase (*gltA*, EC 2.3.3.5) which condenses acetyl-CoA and OAA to produce citrate. The acetyl-CoA participates in the TCA cycle is derived from glycolysis and β-oxidation, whereas the OAA has originated from PEP in an irreversible step mediated by PEPC. OAA serves as a precursor of the biosynthesis of several amino acids including aspartate. It should be pointed out that PEPC lacks oxygenase activity and is therefore more effective in the C assimilation than Rubisco where carboxylase and oxygenase activities compete with each other (Gillion 1998). This fact could explain why C flux, in cyanobacteria, is primarily allocated to the protein biosynthesis. Usually, TCA intermediates cause the suppression of pyruvate kinase activity, the main modulating enzyme in carbohydrate catabolism (Lin et al. 1989).

As stated above, cyanobacteria possess an incomplete TCA cycle where two key enzymes are missing, the 2-oxoglutarate dehydrogenase (*ogdh*, EC 1.2.4.2), involved in the transformation of 2-OG into succinyl-CoA, and the succinyl-CoA synthetase (*sucL*, EC 6.2.1.4, 6.2.1.5), for the formation of succinate (Pearce et al. 1969; Stanier and Cohen-Bazire 1977). Previous modelling studies in *Synechocystis* sp. PCC 6803 evaluated the addition of these missing enzymes to the metabolomic network (Shastri and Morgan 2005). Their results suggested that the lack of a completed TCA cycle does not diminish the growth significantly in this microorganism.

It seems that in cyanobacteria, succinate can originate from 2-OG using a different route (Cooley et al. 2000). In these microorganisms, 2-OG is directly involved in the N_2 fixation via the NH_3-assimilating mechanisms, GDH (*gdhA*, EC 1.4.1.14) (Chávez and Candau 1991) and GS-GOGAT route (Meeks et al. 1978). This process is directly related to amino acid biosynthesis including glutamate and glutamine or indirectly leading (via glutamate) to alanine and aspartate derived from pyruvate and OAA, respectively. The GS-GOGAT system seems to be able to produce 2-OG as well. However, the production of 2-OG by this mechanism is rather insignificant if compared with IDH activity (Muro-Pastor and Florencio 1992).

It has been suggested that 2-OG plays a fundamental role in the amino acid biosynthesis. This theory has been supported in *Synechocystis* by experiments where the external addition of ammonium to N2-free medium enhanced the 2-OG levels as well as the glutamate and glutamine production

(Merida et al. 1991). Furthermore, in cyanobacteria, 2-OG seems to be implicated in the heterocyst differentiation. Previous studies have indicated that the activity of key enzymes in the heterocyst development, including NtcA and PII, is subjected to 2-OG concentrations (Zhang et al. 2005). In the same way, this compound is also considered to modulate the N and C metabolism in non-nitrogen-assimilating cyanobacteria (Eisenhut et al. 2008; Muro-Pastor et al. 2005). However, proving the role of 2-OG as a signalling compound is still challenging since it is quickly diverted into different metabolic processes.

Conclusions

The renewed interest in alternative energies derived from biomass has been recently triggered by the prediction of a reduction in the crude oil production 10 years earlier than speculated by experts (Nashawi et al. 2010). In this context, cyanobacteria have received significant consideration stimulated by the fact that these microorganisms seem able to cope with some of the major difficulties encountered with preceding biofuel generations. Furthermore, cyanobacteria offer a promising biomass feedstock for various organic (ethanol, CH_4 and biodiesel) and inorganic (H_2 and electricity) biofuels. As the examples in "Cyanobacteria as a source of renewable energy" section illustrate, cyanobacteria are a potential source of a wide range of valuable biofuels using different substrates for their production. Many of the cited strategies are still under development and their energy yield may not be economically feasible yet at industrial production levels. Therefore, the metabolic network needs to be optimized to generate an efficient and economic biofuel production system extrapolatable to a commercial scale.

A detailed study of the biosynthetic routes in cyanobacteria would assist us to evaluate the impact of genetic manipulations and its limitations in the entire metabolic network. Thus, this approach would further facilitate the design by genetic engineering of an optimized metabolic network for biofuel production in cyanobacteria.

Previous work has shown that a minimum of the C derived from photosynthesis is directed to pathways involved in the biofuel production (Lindberg et al. 2010). Thus, in order to optimize the energy production by these microorganisms, new strategies of pathway engineering need to be proposed to redistribute the C flux among the biosynthetic pathways of other fuel feedstocks.

Future directions in genetic engineering have been suggested along with this paper not only for an efficient energy production from sugars and lipids but also to expand the spectrum of the products targeted as bioenergy feedstocks (isoprene, propanol and butanol). Both considerations are crucial factors to properly implement cyanobacteria in a future large-scale system of biofuel production.

As indicated previously, metabolic engineering of metabolic pathways could cause unplanned and unforeseen deleterious effects on cellular function. However, engineered organisms could still be a valuable tool for bioenergy production in case the manipulated genes are ligated to specific promoters that can be turned on after the organism reaches some pre-established desirable conditions. Previous studies revealed that temperature-modulated promoters are suitable for controlling ethanol production in *Synechococcus* (Wood et al. 2004).

In conclusion, the success of future generation of biofuels will rely on the advances in metabolic engineering to optimize the existing energy-related biosynthetic pathways and to reduce the stress on the genetically modified organisms. An efficient and cost-effective fuel production from biomass should decrease our current dependence on conventional energies which are both scarce and polluting.

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Chapter 3

Applied and Environmental Microbiology. Submitted.

Effects of the introduction of the *E.coli* thioesterase *tes*A and the knockout of the endogenous acyl-ACP synthase *Δaas* on fatty acid and hydrocarbon production in *Synechococcus elongatus* PCC 7942

Gerben P. Voshol, Vera Meyer, Cees A.M.J.J. van den Hondel

Abstract

To develop a continuous biodiesel production system, which does not require time and cost intensive harvesting processes, cyanobacteria need to be able to sustain a high growth rate and secrete their biofuel precursor into the supernatant. To achieve this, we constructed three mutant strains of *Synechococcus elongatus* PCC 7942 (*'tesA*, *Δaas* and *'tesA*:*Δaas*) with altered fatty acid and alkane production as well as the ability to secrete biofuel precursors into the culture medium. We quantified the amount of extracellular fatty acids, intracellular fatty acids and hydrocarbons produced. Our results show that the thioesterase from *E. coli* ('TesA) might be partially outcompeted by the endogenous acyl-ACP synthetase (Aas), but is still able to significantly influence hydrocarbon production. Disruption of the *aas* gene shows that this enzyme is very important for the synthesis of 8-heptadecene, but not for alkane production. Moreover, the *Δaas* strain shows an increase in the total amount of extracellular fatty acids. Se:ΔA+T, containing the *'tesA* gene and knockout of the *aas* gene, is the only strain which shows a significant increase in the total amount of intra- and extracellular fatty acids. To our knowledge, this is the first study, which provides a comprehensive picture of early effects caused by the introduced mutations on the fatty acid and hydrocarbon profile of PCC 7942.

Introduction

In recent years the need has considerably increased to develop new, clean and sustainable energy sources to complement and eventually replace our reliance on fossil fuels. This increased need is due to concerns about global climate change and diminishing supplies of easily accessible oil reserves. One way to develop such energy sources is to use photosynthetic organisms, which are able to convert solar energy and carbon dioxide into biodiesel and/or biofuel precursors. Cyanobacteria possess several essential traits to develop them as production organisms of biofuels, such as relatively high growth rates, simple nutrient requirements and the susceptibility to genetic modifications (Quintana et al. 2011).

Among cyanobacteria, *Synechococcus elongatus* PCC 7942 is a promising candidate for biofuel production due to its ability to produce relatively large amount of high quality biofuel precursors. These precursors include (i) short to medium length saturated and mono-unsaturated fatty acids which are important for increasing combustibility as well as thermo and oxygen stability of biofuels (Ramos et al. 2009), and (ii) linear alkanes ranging from C14 to C18 (Schirmer et al. 2010). Moreover, one of the competing pathways for fatty acid production, namely the ability to synthesize polyhydroxybutyrate, is not present in this strain (Suzuki et al. 1996).

Biosynthesis of fatty acids starts with the conversion of acetyl-CoA to malonyl-CoA by a multimeric enzyme called acetyl-CoA carboxylase (ACCase) (Fig 1). The malonyl group is subsequently transferred to an acyl carrier protein (ACP) with the help of a malonyl-CoA:ACP transacylase. The formed malonyl-ACP is then elongated in a cyclic manner through its condensation, reduction, dehydration and reduction, by a type II fatty acid synthase, resulting in the final long chain fatty acyl-ACP. Besides the de novo synthesis of fatty acids, long chain fatty acyl-ACP molecules can be synthesized from free fatty acids by the acyl-ACP synthetase. These newly activated fatty acyl-ACPs can then participate in lipid and hydrocarbon synthesis (Kaczmarzyk 2008). In addition, these long chain fatty acyl-ACPs provide feedback inhibition on three of the enzymes involved in its synthesis, namely the ACCase, 3-ketoacyl-ACP synthase and enoyl-ACP reductase (Davis and Cronan 2001; Heath and Rock 1996b; Heath and Rock 1996a; Heath and Rock 1995). A thioesterase is an enzyme, which is able to remove the fatty acyl chain from the ACP molecule. Thereby it can free the fatty acid, which can move through the

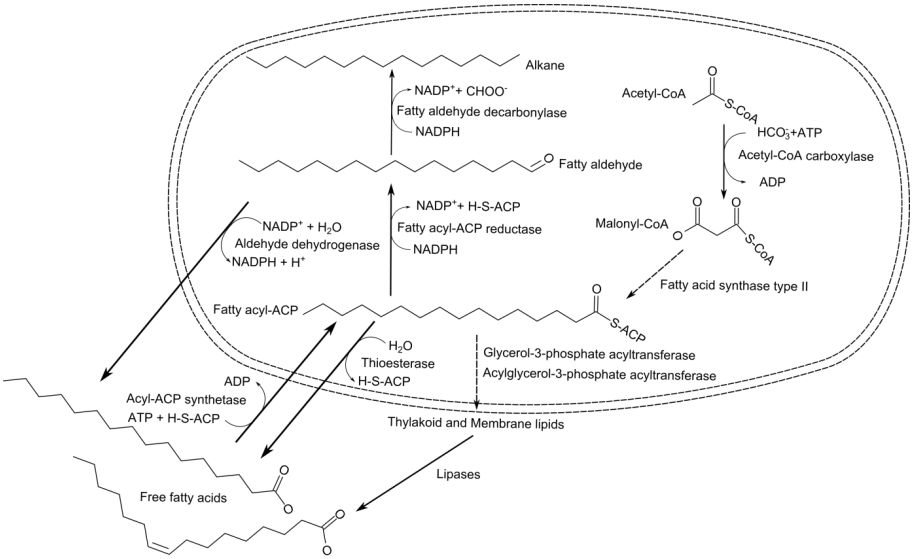

Fig 1 Overview of the lipid metabolism of PCC 7942 and its mutants. Fatty acid biosynthesis starts with the conversion of acetyl-CoA to malonyl-CoA catalyzed by the acetyl-CoA carboxylase (ACCase). The malonyl group is transferred to an acyl carrier protein (ACP) and subsequently elongated in a cyclic manner by a type II fatty acid synthase. This results in the formation of the final long chain fatty acyl-ACP, which can then participate in lipid or hydrocarbon synthesis. Besides the *de novo* synthesis of fatty acids, long chain fatty acyl-ACP molecules can be synthesized from free fatty acids (free fatty acids) by the acyl-ACP synthetase. An enzyme called thioesterase is able to remove the fatty acyl chain from the ACP molecule. Thereby it can free the fatty acid, which can move through the cell membrane.

cell membrane and thus prevents feedback inhibition caused by the long chain acyl-ACP molecule.

The introduction of a heterologous thioesterase ('TesA) in combination with a knockout of the endogenous acyl-ACP synthetase (Aas) can relieve the feedback inhibition and increase the secretion of free fatty acids in *Synechococcus elongatus* PCC 7942 (Ruffing and Jones 2012), *Synechococcus* sp. PCC 7002 (Ruffing 2014) and *Synechocystis* sp. PCC 6803 (Hu et al. 2013; Liu et al. 2011). However, except for one report (Ruffing and Jones 2012), very little is known about the effect of the '*tes*A and Δ*aas* mutations on the fatty acid production of PCC 7942. In particular, while the authors observed an increase in the total amount of secreted free fatty acids, they did not study the influence of the introduced mutations on the absolute quantity and composition of intracellular fatty acids and hydrocarbons. This knowledge, however, is crucial since alterations in the level of certain intracellular fatty acids can enhance the total fatty acid and hydrocarbon productivity. Furthermore, changes in the

intracellular fatty acid pool can have a dramatic effect on thylakoid and cell membrane synthesis and, as a result, on photosynthetic activity and growth of bacterial cells.

In this study, we constructed three mutant strains of PCC 7942 *('tesA, Δaas* and *'tesA:Δaas*) for the production of secreted fatty acids and quantified the amount of secreted fatty acids, as well as the amount of intracellular fatty acids and hydrocarbons produced. Using this approach we addressed the following questions: (i) what are the effects of the introduced mutations (*'tesA*, *Δaas* and *'tesA:Δaas*) on fatty acid and hydrocarbon production in PCC 7942? (ii) Do changes in the intracellular fatty acid pool influence the secreted fatty acid profile? (iii) Do changes in the intracellular fatty acids and/or secreted fatty acids affect the production of hydrocarbons? Based on the results obtained, we have been able to provide a more complete picture of early effect caused by the introduction of '*tes*A , deletion of *aas* and a combination of these two alterations in PCC 7942 on the fatty acid and hydrocarbon profile of this cyanobacterium.

Material and Methods

Strains, Plasmids and Cultivation conditions

Strains and plasmids used in this study are shown in Table 1. Primers used are listed in Table 2.

Wild type *S. elongatus* PCC 7942 as well as its mutants were routinely cultivated in BG-11 medium (Rippka et al. 1979) (pH 7.5) at 30 °C under continuous illumination with cool white light at 60 µEinstein/m2/s. When necessary, antibiotics were added to a final concentration of 25 µg/ml kanamycin and/or 2 µg/ml spectinomycin in combination with 2 µg/ml streptomycin.

For extractions of total fatty acids and hydrocarbons as well as for spectral measurements, bacteria were pre-grown on BG-11 solidified with 1% (w/v) washed agar for seven days. Subsequently, bacteria were harvested from plates and inoculated in duplicate in 50 ml of BG-11 at a starting OD_{750} of 0.05. The liquid bacterial cultures were then cultivated under shaking conditions at 250 rpm under non-CO2 enriched conditions for 120 h. The growth of strains was followed at OD_{750} for 5 days and the specific growth rates were calculated.

Table 1 Strains and plasmids used in this study

	Description	Reference
Strain		
E. coli DH5α	Used for molecular cloning and for the amplification of a truncated thioesterase ('*tesA*)	(Hanahan 1983)
S. elongatus PCC 7942	A freshwater cyanobacterium	Pasteur Institute (Paris, France)
Se:T	*S. elongatus* PCC 7942 expressing a truncated thioesterase from *E. coli* DH5α (*'tesA*)	This study
Se:ΔA	*S. elongatus* PCC 7942 with a disrupted acyl-ACP synthetase (*aas*; Synppc7942_0918)	This study
Se:ΔA+T	*S. elongatus* PCC 7942 expressing a truncated thioesterase from *E. coli* DH5α (*'tesA*) and containing a disrupted acyl-ACP synthetase (Synpcc7942_0918)	This study
Plasmid		
pJet1.2	Used for the cloning of blunt PCR products	Thermo-Fisher Scientific (Waltham, MA, USA)
pBSL128	Contains a Kanamycin resistance disruption cassette, used for the disruption of the *aas* gene	(Alexeyev and Shokolenko 1995)
DS1321	Targets Neutral site 1, confers resistance to spectinomycin (Sp) and streptomycin (Sm) antibiotics and contains a strong isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated lac-trc promoter followed by a ribosome binding site (RBS) and an unique NotI restriction site	(Niederholtmeyer et al. 2010)
pSe1	Derived from DS1321 with the original RBS removed and lacking the ATG start codon near the NotI restriction site	This study
pSe1:T	Derived from pSe1 with a truncated thioesterase from *E. coli* DH5α (*'tesA*) cloned into the unique NotI restriction site behind the inducible lac-trc promoter	This study
pJet1.2:ΔA	Km containing disruption cassette ligated into a EcoRV-SmaI double digested pJet1.2 containing the *aas* gene and flanks	This study
pJet1.2:T	pJet1.2 containing a truncated thioesterase from *E. coli* DH5α (*'tesA*) and a strong RBS	This study

Table 2 Primers used in this study

Primer	Sequence[a]	Remarks
remATGfw	tctagaaagcttgcggcc	Removal of Ribosome binding site from DS1321
remATGrv	gtgagcggataacaatttcacac	Removal of Ribosome binding site from DS1321
5pAASfw	gtcgacttccggcctatgcttaaatg	Amplification of PCC 7942 *aas* gene (=7942_0918), addition of SalI restriction site
3pAASrv	gtcgaccaattccccaaactgttgct	Amplification of PCC 7942 *aas* gene (=Synpcc7942_0918), addition of SalI restriction site
5pTesAfw	gcggccgc**aaggaggaaaaaaaaa**atggcagcggacacgttattgattctgg	Amplification of truncated *tesA* gene for free fatty acid conversion, addition of NotI restriction site
3pTesArv	gcggccgcttatgagtcatgatttactaaaggctgc	Amplification of truncated *tesA* gene for free fatty acid conversion, addition of NotI restriction site
NS1fw	cttcctatggttcgggatca	Amplifies WT Neutral site 1 for segregation check
NS1rv	gtttccaatgccttctccaa	Amplifies WT Neutral site 1 for segregation check
Kmfw	ctagtgacgtcctagactgggcggtttatgga	Verification of presence/absence of Km^R-cassette in PCC 7942 WT and *aas* knockout strains
AASfw	cgatcctgaaacgaaggaaa	Verification of presence/absence *aas* gene in PCC 7942 WT and *aas* knockout strains

[a]Restriction sites are underlined and the ribosomal binding site is indicated in bold.

Genetic engineering of S. elongatus PCC 7942

Plasmid pSe1:T (*'tesA*) was constructed using classical ligation dependent cloning. To obtain the plasmid, the ribosomal binding site of DS1321 (Niederholtmeyer et al. 2010) was removed by amplification of the vector with T4 polynuclease kinase treated primers remATGfw and remATGrv (Table 2) using Phusion polymerase. Subsequently, the amplified product was self-ligated using T4 ligase. This plasmid, pSe1, contains homologous regions to the Neutral site 1 of PCC 7942, a streptomycin (Sm)/spectinomycin (Sp) resistance marker, an *E. coli lacI* gene and a strong trp-lac IPTG inducible promoter. The *tesA* gene was partially amplified from *E. coli* DH5α by colony PCR with primers 5pTesAfw and 3pTesArv (Table 2). The 5' primer contains a NotI restriction site followed by a strong ribosome binding site and a new start codon, while the 3' primer has only a NotI restriction site. The resulting product was subsequently purified from gel and ligated into pJet1.2. Following NotI digestion, the truncated thioesterase gene (*'tesA*) was reisolated and ligated into NotI linearized pSe1 which resulted in the final plasmid pSe1:T.

Plasmid pJet1.2:ΔA for gene knockout of Synpcc7942_0918 was constructed as follows. The *aas* gene including ~1 kb flanks was amplified using primers 5pAASfw and 3pAASrv and ligated into pJet1.2. The resulting

plasmid was digested with EcoRV and SmaI and ligated with the EcoRV digested disruption cassette from pBSL128. This led to the final disruption construct pJet1.2:ΔA.

Extraction of total fatty acids and hydrocarbons

Extraction of total fatty acids and hydrocarbons was performed as follows. Bacteria were grown in BG-11 for 5 days as described above. Subsequently, cells were harvested by centrifugation. The pellet was lyophilized and subsequently used for the extraction of intracellular fatty acids and hydrocarbons. Each 20 mg of freeze-dried biomass was mixed with 50 µl of C10:0 internal standard (5 mg/ml), 1 ml hexane and 2 ml sodium methoxide (0.5 M) in methanol. Subsequently, the samples were sonicated for 5 minutes and incubated at 50 °C for 10 minutes (this gives sufficient time for transesterification, but prevents hydrolysis of the fatty acids). The reaction was stopped by the addition of 3 ml 5% HCl in methanol. The samples were sonicated and incubated at 70 °C for 20 min. After cooling to room temperature, samples were extracted twice with 4 ml of hexane containing 50 mg/L butylated hydroxytoluene to prevent fatty acid oxidation. The samples were then concentrated by vacuum evaporation, dissolved in 1 ml of hexane and analyzed via GC-MS.

For the determination of fatty acids in the supernatant. One volume of chloroform and one volume of methanol were added to each 2 ml supernatant and subsequently an additional volume of chloroform and ultrapure water was added. After vortexing for 30 minutes at room temperature, samples were extracted twice using chloroform, dried and subjected to transesterification as described above. Samples were finally dissolved in 100 µl hexane and GC-MS analysis was performed.

GC-MS analysis

These alkane and fatty acid methyl ester mixtures were analyzed using an Agilent model 7890A gas chromatograph, equipped with a model 7693 autosampler and detected using a model 5975C inert XL mass spectrometer. Separation was performed using a DB-WAX column (10 m, 0.25 mm, 0.25 µm, Agilent). Helium (1 ml/min) was used as the carrier gas. After injecting 1 µl, the oven was initially held at 50 °C for 1 minute, then the temperature was raised with 25 °C/min until the oven reached a temperature of 200 °C, subsequently the temperature was increased with 3 °C/min to 230 °C where it was held for 8

min. Identification was performed based on their retention time and mass spectrum compared with those of authentic reference compounds (Sigma-Aldrich, St. Louis, MO, USA). Fatty acids were quantified based on calibration curves constructed using authentic standards relative to the internal standard. Hydrocarbons were quantified relative to the internal standard.

Statistics

Data analysis was carried out using the R *Statistical* Software (Foundation for *Statistical* Computing, Vienna, Austria). Data obtained for growth as well as quantified fatty acids and hydrocarbons were first tested using Leven's test. All data analyzed were not significantly different in their variances. These data were subsequently compared using ANOVA and post-hoc Tukey Honest Significant Differences test. Changes were considered significant if their corrected p values were smaller than 0.05.

Results

Genetic engineering of free fatty acids secreting S. elongatus PCC 7942

The multifunctional thioesterase I from *Escherichia coli* has a broad *in vitro* substrate specificity which is able to release the C12 to C18 acyl chains from their acyl carrier protein, with highest specificity for C16:0-ACP (Barnes and Wakil 1968). The decrease in the acyl-ACP pool caused by the introduction of this thioesterase is expected to influence both fatty acid and alkane production. For the expression of this protein, plasmid Se:T was constructed by removing the original signal sequence of the *E. coli tesA* gene which normally directs it to the periplasm and inserting the truncated gene (*'tesA*) behind a new ribosomal binding site. This construct, present in plasmid pSe1, was introduced into neutral site 1 of *S. elongatus* PCC 7942. Colony PCR verified that this construct integrated correctly and was fully segregated in strain Se:T .

The acyl-ACP synthase is responsible for the re-thioesterification of free fatty acids and thereby incorporating these into the intracellular acyl-ACP pool (Kaczmarzyk and Fulda 2010). A disruption of this gene leads to the inability of the strain to reuse free fatty acids resulting from the recycling of thylakoid and membrane lipids for subsequent lipid and alkane biosynthesis. For the knockout out of the acyl-ACP synthase, plasmid pJet1.2:ΔA was constructed. This plasmid was made by amplifying the *aas* gene, including 1 kb flanks, of PCC 7942 followed by removal of most of the protein coding sequence

and replacing it by a gene disruption cassette. The final construct was transformed into PCC 7942, leading to strain Se:ΔA. PCR amplification verified the correct integration and complete removal of the *aas* gene. This approach allowed us to study the effect of introduction of the thioesterase without added effects from the *aas* knockout and *vice versa*.

To combine the *E.coli 'tesA* expression and *aas* deletion in one strain pSe1:T was transformed into Se:ΔA and its genotype verified (Se:ΔA+T).

Cultivation of S. elongatus PCC 7942 and its mutants

Difference in growth rates and final biomass accumulation between mutants and wild type might influence the composition of primary lipid metabolites and their final products making interpretation of the results and/or conclusions difficult or even not possible. To minimize these differences, we pre-cultivated mutant and wild type strains on agar plates (this allows *S. elongatus* PCC 7942 to accumulate more carbon storage compounds and photosynthetic pigments, unpublished results) and used the bacterial cells cultivated in this way as an inoculum for liquid cultures (see materials and methods for more details). The resulting specific growth rates of mutant strains (0.044, 0.045 and 0.045 h^{-1} for Se:T, Se:ΔA and Se:ΔA+T respectively) were not significantly different from PCC 7942 (0.045 h^{-1}) (Fig 2).The final biomass of mutant strains was lowered to 374±31 µg/ml (12 %) for Se:T, 385±30 µg/ml (10 %) for Se:ΔA and 368±24 µg/ml (14 %) for Se:ΔA+T in comparison to wild type (427±46 µg/ml). However, these reductions were not statistically significant.

Metabolic profile of S. elongatus PCC 7942

The predominant fatty acids present within PCC 7942 are palmitic (C16:0) and palmitoleic acid (C16:1), which represent 48% and 39% of the total cellular fatty acids, respectively (Supplementary table 1). The remainder is mainly comprised of saturated and unsaturated fatty acids with a chain length of 14 and 18 carbons. Only minor amounts of other fatty acids were detected, including odd chain fatty acids (C9:0, C15:0, C15:1, C17:0 and C17:1) and lauric acid (C12:0). The total amount of intracellular fatty acids was 69.12±1.11 µg/mg dry cell weight and was considerably higher compared to the secreted fatty acids (3.36 ±1.11 µg/mg dry cell weight) (Supplementary table 2).

Secreted fatty acids comprise palmitoleic acid (C16:1, 47%) followed by myristic (C14:0, 23%), palmitic (C16:0, 16%), myristoleic (C14:1, 9%) and oleic acid (C18:1, 4%).

The most predominant alkanes within PCC 7942 are heptadecane (C17:0) (synthesized from C18:0 fatty acyl-ACPs) followed by pentadecane (C15:0) and hexadecane (C16:0) (Supplementary table 3). Furthermore, low amounts of tetradecane (C14:0) and octadecane (C18:0) were detected. Only two alkenes were identified, 8-heptadecene (C17:1) and octadecene, of which C17:1 was the predominant one.

Metabolic profile of 'tesA expressing strain

Strain Se:T, containing a soluble thioesterase I from *E. coli*, did not differ from PCC 7942 in the total amount of intracellular fatty acids (68.31±1.17 µg/mg dry cell weight) and secreted fatty acids (3.08±0.54 µg/mg dry cell weight) (Table 3). Nevertheless, there are some significant changes in the intracellular metabolites of this strain. We observed a substantial increase in intracellular myristoleic acid (C14:1) and a decrease in unsaturated C18:1 fatty acids

Table 3 Summary of changes in total fatty acids, intracellular and secreted fatty acids as well as hydrocarbons

	Se:T vs PCC 7942	**Se:ΔA vs PCC 7942**	**Se:ΔA+T vs Se:T**	**Se:ΔA+T vs Se:ΔA**
Total fatty acids	[a]→IFA & SFA	→IFA ↑SFA	↑IFA & SFA	↑IFA & SFA
Intracellular fatty acids	↑C14:1 ↓C18:1	↑C14:0, C18:0	↑C12:0, C14:0, C14:1, C16:0, C16:1 ↓C18:1	↑C12:0, C14:0, C14:1, C16:0, C16:1 ↓C18:1
Secreted fatty acids		↑C16:1, C18:1	→C16:0, C16:1, C18:1	↑C16:0, C16:1
Hydrocarbons	↓C17:0 ↑C17:1	↓C15:0, C17:1	↓C17:1	↑C15:0

[a]→ indicates no significant change, ↑ indicates a significant increase and ↓ indicated a significant decrease compared to the indicated strain. IFA = intracellular fatty acids and SFA = secreted fatty acid.

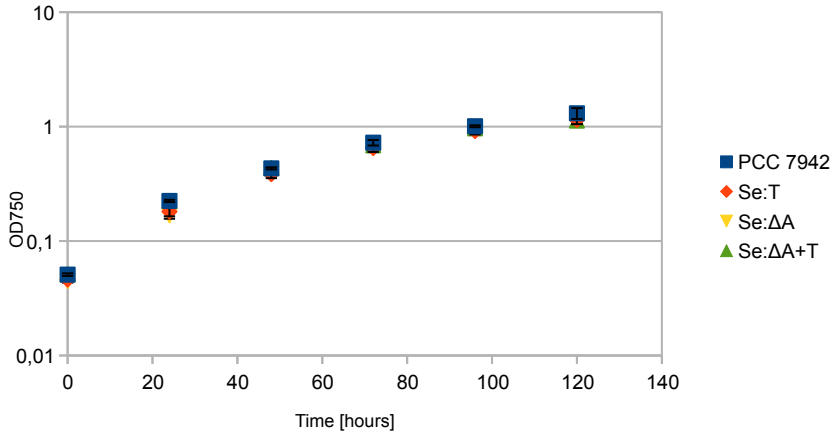

Fig 2 Cellular growth of wild type PCC 7942, strain Se:T, Se:ΔA and Se:ΔA+T. Growth was measured at 24 hour intervals using the optical density at 750 nm. Error bars represent the standard deviation of four replicates.

compared to PCC 7942, of 39% and 23%, respectively (Supplementary table 1 and Fig 3a).

Also, the secreted fatty acids were not significantly different from those of PCC 7942 (Supplementary table 2 and Fig 3b).

However, we did find significant changes in the hydrocarbon profile of this strain. There was a decrease in the amount of heptadecane (C17:0, 20%) and a considerable increase of 8-heptadecene (C17:1, 35%) (Supplementary table 3 and Fig 3c).

Metabolic profile of Δaas

Strain Se:ΔA, which is incapable of reactivating free fatty acids, showed an increase in the amount of intracellular myristic acid (C14:0) by 28% and a substantial increase (more than 60%) of stearic acid (C18:0) compared to PCC 7942. However, strain Se:ΔA did not show a significant increase in the total amount of intracellular fatty acids (69.29±1.92μg/mg dry cell weight) (Table 3).

Nevertheless, we observed a substantial increase (~70% compared to PCC 7942) in the total amount of secreted fatty acids (5.71±1.85 μg/mg dry cell weight). This increase is mostly due to the secretion of palmitoleic acid (C16:1)

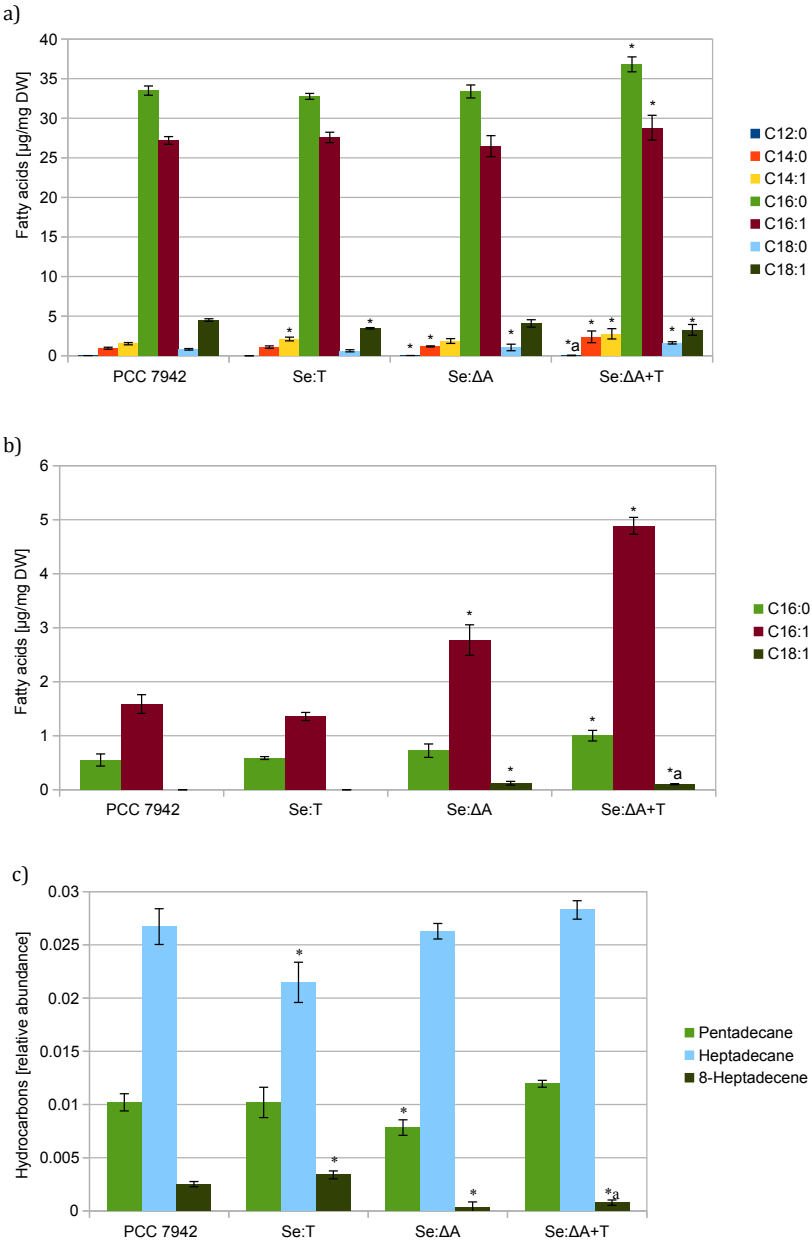

Fig 3 Quantitative measurement of fatty acids and hydrocarbons in PCC 7942, strain Se:T, Se:ΔA and Se:ΔA+T a) intracellular fatty acids, b) secreted fatty acids and c) intracellular hydrocarbons. Only those compounds are shown which are differentially produced among strains. Metabolites indicated with an asterisk * are significantly different ($p < 0.05$) from those of other strains. Compounds indicated with *a are significantly different from those of wild type and Se:T, but not Se:ΔA. Error bars represent the standard deviation of four replicates.

which represents 49% of the total secreted fatty acids (Table 3 and Fig 3b). Although other free fatty acids contribute to the total increase of secreted fatty acids the only other significant increase was observed for C18:1.

Intracellular hydrocarbons showed a reduced amount of pentadecane (C15:0) and a remarkable decrease of 8-heptadecene (C17:1) (89%).

Metabolic profile of 'tesA:Δaas

Analysis of PCC 7942 *('tesA*:Δ*aas)* revealed a similar pattern as those observed in the *'tesA* and Δ*aas* strains, such as an intracellular increase in C14:0 (Se:ΔA), C18:0 (Se:ΔA), C14:1 (Se:T) and a decrease in C18:1 (Se:T) (Table 3 and Fig 3a). However, the amounts of these fatty acids were significantly higher/lower than in both wild type PCC 7942 and the single mutants. Furthermore, there was a significant increase in C12:0, C16:0 and C16:1, by at least 1300%, 12% and 10% compared to PCC 7942 respectively. Moreover, this strain was the only mutant which showed a significant increase of total intracellular fatty acid from 69.12±1.11 μg/mg dry cell weight in PCC 7942 to 79.04±2.11 μg/mg dry cell weight in Se:ΔA+T (14%) (Supplementary Table 1).

The amount of secreted fatty acids also increased significantly from 3.36 ±1.11 μg/mg dry cell weight to 8.02±0.32μg/mg dry cell weight in this strain (139% increase compared to PCC 7942). The composition of secreted fatty acids was similar to that observed in strain Se:ΔA, with palmitoleic acid (C16:1) being the most abundant (60% of total secreted fatty acids). In addition, Se:ΔA+T showed a significant increase in the amount of palmitic acid (C16:0) which increased by almost 82% compared to PCC 7942 (Fig 3b).

The production of 8-heptadecene was similarly reduced in strain Se:ΔA+T as it was shown for the *Δaas* mutant. However, the double mutant did not show a decrease in pentadecane observed in strain Se:ΔA or a decrease in heptadecane detected for strain Se:T. Both these hydrocarbons were present at wild type levels (Fig 3c).

Discussion

Genetic engineering by introduction of a thioesterase and knockout of *aas* has been used in several studies aimed at generating strains with enhanced biodiesel production (Hu et al. 2013; Liu et al. 2011; Ruffing and Jones 2012). However, a general overview of the changes in the fatty acid and hydrocarbon pool by this engineering and their significance for biotechnological application

is still missing. In this study we have described experiments, which allow us to provide a comprehensive picture of early effects on the fatty acid and hydrocarbon content within and in the cultivation medium of PCC 7942 caused by the introduced mutations.

Unlike a previous study, in which a more than 20% growth reduction in a similarly engineered double mutant was reported (Ruffing and Jones 2012), we showed that using pre-growth culture conditions, the mutants were not affected in their growth rate and total biomass accumulation (Fig 2). This allowed us to better interpret the individual strain performances.

In general, the secreted fatty acids profile of PCC 7942 and its mutants is similar to earlier reports with respect to the presence of unsaturated C16 as the major fatty acid outside the cell (Kaczmarzyk and Fulda 2010). Also similar to earlier reports the intracellular fatty acids profile of wild type and engineered strains is characterized by almost an equal ratio of saturated and unsaturated C16, which builds up nearly the entire intracellular fatty acid pool. With respect to hydrocarbons, we found mostly C17:0 followed by C15:0 alkanes and this finding is in agreement with previous work (Schirmer et al. 2010). However, our detailed analysis also clearly indicated interesting differences between the wild type and mutant strains discussed below.

The effect of expressing 'tesA in S. elongatus PCC 7942

Strain Se:T, expressing a cytosolic *E. coli* thioesterase, does not have a significant change in total fatty acid production compared to the wild type. Our result is similar to those obtained for *Synechocystis* sp. PCC 6803, where the introduction of a thioesterase (*fatB*) from *Arabidopsis* also failed to lead to a significant increase in the total secreted and intracellular free fatty acids (Hu et al. 2013). Hu et al. suggested that the endogenous AAS enzyme is able to outcompete the introduced thioesterase and, as a result, masks the liberation of fatty acids. However, since we do see significant changes in intracellular amount of C14:1 and C18:1 fatty acids in the *'tesA* expressing strain, this suggests that 'TesA is outcompeted only for some fatty acids. This is further supported by the observation, that in the *'tesA:Δaas* double mutant we see an almost 50% increase in the amount of C14:1 compared to the *Δaas* mutant. Moreover, some of the fatty acids released by 'TesA can be converted to hydrocarbons. For example, we observe a decrease in C18:1 intracellular fatty acids which is most likely linked to an increase in C17:1 hydrocarbons.

Since (i) acyl-ACPs are synthesized saturated and (ii) desaturation of fatty acids occurs exclusively in lipids (Higashi and Murata 1993), we would expect that the introduction of *'tesA* would only influence the production of saturated acyl-ACPs. However, we also observed changes in the amount of unsaturated fatty acids. A possible explanation could be that unsaturated fatty acids are released by lipases in the process of membrane recycling. However, since there is (i) no increase in C14:1 in *Δaas* mutant compared to wild type (indicating that this fatty acid is not released by lipases under tested conditions) and (ii) an almost 50% increase of C14:1 in the double mutant compared to *Δaas*, another explanation could be that the release of unsaturated fatty acids is mediated by *lysophospholipase* activity of thioesterase I (Doi and Nojima 1975). Of course, the changes in the amount of unsaturated fatty acids could also be a combination of these two processes.

The effect of deleting the aas gene in S. elongatus PCC 7942

Similar to previous reports (Kaczmarzyk and Fulda 2010; Ruffing and Jones 2012), we found a significant increase in the amount of total secreted fatty acids in the *Δaas* strain. This is mainly due to an increase in unsaturated C16:1 and C18:1 free fatty acids which are favorable for biodiesel production (Quintana et al. 2011).

Although we did not see a significant change in the total intracellular fatty acids, there was an increase in individual saturated fatty acids (C14:0 and C18:0). These are most likely either synthesized *de novo* to compensate the loss of Aas activity, or released during membrane recycling.

The inability of *Δaas* to reactivate free fatty acids caused a dramatic decrease in 8-heptadecene (C17:1), one of the major constituent of alkenes in cyanobacteria (Wang et al. 2013). This indicates that Aas activity is important for alkene biosynthesis in PCC 7942. In contrast to alkenes, alkane biosynthesis was practically not affected. Interestingly, in another cyanobacterium, *Synechocystis* sp. PCC 6803, knockout of *aas* caused a decrease in both alkane and alkene biosynthesis, indicating that in this strain hydrocarbon production mostly relies on the extracellular free fatty acid pool (Gao et al. 2012). Taken together, our results suggest that unlike PCC 6803, PCC 7942 has sufficient *de novo* synthesis of acyl-ACPs to produce both fatty acids and alkanes.

The effect of expressing 'tesA in Se:ΔA

Strain Se:ΔA+T, shows the largest increase in total fatty acid production. The secreted fatty acids increased by more than 40% compared to strain Se:ΔA. It is interesting to note, that nearly 90% of this increase was due to unsaturated fatty acids, while the intracellular fatty acids pool consisted of almost equal amounts of saturated and unsaturated fatty acids. These results suggest that saturated fatty acids are either not secreted as efficiently as unsaturated ones, or more readily taken up by the cell (Kaczmarzyk 2008).

Unlike the single mutants, which did not show a change in intracellular fatty acids, the double mutant had a significant increase of intracellular fatty acids which totals 55% of the entire rise in fatty acids. This indicates that neither of the single alterations was able to change the intracellular acyl-ACP pool enough to cause changes in the total amount of intracellular fatty acids. TesA was not able to release enough fatty acids to compete with the Aas and the loss of Aas activity, although leading to an increase in secreted fatty acids, was fully complemented by *de novo* synthesis.

While the alkene biosynthesis of Se:ΔA+T was reduced similar to Se:ΔA, the alkane profile (C15:0 and C17:0) was fully restored to wild type levels. This is most likely caused by an increase in the acyl-ACP pool due to a reduction in feedback inhibition by long fatty acyl-ACP molecules. The latter was achieved by both introducing 'TesA, which liberates these fatty acids, and making an *aas* knockout (this prevents reactivation of free fatty acids).

Concluding remarks and future prospects

Our results indicate that the fate of the synthesized acyl-ACP molecule is determined by a complex interplay between the acyltransferase, lipases, fatty acyl reductase, Aas and 'TesA.

The Se:ΔA+T mutant proved to be the best strain for the enhanced fatty acid and alkane production, while *'tesA* showed an increase in alkene biosynthesis. Although total strain productivity is still low with respect to large-scale production, the presence of both saturated and monounsaturated fatty acids in the metabolic profile of engineered strains is an important prerequisite for making high quality biodiesels.

Further optimization of fatty acid production can be achieved by overexpressing the acyl-Coa carboxylase, which should direct more carbon to fatty acids and hydrocarbons. Moreover, since the release of free fatty acids by 'TesA might have been partially masked by hydrocarbon synthesis, it would be

interesting to investigate the effect of 'TesA in combination with a deletion of the native fatty acyl–reductase. Furthermore, reducing the polarity of the cell membrane, or introducing fatty acid exporters might enhance the secretion of fatty acid.

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Chapter 4

Effects of the introduction of the Acetyl-CoA Carboxylase from *Synechococcus elongatus* PCC 7942 in *E. coli* DH5α and *S. elongatus* PCC 7942 Se:ΔA+T on the fatty acid and hydrocarbon profile

Gerben P. Voshol, Vera Meyer, Cees A.M.J.J. van den Hondel

Abstract

In this study we introduced the acetyl-CoA carboxylase (ACCase) from *S. elongatus* PCC 7942 and expressed it in both *E. coli* DH5α and *S. elongatus* PCC 7942 strain Se:ΔA+T. These strains were subsequently analysed for changes in their intracellular fatty acids (*E. coli* and *S. elongatus*), hydrocarbons (*S. elongatus*) and extracellular fatty acids (*S. elongatus*). Introduction of the ACCase alone did not cause an increase in intracellular fatty acids in both strains (*E. coli* and *S. elongatus*). However, in *S. elongatus* PCC 7942 engineered for fatty acid secretion (Se:ΔA+T) there was a significant increase in the amount of extracellular fatty acids in both absolute and per dry cell weight basis. Induction with Isopropyl β-D-1-thiogalactopyranoside resulted in an increase in the amount of extracellular fatty acids on a per cell basis, but not in absolute amounts. This is the first report that shows that the introduction of the ACCase from PCC 7942 allows for additional enhancement of biodiesel precursor production. Further optimization of the ACCase containing construct (stability, transcription) might help to enhance the extracellular fatty acid production.

Introduction

Concerns about peak oil, geopolitical instabilities and environmental impact drive the search for new renewable energy sources. This search has led to the development of first and second-generation biofuels, which are derived from crops and/or agricultural waste. However, these biofuels have several disadvantages, such as competition for arable land and fresh water, which make them undesirable alternative energy sources. Currently third generation biofuels are being developed using eukaryotic and prokaryotic microalgae.

Due to the relatively high growth rate, amenability for genetic transformation and simple nutrient requirements, cyanobacteria have received a lot of focus from the scientific community for the production of biofuels. The most promising biofuel precursor synthesized by cyanobacteria, are fatty acids (FA). The synthesis of FA takes place in the cellular membranes and starts with the carboxylation of acetyl-CoA to malonyl-CoA. This reaction is catalyzed by the acetyl-CoA carboxylase (ACCase) enzyme and is proposed to be the first rate-limiting step in FA synthesis (Davis and Cronan 2001). Malonyl-CoA is not only an important intermediate for fatty acid synthesis (Lu et al. 2008), but also for polyketide (Miyahisa et al. 2006) and flavonoid (Leonard et al. 2007) production. Moreover, the concentration of acetyl-Coa and malonyl-CoA is suggested to be the key step coordinating the rate of fatty acid biosynthesis and competing pathways such as cell growth and macromolecule synthesis (Davis et al. 2000).

Since the ACCase plays a central role in the production of fatty acids, it is a commonly investigated step in optimizing biodiesel precursor production. In general, there are two approaches for increasing the ACCase activity inside the cell. This is done either by overexpression of the native ACCase or by identifying an ACCase from another organisms and optimizing its expression. While the later approach resulted in a 3-fold increase in intracellular lipids in Escherichia coli (Meng et al. 2011), it did not lead to any significant changes in FA secretion of *Synechococcus elongatus* PCC 7942 (Ruffing 2013b).

In this study we expressed the ACCase from PCC 7942 in *E. coli* DH5α to study the effect of this enzyme on the growth and intracellular fatty acids of DH5α. We also expressed the ACCase in a mutant PCC 7942 (Se:ΔA+T) which contains a knockout of the *aas* gene and expresses a soluble thioesterase from E. coli (Voshol et al., 2014). This allowed us to elucidate the effect of expression of the ACCase not only on the intracellular fatty acids and hydrocarbons, but also on the extracellular fatty acids in PCC 7942. Furthermore, we investigated

the effect of induction of the ACCase with Isopropyl β-D-1-thiogalactopyranoside (IPTG) in both *E. coli* DH5α and Se:ΔA+T.

Materials and Methods

Bacterial strains and culture conditions

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was routinely cultured using LB throughout this study. Unless stated otherwise, *E. coli* cultures were inoculated to a starting density of 0.03 (OD600) and incubated on an orbital shaker (250 rpm) at 37 °C. Bacterial growth was monitored by measuring the optical density at 600nm for *E.coli*. When plasmid bearing *E. coli* strains were cultured, the appropriate antibiotics were added to a final concentration of 100 μg/ml Carbenicillin (Cb), 50 μg/ml Kanamycin (Km) and/or 10 μg/ml Chloramphenicol (Cm). If needed, cultures were induced by the addition of IPTG to a final concentration of 1 mM when they reached an OD600 of approximately 1.0 and growth was continued for another 3 to 16 hours.

S. elongatus PCC 7942, the donor organism for the ACCase, was maintained in BG11 medium shaking at 250 rpm with continuous illumination (60 μE/m2/s) at 30 °C. For the extraction of hydrocarbons, fatty acids and spectral measurements, cyanobacteria were pre-grown for 7 days on BG-11 agar (1% w/v) as previously reported (Voshol et al. 2014). Subsequently, inoculated in 2 times 50 ml of BG-11 medium (treated as a single 100 ml culture) at a starting OD750 of 0.05. The liquid bacterial cultures were then cultivated under non-CO2 enriched conditions. When needed, cultures were induced with 1 mM IPTG when the OD750 was approximately 0.2 and growth was continued for another 4 days.

Plasmid construction

All plasmids were constructed using the three-fragment ligation kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers protocol with slight modifications. Briefly, to allow expression of the multimeric ACCase protein from PCC 7942, chromosomal DNA was extracted according to Tillett and Neilan (2000). Followed by PCR amplification of all four subunits using primers listed in Table 2 to amplify (1) the biotin carboxylase (Synpcc7942_1379), (2) the carboxyltransferase subunit alpha (Synpcc7942_1595), (3) the beta

Table 1 Strains and plasmids used in this study

	Description	Reference
Strain		
E. coli DH5α	Used for molecular cloning and for the amplification of a truncated thioesterase ('*tesA*)	(Hanahan 1983)
S. elongatus PCC 7942	A freshwater cyanobacterium	Pasteur Institute (Paris, France)
GV	*E. coli* DH5α control strain containing an empty pMMB67HE vector	This study
GV2.6	*E. coli* DH5α containing vector pGV2.6 to express all 4 subunits of the ACCase from *S. elongatus* PCC 7942	This study
Se:ΔA+T	*S. elongatus* PCC 7942 expressing a truncated thioesterase from *E. coli* DH5α (*'tesA*) and containing a disrupted acyl-ACP synthetase (Synpcc7942_0918)	(Voshol et al. 2014)
Se:ACCase	Strain Se:ΔA+T containing pSe:ACCase integrated into the neutral site 3, to allow expression of all 4 subunits of the ACCase enzyme	This study
Plasmid		
pMMB67HE	Contains a Carbenicillin resistance (Cb) marker, an isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated trp-lac promoter followed by a ribosome binding site (RBS) and a polyclonal site	(Fürste et al. 1986)
pHN1-LacUV5	Targets Neutral site 3, confers resistance to Chloramphenicol (Cm) and contains a strong isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated lacUV5 promoter followed by a ribosome binding site (RBS) and an unique NotI restriction site	(Niederholtmeyer et al. 2010)
pNS3	Derived from pHN1-LacUV5 with the original RBS removed and lacking the ATG start codon near the NotI restriction site	This study
pGV2.6	Vector pMMB67HE containing the acetyl-CoA carboxylase biotin carboxylase subunit (EC:6.3.4.14), acetyl-CoA carboxylase carboxyltransferase subunit alpha (EC:6.4.1.2), acetyl-CoA carboxylase subunit beta (EC:6.4.1.2) and biotin carboxyl carrier protein of PCC 7942 cloned behind the IPTG inducible promoter.	
pSe:ACCase	Derived from pNS3 with the HindIII fragment containing the ACCase subunits from pGV2.6 cloned into the unique HindIII restriction site behind the inducible lacUV5 promoter	This study

carboxylase (Synpcc7942_1956) and (4) the biotin carboxyl carrier protein (Synpcc7942_2564) subunit. The PCR products of subunits one and two were subjected to BP recombination into pDONR™ P4-P1R and pDONR™ 221, respectively. Subunits three and four were first amplified using a phusion PCR followed with performing the BP recombination using the final purified PCR product into pDONR™ P2R-P3. All three entry clones, containing the amplified genes and preceded by a ribosome-binding site, were subjected to the LR recombination reaction into the gateway ready pMMB67HE destination vector. Since the gateway ready pMMB67HE destination vector contained an inactive ccdB gene, an additional digestion with NcoI was performed and this mixture was subsequently transformed and screened for the presence of the construct by replica plating on LB containing Cb and LB containing Cb and Cm. Subsequently, mini-prep and digestion was performed on the clones which failed to grow on LB plates containing both antibiotics to verify the orientation and presence of the construct. Three clones were selected for sequencing using primers listed in Table 2. One of three clones obtained, pGV2.6, was selected for subsequent experiments.

For expression of the ACCase in *S. elongatus* PCC 7942, construct pGV2.6 was digested with HindIII (liberating the ACCase) and subsequently inserted in vector pNS3.

Total fatty acid analysis using GC-MS

Total fatty acids were determined as previously described (Voshol et al. 2014). Briefly, a 50 ml aliquote of *E. coli* cell suspension was removed for the Erlenmeyer flask, chilled on ice and centrifuged at 4 °C for 10 minutes. *S. elongatus* cells were harvested by taking a 100 ml and centrifuged for 20 minutes at 4500 rcf. The supernatant was discarded and the cell pellet was lyophilized. Twenty mg of freeze-dried biomass was weighed and 250 µg internal standard (IS; C10:0) was added and allowed to become part of the cellular matrix by drying it under a stream of nitrogen. Subsequently, 1 ml hexane and 2 ml sodium methoxide (0.5 M) in methanol was added. After briefly vortexing, samples were sonicated for 5 minutes and transesterification was performed by incubating at 50 °C. After 10 minutes the reaction was stopped by the addition of 3 ml concentrated HCl in methanol (5% v/v). The samples were then vortexed for 30 seconds, sonicated for an additional 5 minutes and incubated at 70 °C for 20 min. After the samples reached room

Table 2 Primers used in this study

Primer	Sequence[a]	Remarks
REMatgfw	tctagaaagcttgcggcc	Removal of Ribosome binding site from pHN1-LacUV5
REMatgrv	gtgagcggataacaatttcacac	Removal of Ribosome binding site from pHN1-LacUV5
AttR4 fw	cccaagcttccatgattacgccaagc tatc	Amplifies the AttR4, Ccdb, CmR, AttR3 fragment of pDestR4-R3
AttR3 rev	cccaagcttacgacggccagtgaatt atc	Amplifies the AttR4, Ccdb, CmR, AttR3 fragment of pDestR4-R3
Synpcc7942_137 9 attB4 Fwd	ggggacaactttgtatagaaaagttg **gaaggagatatacat**atgcgtttca acaagatcct	Amplifies the acetyl-CoA carboxylase biotin carboxylase subunit (EC:6.3.4.14) of *S. elongatus* PCC 7942
Synpcc7942_137 9 attB1r Rev	ggggactgctttttttgtacaaacttgc taggacttgaggatccgag	Amplifies the acetyl-CoA carboxylase biotin carboxylase subunit (EC:6.3.4.14) of *S. elongatus* PCC 7942
Synpcc7942_159 5 attB1 Fwd	ggggacaagtttgtacaaaaaagca ggct**gaaggagatatacat**atggct gcacctgtcacgaag	Amplifies the acetyl-CoA carboxylase carboxyltransferase subunit alpha (EC:6.4.1.2) of *S. elongatus* PCC 7942
Synpcc7942_159 5 attB2 Rev	ggggaccactttgtacaagaaagctg ggtttagtcactgctttccagaaac	Amplifies the acetyl-CoA carboxylase carboxyltransferase subunit alpha (EC:6.4.1.2) of *S. elongatus* PCC 7942
Synpcc7942_195 6 attBr2 Fwd	ggggacagctttcttgtacaaagtgg **gaaggagatatacat**atgtcgctct tggactggtttg	Amplifies the acetyl-CoA carboxylase subunit beta (EC:6.4.1.2) of *S. elongatus* PCC 7942
Synpcc7942_195 6 Reverse	gttgcacgtgtatatctccttcctaga ggggtgcggtggcag	Amplifies the acetyl-CoA carboxylase subunit beta (EC:6.4.1.2) of *S. elongatus* PCC 7942
Synpcc7942_256 4 Forward	**gaaggagatatacac**gtgcaactg aacttcagccaac	Amplifies the biotin carboxyl carrier protein of *S. elongatus* PCC 7942
Synpcc7942_256 4 attB3 Rev	ggggacaactttgtataataaagttgt cagagaggccgcaaccggaac	Amplifies the biotin carboxyl carrier protein of *S. elongatus* PCC 7942
seq1	tcatcggctcgtataatgtgtg	Used for sequencing the ACCase subunits
seq2	ggagaatgcccgctttgcag	Used for sequencing the ACCase subunits
seq3	tgtagtgcatctaggcgagc	Used for sequencing the ACCase subunits
seq4	gccgcattacaggctatttac	Used for sequencing the ACCase subunits
seq5	agctggaggaacggatcacg	Used for sequencing the ACCase subunits
seq6	ggcactgcgtttgatggagc	Used for sequencing the ACCase subunits
seq7	cgcatcaattctctggcgtg	Used for sequencing the ACCase subunits
seq8	tggtctctggacgaagtgtg	Used for sequencing the ACCase subunits
seq9	cagcatgggttctgtcgtcg	Used for sequencing the ACCase subunits
seq10	tgaagaagaccttggcgcaac	Used for sequencing the ACCase subunits
seq11	caccttctatcgcgctccag	Used for sequencing the ACCase subunits

[a]Restriction sites are underlined and the ribosomal binding site is indicated in bold.

temperature, they were extracted twice with 4 ml of hexane containing butylated hydroxytoluene as an anti oxidant. After concentrating the samples by vacuum evaporation, methylated fatty acids were dissolved in 1 ml of hexane and transferred to GC-MS vials closed with Teflon coated caps.

These samples were subsequently analysed using an Agilent model 7890A gas chromatograph as previously described (Voshol et al. 2014). Briefly, samples were separated using a DB-WAX column (10 m, 0.25 mm, 0.25 µm) and detected using an inert XL mass spectrometer (model 5975C) using helium as carrier gas. The retention times and the mass spectrum of authentic samples were used for identification. Hydrocarbons were quantified relative to the internal standard while absolute quantities of fatty acids were determined based on calibration curves constructed using authentic standards.

Results

Genetic engineering of E. coli DH5α and S. elongatus PCC 7942 strain Se:ΔA+T

The multimeric Acetyl-CoA carboxylase from *S. elongatus* PCC 7942 is proposed to be the rate-limiting step determining the carbon flow towards fatty acid synthesis and other macromolecules. Overexpression of this enzyme was obtained by amplifying the individual subunits of this enzyme (Synpcc7942_1379 (BC), Synpcc7942_1595(CT-α), Synpcc7942_1956(CT-β) and Synpcc7942_2564 (BCCP)) with primers containing a new ribosomal binding site (Table 2). This artificial operon (AccCABD) was inserted behind the IPTG inducible promoter of plasmid pMMB67HE for expression in *E. coli* DH5α (strain GV2.6) and behind the lacUV5 promoter of integrative vector pNS3 for expression in *S. elongatus* PCC 7942 strain Se:ΔA+T (strain Se:ACCase) (Fig 1).

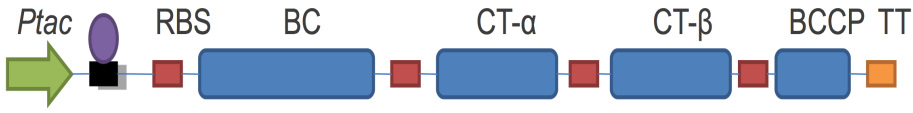

Fig 1. Schematic overview of the Acetyl-CoA carboxylase construct of pGV2.6 and pSe:ACCase. The green arrow, black box and purple oval indicates the IPTG inducible promoter, the red boxes indicate the ribosomal binding sites, the blue boxes indicate the individual subunits and the orange box indicated the rho independent transcriptional terminator.

Growth of E. coli GV2.6 and S. elongatus Se:ACCase

Specific growth rate of ACCase expressing strains is reduced in both *E. coli* GV2.6 and *S. elongatus* strain Se:ACCase. In *E. coli*, the specific growth rate of GV2.6 was reduced to 0.705±0.005 h^{-1} uninduced and 0.717±0.027 h^{-1} induced compared to 1.130±0.018 h^{-1} and 1.126±0.033 h^{-1} for strain GV, uninduced and induced respectively (Fig 2a). In PCC 7942, the specific growth rates of strain Se:ACCase were 0.034±0.001 (uninduced) and 0.034±0.003 (induced) h^{-1} and were significantly lower than the specific growth rates of strain Se:ΔA+T (0.045±0.001 uninduced and 0.045±0.002 h^{-1} induced) (Fig 2b). Moreover, strain Se:ACCase and GV2.6 both showed an extended lag phase not observed in the control strains and in the other engineered PCC 7942 strains.

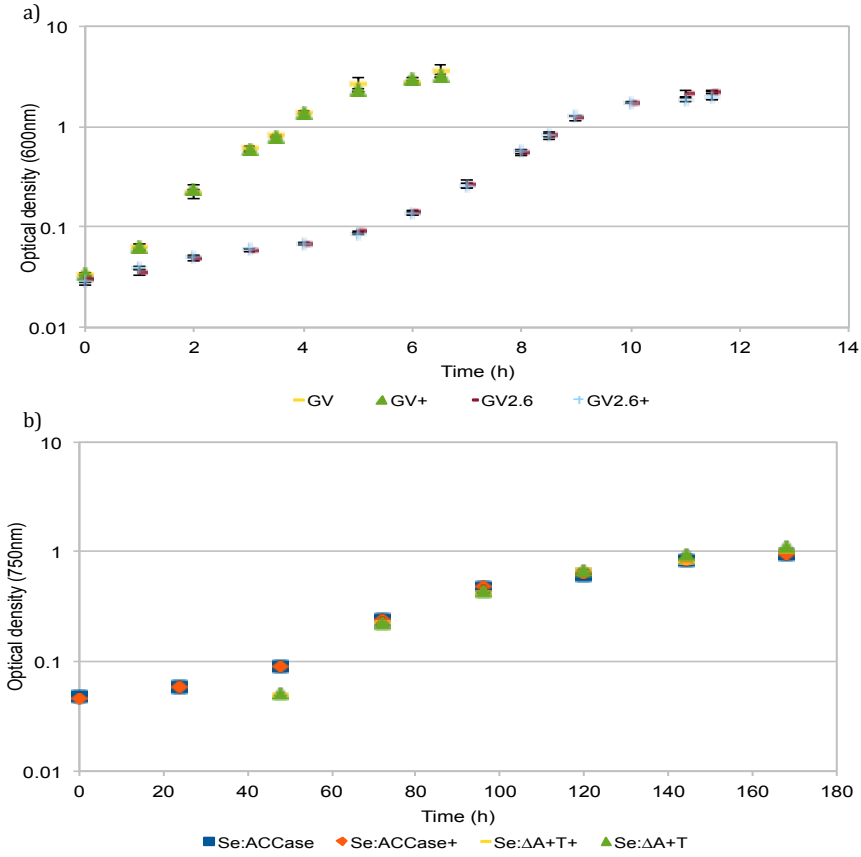

Fig 2 Cellular growth measured using the optical density of a) *E. coli* strain GV and GV2.6 (600nm) and b) *S. elongatus* PCC 7942 strain Se:ΔA+T and Se:ACCase (750nm) either induced with IPTG (+) or uninduced. Error bars represent the standard deviation of three (a) or four replicates (b).

GC-MS *E. coli GV2.6 uninduced*

Strain GV2.6, containing the plasmid for expression of the ACCase subunits of *S. elongatus* PCC 7942, does not contain a significantly different amount of total fatty acids at 3 and 16 hours. Three hours after this strain reached exponential phase of growth, the relative abundance of C18:1 was decreased in this strain compared to strain GV (from 11.8±0.4% to 9.9±0.5%) and the amount of C14:0 and C16:0 was increased from 5.8±0.0% to 6.6±0.2% and from 48.3±0.3% to 49.7±0.5%, respectively (Fig 3a). After 16 hours, the amount of C18:1 was still lower than that of strain GV (3.6±0.1% in strain GV2.6 compared to 5.8±0.3% in strain GV) and the relative amounts of C14:0 and C16:0 were still higher (Fig 3b). The C14:0 content of GV2.6 was 6.8±0.2% compared to 5.6±0.1% in GV and the amount of C16:0 increased from 47.1±0.4% in GV to 48.0±0.1% in strain GV2.6. Moreover, the amount of C16:1 was slightly reduced compared to the negative control (from 3.9±0.1% to 3.4±0.0%) and C17:0 was increased from 0.8±0.1% to 1.1±0.1% of total fatty acids.

GC-MS *E. coli GV2.6 induced*

Induction of the ACCase containing strain GV2.6 does not cause an increase in the total amount of intracellular fatty acids neither at 3 hours nor at 16 hours. Three hours post induction, the relative amount of C16:1 increased from 11.7±0.8% in strain GV to 13.5±0.2% in strain GV2.6 and the amount of C14:0 increased from 5.8±0.2% in strain GV to 6.7±0.1% in strain GV2.6 3 hours after induction with IPTG (Fig 3a). The relative quantities of intracellular fatty acids C16:2 and C18:2 observed after 16 hours of induction were reduced compared to the control strain GV from 22.8±0.3% to 21.5±0.2% and from 6.9±0.2% to 4.3±0.1%, respectively (Fig 3b). The amount of C15:0, C16:1, C17:0 and C18:0 were all slightly, but significantly increased in the induced strain after 16 hours. The relative amount of C15:0, C16:1, C17:0 and C18:0 increased from 1.4±0.2%, 3.9±0.2%, 0.8±0.1% and 0.9±0.0% in strain GV to 2.8±0.1%, 5.1±0.2%, 1.5±0.1% and 1.1±0.0% in strain GV2.6.

GC-MS *Se:ACCase uninduced vs Se:ΔA+T*

Strain Se:ACCase, does not shown a significant change in the total amount of intracellular fatty acids. However, there are some significant reductions in the amount of individual fatty acids compared to the strain Se:ΔA+T. The amount of intracellular C9:0, C12:0, C14:0, C14:1, C17:0, C17:1, C18:0 and C18:1 is

reduced in the uninduced Se:ACCase strain (Fig 4a and Supplementary Table

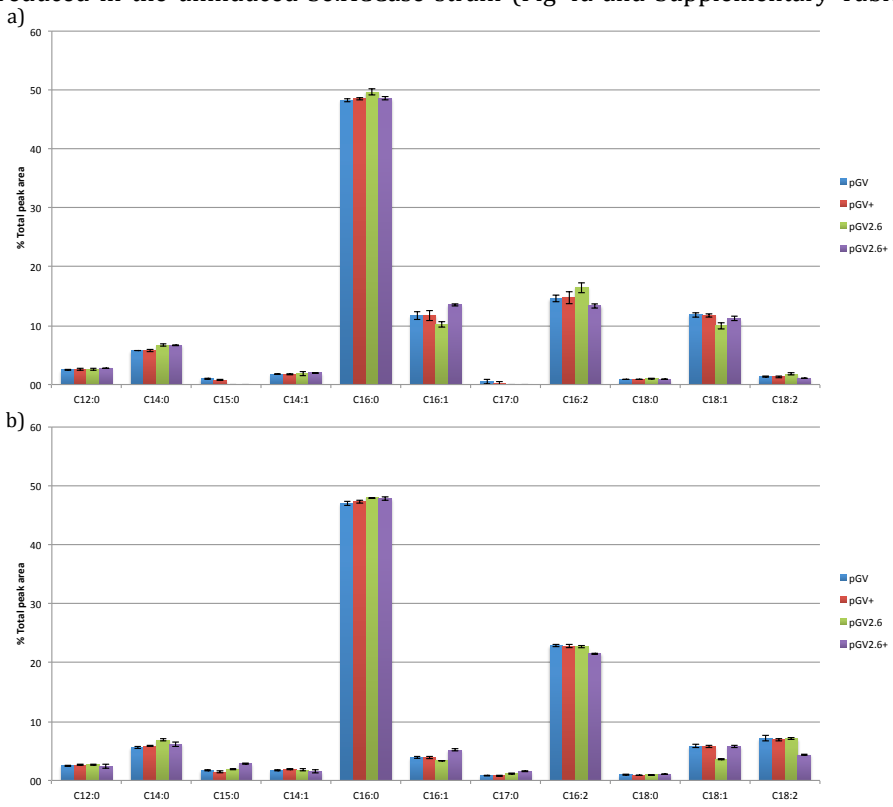

Fig 3 Relative amount (% total peak area) of intracellular fatty acids of strains GV and GV2.6 uninduced and a) 3 hours or b) 16 hours after induction with IPTG (indicated with +). Error bars represent the standard deviation of three replicates.

2). There was an increase in the relative abundance of C16:0 fatty acid from 48% of total fatty acids to 50% in Se:ACCase.

The total amount of extracellular fatty acids is increased in the uninduced strain from 8.0±0.3µg/mg DCW for strain Se:ΔA+T to 10.1±1.0µg/mg DCW in the ACCase expressing strain (~27% increase)(Fig 4b and Supplementary Table 1). This increase is due to a significant rise in C14:1, C16:0 and C18:0. Together these fatty acids account for 53% of the total increase, C14:1 accounts for 25%, C16:0 for 24% and the remainder (4%) is accounted for by C18:0.

The intracellular hydrocarbons of Se:ACCase showed a slight, but significant reduction in the amount of n-Heptadecane (Fig 4c and Supplementary Table 3). Moreover, n-Octadecane which was detected in trace amounts in strain Se:ΔA+T was not detected for strain Se:ACCase.

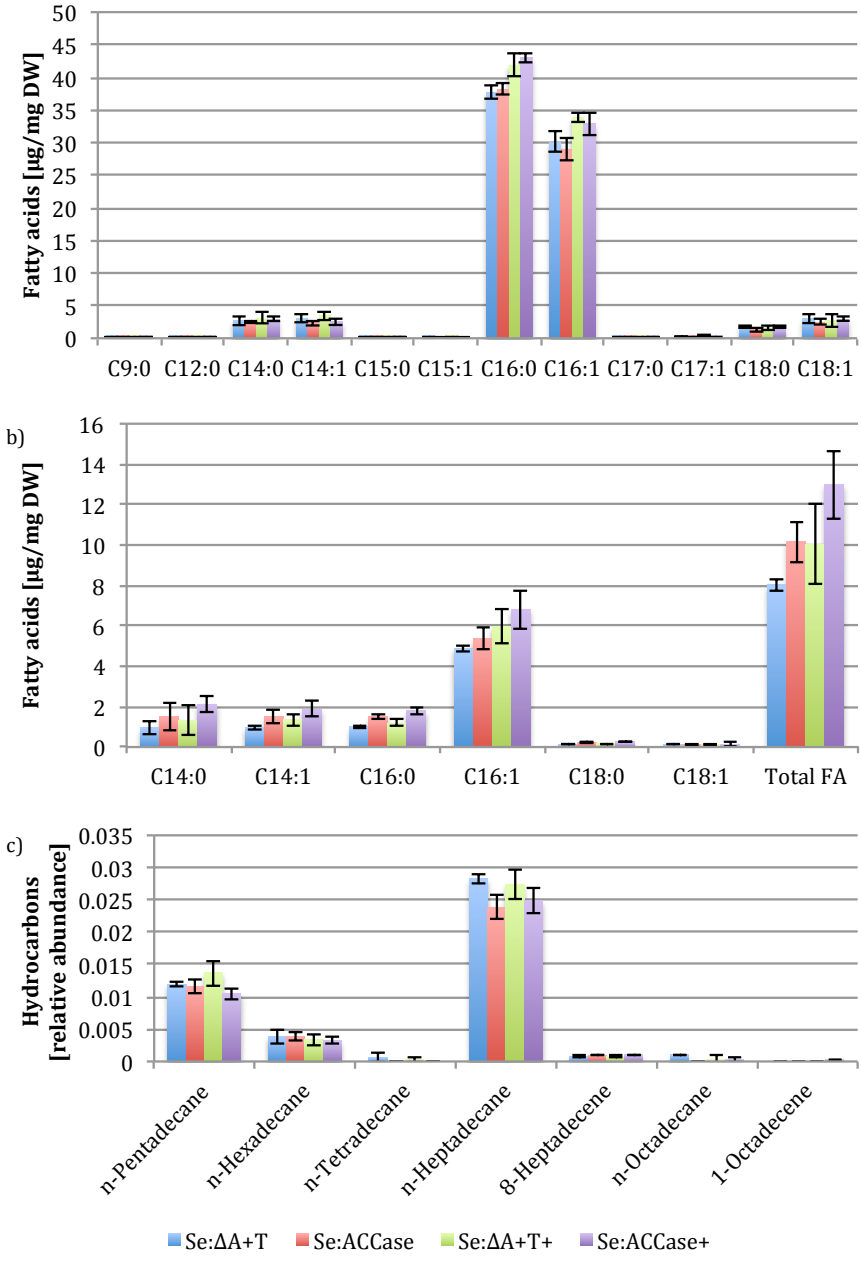

Fig 4 Quantitative measurement of fatty acids and hydrocarbons in *S. elongatus* PCC 7942, strain Se:ΔA+T and Se:ACCase induced (+) or uninduced a) intracellular fatty acids, b) secreted fatty acids and c) intracellular hydrocarbons Error bars represent the standard deviation of four replicates.

GC-MS *Se:ACCase induced vs Se:ΔA+T*

Comparing the induced ACCase expressing strain (Se:ACCase) with the induced Se:ΔA+T strain shows similar results as those described above. The total amount of fatty acids, although significantly higher than uninduced, is not changed between these two strains. The amount of intracellular C12:0, C14:1, C15:1 and C17:1 is reduced in the IPTG induced ACCase expressing strain (Se:ACCase) compared to strain Se:ΔA+T (Fig 4a and Supplementary Table 2). Similar to the results obtained for the uninduced strains, the relative abundance of C16:0 increased significantly from 48% to 50% of total fatty acids in Se:ACCase.

The total amount of extracellular fatty acids increased in the ACCase expressing strain from 10.0±2.0 μg/mg DCW to 13.0±1.7 μg/mg DCW (Fig 4b and Supplementary Table 1). This ~30% increase in total extracellular fatty acids is due to an increase in C14:1 (18% of increase), C16:0 (21% of increase) and C18:0 (3% of increase).

Except for a reduction in the amount of n-Pentadecane there are no significant differences in the amount of intracellular alkanes and alkenes (Fig 4c and Supplementary Table 3).

Discussion

Growth of E. coli GV2.6 and S. elongatus Se:ACCase

Both *E. coli* strain GV2.6 and *S. elongatus* strain Se:ACCase containing the ACCase construct show an extended lag phase and a reduction in their growth rate. This observed reduction in growth is absent in an *E. coli* strain carrying a similar ACCase construct, which has a truncated biotin carboxylase subunit of the ACCase. This suggests that either the first subunit (biotin carboxylase) or the presence of the complete construct (ACCase) is responsible for the observed differences in growth of strain GV2.6. In the latter case, the observed differences in growth might be due to the effect of the introduced ACCase on the intracellular concentration of acetyl-CoA and manolyl-CoA. These concentrations are proposed to determine the allocation of carbon to cellular growth or fatty acid synthesis. However, supplementation of strain GV2.6 with acetate, which can readily be converted to acetyl-CoA by acetate kinase and phosphate acetyltransferase (Underwood et al. 2002), failed to enhance the growth of this mutant (data not shown). It should be noted, that in contrast to

E. coli, no differences were observed in strain PCC 7942 expressing the complete ACCase construct versus parental PCC 7942. This suggests that the growth reduction observed in strain Se:ACCase is not a direct effect of the ACCase, but a combined result of the introduction of three different mutations (ACCase, Δaas and 'tesA).

Fatty acids of E. coli GV2.6 and S. elongatus Se:ACCase

Introduction of the ACCase in E. coli or PCC 7942 strain Se:ΔA+T did not lead to an increase in the total amount of intracellular fatty acids. Moreover, the introduction of the ACCase construct in PCC 7942 did not have a significant effect on intracellular fatty acids (data not shown). This observation is in agreement with previous reports, which showed that the introduction of an ACCase without other genetic modifications does not lead to an increase in fatty acid synthesis (Davis et al. 2000; Lennen et al. 2010). This finding might be explained by feedback inhibition of the ACCase by long chain acyl-ACP molecules (Davis and Cronan 2001). The enzyme concentration and/or activity of the ACCase might not have been high enough to cause a significant increase in fatty acids, or the feedback on the other enzymes in the fatty acid synthesis pathway is too high to cause an increase in the total amount of fatty acids. Both these scenarios are possible since there is often an increase in malonyl-CoA observed without a significant increase in fatty acid synthesis. However, (Meng et al. 2011) did observe an increase in fatty acid synthesis in *E. coli*, which suggests that expression of a single ACCase in a sufficiently high concentration and activity should be able to at least overcome part of the feedback inhibition.

Although the total amount of intracellular fatty acids does not increase significantly, there are changes in the amount of individual fatty acids. The main change in both Se:ΔA+T and E. coli is in the relative amount of C16 and its direct derivatives (C16:1, C16:2). It has been shown that C16 is the primary end product of fatty acid synthesis (Dijkstra et al. 2008). This suggests that the rate of *de novo* synthesis of fatty acids might be increased in these strains.

The total amount of extracellular fatty acids of strain Se:ACCase is significantly increased compared to strain Se:ΔA+T. This observation supports the hypothesis that partial removal of the acyl-ACP molecules allows an increase in the amount of fatty acid synthesis. We also found that both inducing and uninducing conditions lead to a significant increase in the amount of extracellular fatty acids. This might indicate that the release of free fatty acids by the thioesterase is not a rate-limiting step since the enzyme is not

completely saturated and might be able to handle more substrate. However, we can not draw strong conclusions, since both the growth rate and the amount of biomass was reduced compared to strain Se:ΔA+T. Under uninduced conditions, the absolute amount (not corrected for biomass) of extracellular fatty acids was also significantly increased ($p = 0.0453$) in strain Se:ACCase compared to Se:ΔA+T. However, under induced conditions, the absolute amount of extracellular fatty acids was not significantly changed in strain Se:ACCase compared to Se:ΔA+T. It is interesting to note that induction of the ACCase in *E. coli* leads to an increase in degradation of the RNA and very little to no additional full length transcript was observed on the northern blot (data not shown).

Concluding remarks and future prospects

Our study shows that the introduction of the ACCase from *S. elongatus* PCC 7942 is unable to enhance fatty acid production alone. However, in combination with the expression of an *E. coli* thioesterase and removal of endogenous acyl-ACP synthetase there is a significant increase in the amount of extracellular fatty acids. To our knowledge this is the first report in which introduction of the ACCase in PCC 7942 shows a significant increase in the amount of extracellular fatty acids on a per cell basis as well as an increase in the absolute amount (not corrected for biomass) of extracellular fatty acids in this strain.

It would be of interest to measure the changes in the amount of acetyl-CoA and manolyl-CoA. This would allow us to directly study the effect of the introduction of the ACCase construct and the thioesterase. Furthermore, it might allow us to elucidate why there is such a dramatic effect of introduction of the ACCase on growth both in *E. coli* and in Se:ΔA+T, but not in the strain containing the truncated ACCase construct or in PCC 7942.

Induction of the ACCase in *E. coli* and Se:ΔA+T also suggests that there might be room to enhance the stability, transcription and translation of the construct. Choosing a stronger promoter such as the psbA promoter might enhance transcription. Translation might be enhanced by choosing a different ribosome binding site, such as those chosen for the expression of the thioesterase (data no shown). More ribosome loading might also enhance the stability of the RNA (Deutscher 2006).

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Chapter 5

Biotechnology for Biofuels. Submitted.

GTP-binding protein Era: a novel gene target for biofuel production

Gerben P. Voshol, Vera Meyer, Cees A.M.J.J. van den Hondel

Abstract

Biodiesel production using cyanobacteria is a promising alternative to fossil fuels. In this study we created a transposon library of *Synechococcus elongatus* PCC 7942 in order to identify novel gene targets for enhanced fatty acid and hydrocarbon production. The transposon library was subsequently screened for desirable traits using macro- and microscopic observations as well as staining with the lipophilic dye Nile Red. Based on the screening results, we selected a single mutant, which has an insertion in the gene encoding for the GTP-binding protein Era. We subsequently verified the phenotype-genotype relation by overexpression, reintroducing and complementing the mutation. The GTP-binding protein Era has never been studied in cyanobacteria and proved to be an essential gene for *S. elongatus* PCC 7942. We also found that this protein is important for hydrocarbon and fatty acid metabolism as well as determination of the cell size in PCC 7942. Our results suggest that the GTP-binding protein Era can be used as a novel target to further improve the production of biofuel precursors in promising strains.

Introduction

Biodiesel produced by photosynthetic microorganisms, such as eukaryotic algae and cyanobacteria provides a promising alternative to reduce our reliance on fossil fuels. Biodiesel can be produced either directly by these organisms or from their biomass. This fuel is carbon neutral, renewable and its use requires minimal changes in the current structure of fuel delivery and consumption. However, the relatively low productivity and the high cost of harvesting the biomass present mayor limitations for commercialization of cyanobacteria-derived biodiesels (Ruffing and Jones 2012; Slade and Bauen 2013).

These limitations have been mainly addressed by using a rational approach (Hu et al. 2013; Liu et al. 2011; Ruffing 2013a). The rational approach involves designing strains with enhanced biodiesel precursor production (e.g. fatty acids, hydrocarbons) by modifying and/or introducing known metabolic pathways. The most common modifications include the introduction of a heterologous thioesterase and removal of the endogenous acyl-ACP synthetase. The introduced thioesterase is capable of hydrolysing the acyl-ACP molecule and thus releases the fatty acid, the major precursor of biodiesel (Cho and Cronan 1995). The acyl-ACP synthetase can reactivate free fatty acids by attaching them to the ACP-molecule (Kaczmarzyk and Fulda 2010). By introducing a thioesterase in combination with disrupting the endogenous acyl-ACP synthetase, one can significantly enhance fatty acid production and secretion in cyanobacteria (Hu et al. 2013; Liu et al. 2011; Ruffing and Jones 2012).

However, despite some success, the reported fatty acid yields are still not sufficient for large-scale production. This is partly due to the fact that the bulk of the fatty acids remain within the cell. Changing the cell structure in such a way that the cells can auto-flocculate or elongate might help to harvest the remaining biomass more efficiently (Markou and Georgakakis 2011). Since the rational approach is guided by existing knowledge about the cellular processes involved in fatty acid biosynthesis and/or transport, it can limit strain improvement due to missing information on key intermediates, regulators, competing pathways etc. Therefore, combining the rational approach with random approaches (e.g. transposon mutagenesis) may result in the identification of novel genes involved in efficient biodiesel precursor production.

Random mutagenesis has been successfully used in cyanobacteria to isolate filamentous (Campellone and Leong 2005) and grazing resistant mutants (Simkovsky et al. 2012) as well as to identify environmentally responsive genes (Wolk 1991) and genes involved in polyhydroxybutyrate synthesis (Miyake et al. 2000). Furthermore, this approach was previously applied to isolate genes involved in fatty acid production in *Escherichia coli* (Hoover et al. 2012). However, genes identified by Hoover and colleagues (2012) in *E. coli* lack obvious orthologous genes in cyanobacteria. Moreover, to our knowledge, random mutagenesis has never been applied to isolate lipid-overproducing mutants in cyanobacteria.

In this study we used *Synechococcus elongatus* PCC 7942 which is amenable to genetic modification and has a fully sequenced genome. This strain produces the major biodiesel precursors and lacks the ability to synthesize polyhydroxybutyrate (PHB; a competing pathway for biofuel production) (Miyake et al. 2000). These properties make *S. elongatus* PCC 7942 a good model strain to isolate mutants with an enhanced fatty acid production. The construction and analysis of a transposon generated mutant library, resulted in the identification of a gene that directly or indirectly affects fatty acid and hydrocarbon production. Moreover, this gene, named *era*, is also involved in determining the cellular morphology of PCC 7942. To our knowledge this gene has never been analysed in cyanobacteria.

Materials and Methods

Bacterial Strains and Plasmids

Wild-type and mutant *E. coli* and *S. elongatus* PCC 7942 strains are shown in Table 1. Table 1 further lists the plasmids that were used in this study. Primers used for vector construction and verification are listed in Table 2.

Culture Conditions

PCC 7942 and its mutants were cultured in BG-11 medium at 30 °C. Liquid cultures were incubated on a rotary shaker at 250 rpm under continuous light (60 $\mu E/m^2/s$). When needed antibiotics were added to a final concentration of 25 µg/ml kanamycin and/or 10 µg/ml chloramphenicol. Cellular growth was routinely determined by taking an aliquot of 1 ml every 24 hours for 5 days, diluted to an appropriate optical density and the absorption was determined at 750 nm.

Table 1 Strains and plasmids used in this study

	Description	Reference
Strain		
E. coli DH5α	Used for molecular cloning	(Hanahan 1983)
S. elongatus PCC 7942	Wild-type freshwater cyanobacterium	Pasteur Institute (Paris, France)
2A01	*S. elongatus* PCC 7942 containing transposon pRL1063a	This study
PCC 7942	*S. elongatus* PCC 7942 containing pNS3	This study
Se:era	*S. elongatus* PCC 7942 expressing the *era* gene from neutral site 3	This study
Se:Δera	*S. elongatus* PCC 7942 with a insertion of the inactivate transposon (pSE3) and expressing the *era* gene from neutral site 3	This study
Se:Δera+era	*S. elongatus* PCC 7942 expressing a truncated thioesterase from *E. coli* DH5α (*'tesA*) and containing a disrupted acyl-ACP synthetase (Synpcc7942_0918)	This study
Plasmid		
pJet1.2	Used for the cloning of blunt PCR products	Thermo-Fisher Scientific (Waltham, MA, USA)
pRL1063a	Contains a transposon based on Tn5, which bears several antibiotic resistance genes (kanamycin (Km), bleomycin (Ble) and streptomycin (Sm)), promoterless luciferase (*luxAB*) reporter genes, an oriV not recognized by PCC 7942 and a transposase gene. The oriT for conjugative transfer is present on the plasmid but is not part of the transposon.	(Wolk 1991)
pHN1-LacUV5	Targets Neutral site 3, confers resistance to chloramphenicol antibiotic (Cm) and contains a strong isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated lacUV5 promoter followed by a ribosome binding site (RBS) and an unique HindIII restriction site	(Niederholtmeyer et al. 2010)
pNS3	Derived from pHN1-LacUV5 with the original RBS removed and lacking the ATG start codon near the multiple cloning site	This study
pNS3:ERA	Derived from pNS3 with *era* gene (Synpcc7942_0160) cloned into the unique HindIII restriction site behind the inducible lacUV5 promoter	This study
pSe:Δera	Inactivate transposon TN5-1063a originally isolated from strain 2A01, with the transposase mutate by digestion with NotI and blunting using T4 DNA polymerase	This study

Table 2 Primers used in this study

Primer	Sequence[a]	Remarks
5pERAfw	ctcgaggtaggggt tgatctcgtgga	Used in combination with pRL1063a_rev and ERA3pRev2 for segregation check
ERA3pRev2	ggcaaacgctgaaa gtcttc	Amplifies the 3 prime region outside the inactive transposon construct pSe:Δera. Used to check the segregation in combination with pRL1063a_fw and 5pERAfw
ERAfw	aagctt**aaggagg aaaaa**atgtccga ccttttcaccac	Primer for amplifying the *era* gene, addition of HindIII restriction site and ribosome binding site
ERArv	aagcttttactcact ctcaggtcggtagc	Primer for amplifying the *era* gene, addition of HindIII restriction site
pRL1063a_fw	aggaggtcacatgg aatatcagat	Used for sequencing flanking regions of TN5-1063a
pRL1063a_rev	tactagattcaatgc tatcaatgag	Used for sequencing flanking regions of TN5-1063a

[a]Restriction sites are underlined and the ribosomal binding site is indicated in bold.

Transposon Library Construction

To perform transposon mutagenesis, plasmid pRL1063a was used (Wolk 1991). Plasmid pRL1063a contains a transposon based on Tn5, which bears several antibiotic resistance genes (kanamycin (Km), bleomycin (Ble) and streptomycin (Sm)), promoterless luciferase (*luxAB*) reporter genes, an oriV not recognized by PCC 7942 and a transposase gene. The oriT for conjugative transfer is present on the plasmid but is not part of the transposon. Conjugation was used to introduce pRL1063a into PCC 7942, using a method similar to that of Clerico et al. (2007). After 3 days of growth under low light conditions (5 $\mu E/m^2/s$), kanamycin was added underneath the plates to a final concentration of 25 µg/ml and plates were re-incubated at 30 °C under normal light conditions (60 $\mu E/m^2/s$) until colonies formed.

Screening for increased lipids using Flow cytometry and Nile Red

To get a qualitative indication of lipid content, mutant cells were stained using the lipophilic dye Nile Red (Hoover et al. 2012). Nile Red is a dye that becomes strongly fluorescent when present in a hydrophobic environment (e.g. membrane lipids). Depending on the hydrophobicity of the compounds, the dye emits either a yellow or red fluorescence. The intensity of the signal is an indication for the amount of lipids present. An aliquot of cells (1 ml) from an exponentially growing culture was taken, transferred to an eppendorf tube and Nile red (1 mg/ml in DMSO) was added to a final concentration of 1 µg/ml. Cells were examined using a guave easyCyte flow cytometer (Merck), by exciting with a 485nm laser and determining several parameters such as

cellular size (forward scatter), complexity (side scatter) and yellow fluorescence (583/26 nm).

Determination of Transposon Insertion Site

To determine the site of transposition, cyanobacterial genomic DNA from mutant 2A01 was extracted using the method of Clerico et al. (2007). Subsequently, 10 µg of chromosomal DNA was digested overnight with EcoRI, re-ligated using T4 ligase and 5 µg DNA from this mixture was transformed into chemically competent *E. coli* DH5α cells. After plaiting the cells on LB medium containing 50 µg/ml kanamycin, colonies that formed after overnight cultivation at 37 °C were inoculated into liquid LB medium and plasmid DNA was isolated and analysed by restriction analysis and sequencing.

Construction of Era overexpression mutant and control

To elucidate the function of Era for *S. elongatus* PCC 7942, an overexpression vector (pNS3:ERA) was constructed. This vector was constructed using vector pNS3, which contains amongst others a chloramphenicol resistance marker, IPTG inducible promoter and homologous regions for integration at neutral site III. The *era* gene was amplified from PCC 7942 genomic DNA with primers ERAfw (containing a HindIII restriction site and ribosomal binding site) and ERArv (containing a HindIII restriction site) (Table 2). The resulting product was digested and ligated into a pNS3 vector, which resulted in the final plasmid pNS3:ERA. Transformation of this vector into PCC 7942 resulted in strain Se:era. At the same time, transformation of pNS3 into PCC 7942 was done to create a negative control.

Construction of Era disruptions and complementation

To reconstruct the original phenotype caused by the transposon found in 2A01, the re-isolated transposon of 2A01 was inactivated and reintroduced into PCC 7942 as follows. The plasmid was digested using EcoRV to reduce the size of the vector (from app. 20 kb to 11 kb by removing part of the genomic DNA from this plasmid) and thereby making subsequent cloning steps easier. This smaller plasmid was linearized using NotI, which cuts in the transposase gene blunted using T4 DNA Polymerase and re-ligated. This vector (pSe:Δera) was transformed into strain Se:era, creating strain Se:Δera+era and PCC 7942 containing pNS3 creating strain Se:Δera.

Analysis of Cellular length

To see the effect of the introduced mutations on bacterial cell length, pictures were taken with a phase contrast microscope (Carl-Zeiss, Sliedrecht, The Netherlands) and analysed using ImageJ (Schneider et al. 2012) with the Coli-Inspector plugin (Norbert Vischer, Bacterial Cell Biology, University of Amsterdam). This plugin is able to determine the cell length and diameter of individual cells in an automated manner. From this data a histogram was constructed and the median cell length was determined.

Extraction of hydrocarbons and fatty acids for GC-MS

Extraction of hydrocarbons and fatty acids was performed as previously described in Voshol et al. 2014. Briefly, cells from a 5 day old culture were diluted to a starting optical density (750nm) of approximately 0.05 and grown in BG-11 for 5 days as described above (in quadruple). Bacterial cells were subsequently harvested by centrifugation (20 min, 4500 rcf), the supernatant was discarded and the pellet was freeze-dried. To each 20 mg of lyophilized biomass, 50 µl of internal standard (C10:0, 5 mg/ml), 1 ml hexane and 2 ml methanol containing 0.5 M sodium methoxide was added. The samples were vortexed for 30 seconds and sonicated for 5 minutes. Subsequently they were incubated at 50 °C for 10 minutes after which 3 ml 5% HCL in methanol was added to stop the reaction. The samples were then vortexed (30 seconds), sonicated (5 minutes) and incubated at 70 °C (20 minutes). Samples were allowed to cool to room temperature and were extracted twice with 4 ml of hexane containing 50 mg/L butylated hydroxytoluene. The samples were then vacuum evaporation, dissolved in 1 ml of hexane and transferred to GC-MS vials.

GC-MS analysis

These extracted hydrocarbons and fatty acids mixtures were analysed using an Agilent model 7890A gas chromatograph as previously described (Voshol et al. 2014). Briefly, samples were inserted into a model 7693 autosamples, separated using a DB-WAX column (10 m, 0.25 mm, 0.25 µm) and detected using an inert XL mass spectrometer (model 5975C) using helium as carrier gas. The initial oven temperature was 50 °C for 1 minute, then the temperature was increased to 230 °C (25 °C/min until 200 °C and 3 °C/min until 230 °C) after which this temperature was held for 8 minutes. Retention time and the mass spectrum of authentic samples were used for identification.

Hydrocarbons were quantified relative to the internal standard while absolute quantities of fatty acids were determined based on calibration curves constructed using authentic standards.

Statistics

R *Statistical* Software (Foundation for *Statistical* Computing, Vienna, Austria) was used to carry out the data analysis. Data were first tested using Leven's test to indicate whether their variances were significantly different. Data that did not show a significant difference using Leven's test were subsequently compared using ANOVA and post-hoc Tukey Honest Significant Differences test. Data that were not normally distributed or showed unequal variances were analysed using the nonparametric Mann-Whitney-Wilcoxon test. Differences were considered significant if their p values were smaller than 0.05.

Results

Isolation of a putative fatty acid overproducing mutant by random mutagenesis

To obtain a mutant library, we haven chosen to construct a transposon library using plasmid pRL1063a, which contains a Tn5 transposon with a kanamycin resistance marker, promoterless *luxAB* reporter genes and an origin of replication which functions in *E. coli*, but not in PCC 7942. This library, contained approximately 600 individual mutants, was subsequently screened for mutants with beneficial phenotypes such as filamentous and auto-flocculating growth, higher lipid content and phenotypic stability. Out of the initial 600 mutants a single strain was selected for further study based on its (i) elongated cell morphology, (ii) enhanced fluorescence after staining with the lipophilic dye Nile Red and (iii) phenotypic stability.

The selected mutant, 2A01, was both phenotypically and genotypically stable for a period of at least 3 months (verified by liquid to liquid sub-cultivation and PCR). This mutant showed a different colony morphology compared to PCC 7942 (Fig. 1A and B). 2A01 has a round, slightly elevated colony morphology with irregular shaped edges on plate (Fig. 1B) while PCC 7942 is round, raised with an entire smooth edge (Fig. 1A). Phase contrast microscopy revealed that cultures of mutant 2A01 contained a heterogeneous mix of short cells similar in size to PCC 7942 and long elongated cells some more than 30 times the length of PCC 7942 (Fig. 1 C and D).

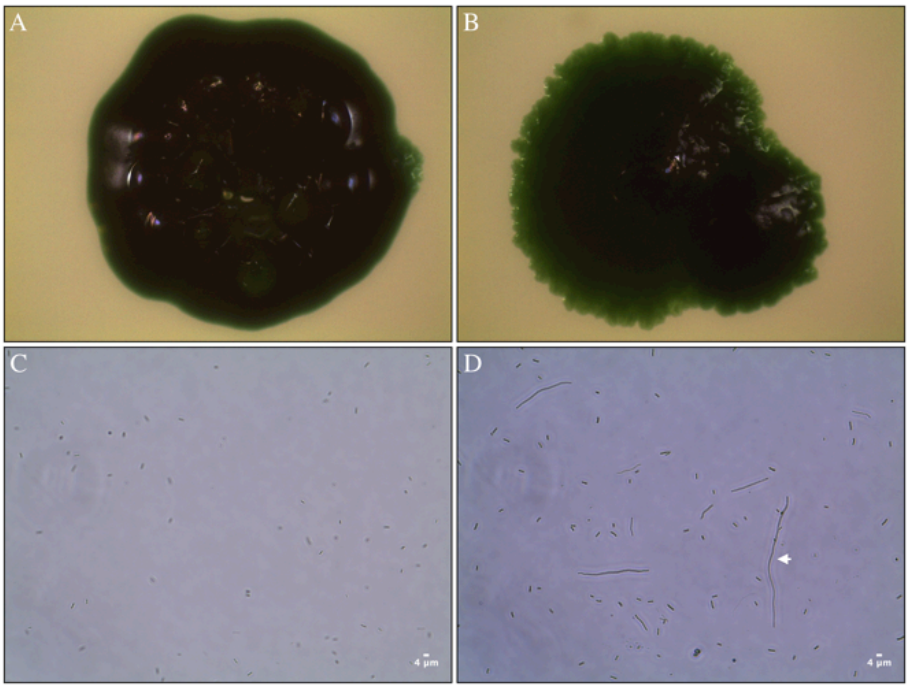

Figure 1 Macro (A and B) and microscopic morphology (C and D) of wild-type PCC 7942 (A and C) and mutant 2A01 (B and D). Macroscopic morphology was visualized after growth on plate using a stereomicroscope. Microscopic morphology was visualized by phase-contrast microscopy. Scale bars represent 4 µm. Example of an elongated cell is indicated with an arrow (97 µm long).

We also used flow cytometry to determine cellular morphology (size and complexity) of PCC 7942 and 2A01 stained with Nile Red (Fig. 2B and D) or unstained (Fig. 2A and A). Stained cells are very similar to unstained cells in respects to their cellular size (forward scatter), however the complexity (side scatter) after staining with Nile Red is slightly, but significantly higher. Comparison of cells of PCC 7942 (Fig. 2A) and 2A01 (Fig. 2C), furthermore indicates that cells of 2A01 are both larger in overall size and complexity than PCC 7942.

The largest differences between PCC 7942 and 2A01 were observed when comparing the yellow fluorescence (indication of lipid content) of Nile Red stained cells. In general, unstained cells showed a low amount of yellow fluorescence compared to both red and green fluorescence (data not shown). Cells stained with Nile Red showed an increase of yellow fluorescence (Fig. 3A and C versus B and D) and 2A01 showed a higher increase in yellow fluorescence even at comparable cellular size (forward scatter) than PCC 7942.

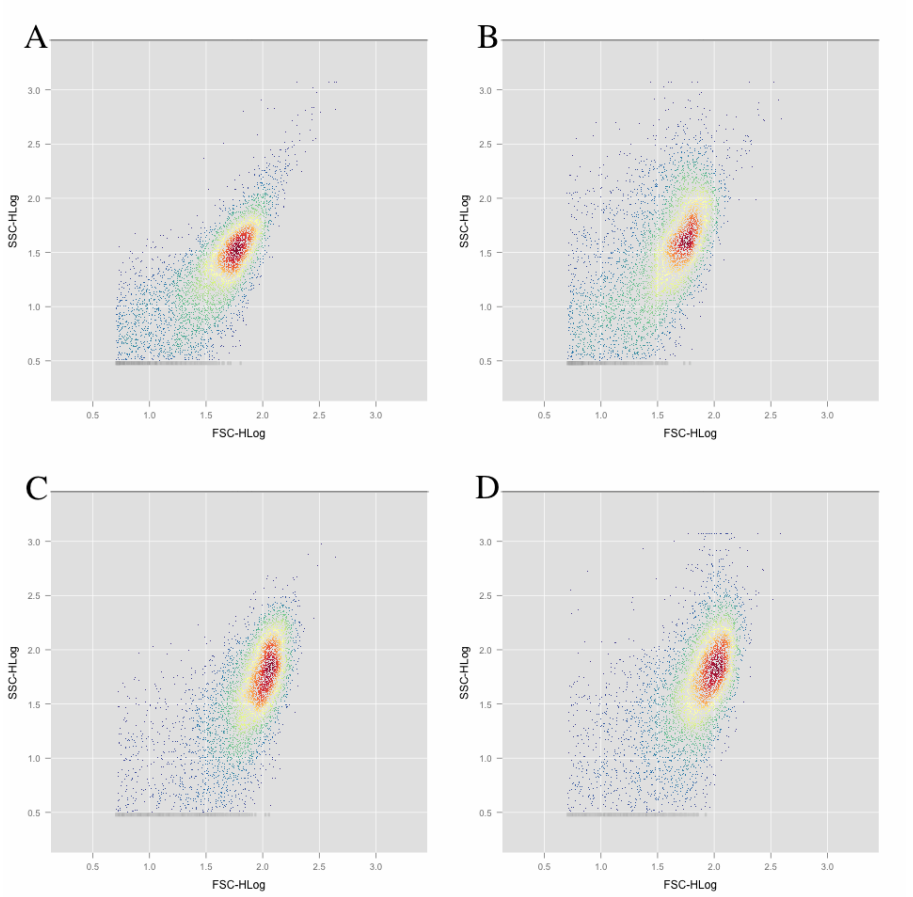

Figure 2 Side scatter (cellular complexity) versus front scatter (cell length) as determined using flow cytometry of PCC 7942 (A and B) and mutant 2A01 (C and D) grown in liquid media for 48 hours either unstained (A and C) or stained (B and D) with the lipophilic dye Nile red.

Transposon Insertion Site

To identify the insertion site of the transposon, genomic DNA was isolated, digested, re-ligated and transformed into *E. coli* DH5α. Plasmid DNA was extracted from colonies that acquired antibiotic resistance and restriction patterns were compared to identify additional transposition events. Based on their distinct restriction patterns, DNA of two isolated plasmids was sequenced. Both sequences showed the insertion of the transposon into the 3 prime region of gene Synpcc7942_0160 annotated as a GTP-binding protein Era. The insertion caused the formation of an early stop codon leading to a truncated protein lacking the last 20 amino acids. The GTP-binding protein era of PCC

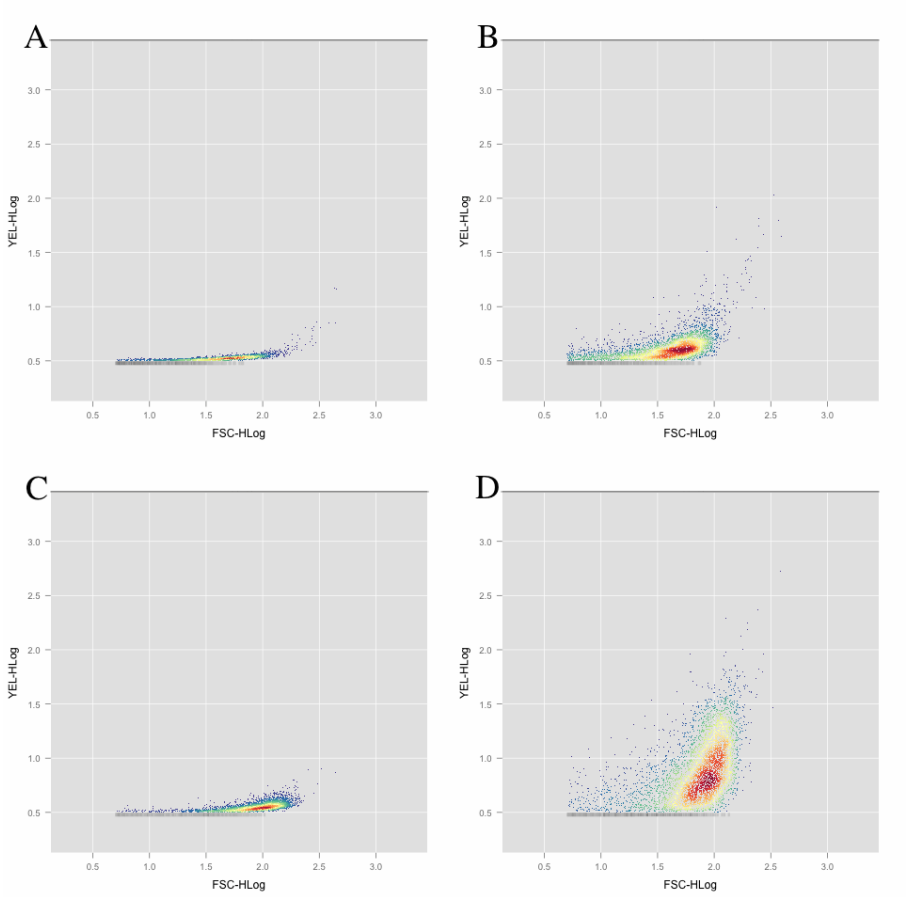

Figure 3 Yellow fluorescence versus front scatter (cell length) as determined using flow cytometry of PCC 7942 (A and B) and mutant 2A01 (C and D) grown in liquid media for 48 hours either unstained (A and C) or stained (B and D) with the lipophilic dye Nile red.

7942 contains both a GTP binding and ATP hydrolysing domain at its N terminus and a type 2 K homology (KH) domain at its C-terminus. In PCC 7942, this gene is the first gene in an operon containing a total of 8 genes (Vijayan et al. 2011).

Verification genotype-phenotype relationship

To identify the genotype, phenotype relationship of mutant 2A01 and to elucidate the function of the GTP-binding protein Era in PCC 7942, several rational approaches were used.

To test the effect of overexpression of the GTP-binding protein Era in PCC 7942, a construct containing an IPTG inducible promoter followed by a ribosomal binding site and the full-length wild type era gene was made

(pNS3:era). Insertion of this construct into the neutral site 3 from PCC 7942 (Niederholtmeyer et al. 2010) was obtained after transformation, resulting in strain Se:era. Full segregation was verified by PCR amplification. As a negative control, a similar construct lacking the ribosomal binding site and era gene (pNS3) was inserted into the neutral site 3 from PCC 7942 (PCC 7942).

To reconstruct the original phenotype, the plasmid containing the transposon flanked by genomic DNA isolated from 2A01 was reinserted into the genome of PCC 7942 after inactivating the transposase by introducing a frame shift mutation. This mutant showed the correct insertion and was fully segregated (as shown by PCR amplification). This mutant was complemented by introducing the overexpression construct pNS3:era, resulting in strain se:Δera+era and as a negative control the construct lacking the ribosomal binding site and era gene was inserted into neutral site 3, mutant se:Δera.

In addition, a knockout construct was made in which the coding region of the era gene was replaced by a Km resistance cassette. Mutants obtained revealed an elongated phenotype (data not shown). However after subcultivation, these initial mutants either lost viability, or the wild type phenotype was restored.

Growth

All strains show similar specific growth rates as measured using OD750 (Fig. 4). The growth rates of mutant strains were 0.041±0.002, 0.042±0.001 and 0.039±0.001 h^{-1} for Se:era, Se:Δera and Se:Δera+era respectively. These specific growth rates were not significantly different from that of PCC 7942 (0.041±0.002 h^{-1}). The final biomass of mutant strains at time of harvesting was also not significantly different compared to PCC 7942.

Cellular morphology: length distribution during growth

To monitor changes in cell size, aliquots from cultures were examined using a phase contrast microscopy (Fig. 5).

Strain se:era, containing an additional copy of era, is significantly smaller at the start of the experiment (at 0 hours) compared to WT with an median of 3.3 and 3.8 μm, respectively. At time points 24, 48 (exponential phase) and 72 hours (early stationary phase) both PCC 7942 and se:era have a similar median cell length of 3.1 μm, 3.3 μm and 3.4-3.5 μm (se:era-PCC 7942), respectively. At 96 hours, the median cell length of PCC 7942 is significantly larger compared to se:era (3.5 μm versus 3.3 μm). At the time of harvesting

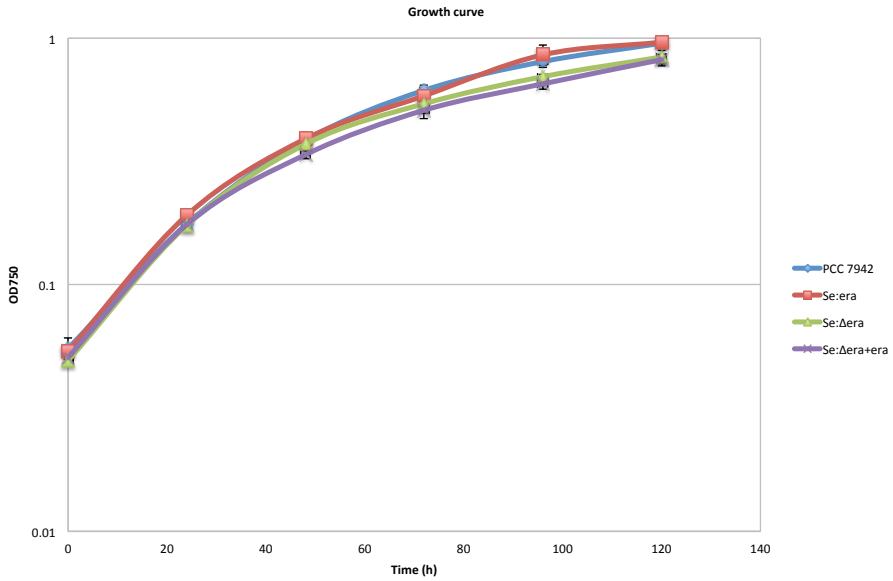

Figure 4 Cellular growth of wild type PCC 7942, strain Se:era, Se:Δera and Se:Δera+era. Growth was measured at 24 hour intervals using the optical density at 750 nm. Error bars represent the standard deviation of four replicates.

(120 hours) the cell length of PCC 7942 elongated further to 3.8 µm while cells from se:era had a median cell length of 3.4 µm.

Strain se:Δera has a significantly larger median cell size compared to PCC 7942 at all time points. Se:Δera had a median cell length of 6.4 µm at the time of inoculation (0 hours). This cell length decreased in the first 24 hours of growth to 5.2 µm. After this time point the cell length increase to 5.3 µm, 5.6 µm, 6.0 µm and 7.0 µm at 48, 72, 96 and 120 hours, respectively.

Strain se:Δera+era has a significantly reduced cell length compared to se:Δera, but is larger than PCC 7942 at all time points (Fig. 5 and supplementary data).

Cellular morphology: microscopy

Differential interference microscopic analysis of the mutant and wild-type strains revealed that strain PCC 7942, se:era and se:Δera:era appear very similar to each other in cell size at 48 hours (Fig. 6A, B and F). This result is similar to the results obtained by measuring the cell size (Fig. 5C). Furthermore, septum formation occurs in a symmetric manner for all these mutants.

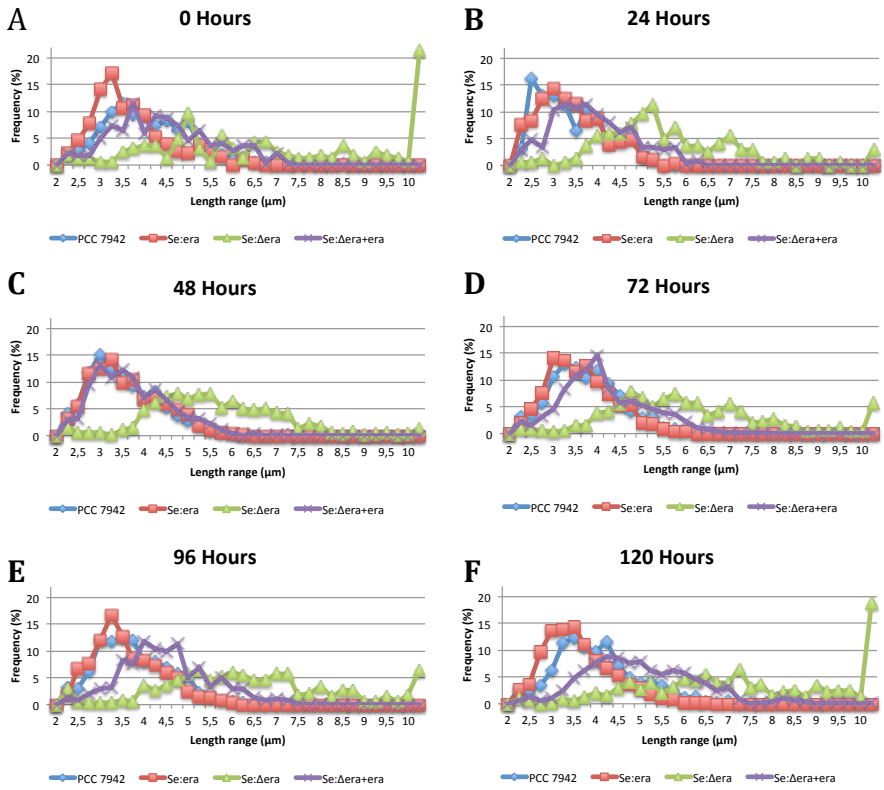

Figure 5 Distribution of cell lengths in liquid cultures of PCC 7942 and mutant Se:era, Se:Δera and Se:Δera+era . Lengths of cells of each strain was determined at 24 hour intervals by taking pictures using a phase contrast microscope and using imageJ to determine their cell size.

Mutant se:Δera however has both elongated cells and cell similar in size to PCC 7942. Constrictions can occur similar to PCC 7942, at the middle of the cell (Fig. 6D). However, constrictions are often observed more towards the cellular pole (Fig. 6E) or sometimes multiple times within a single cell (Fig. 6C). Furthermore, full separation of the daughter cells does also not always occur leading to the formation of cells arrested at the pre-divisional two-cell stage.

Fatty acid and Hydrocarbon profile PCC 7942

PCC 7942 contains a mixture of saturated and unsaturated fatty acids ranging from C14 to C18. Palmitic (C16:0) and palmitoleic acid (C16:1) represent the main intracellular fatty acids of PCC 7942 (Fig. 7). Together these fatty acids represent approximately 89% (35.7±1.2 µg C16:0/mg DW and 30.1±1.2 µg C16:1/mg DW) of the total fatty acids (73.6±2.7 µg/mg DW). The remainder

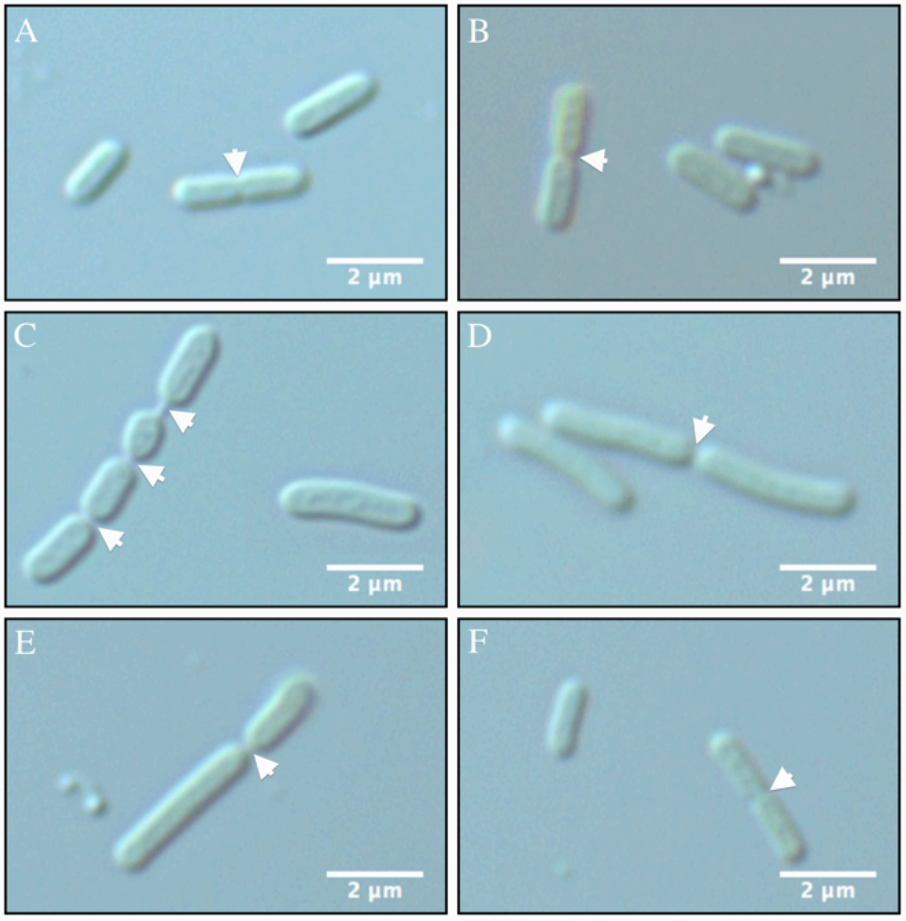

Figure 6 Morphology of PCC 7942 (A), mutant Se:era (B), Se:Δera (C, D and E) and Se:Δera+era (F) grown in liquid media for 48 hours. Scale bars represent 2 µm. The site of septa formation are indicated with arrows

mainly consists of saturated and unsaturated C14 and C18 fatty acids followed by trace amounts of odd chain C15:0, C17:0 and C17:1.

PCC 7942 is able to synthesize both alkanes and alkenes (Fig. 8). The alkanes synthesized by PCC 7942 are mostly heptadecane (C17:0), followed by pentadecane (C15:0) and hexadecane (C16:0). The only alkene that we detected was 8-heptadecene at levels comparable to those of hexadecane.

Fatty acid and Hydrocarbon profile Se:era

Strain Se:era, which contains an additional *era* gene inserted at neutral site 3, has a significant increase in the total amount of fatty acids. The amount

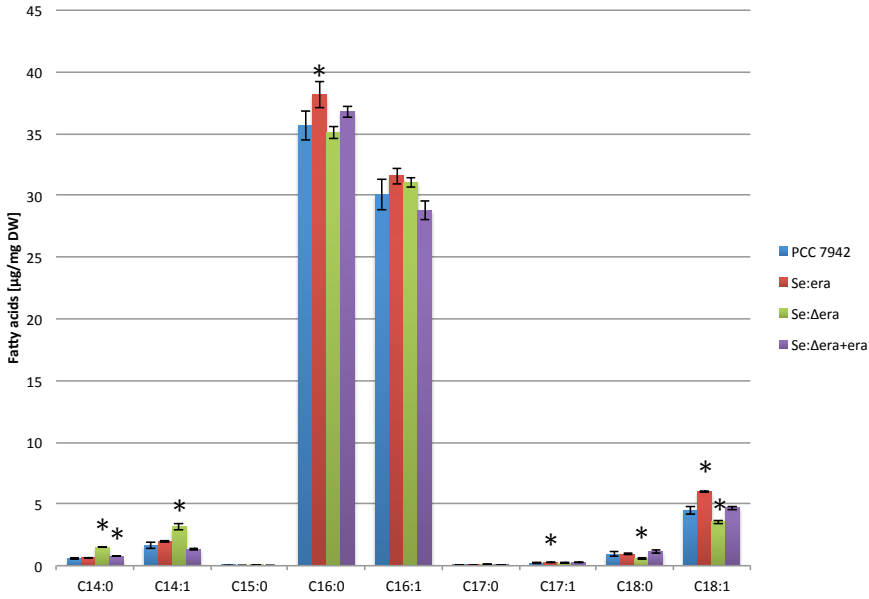

Figure 7 Quantitative measurement of intracellular fatty acids in PCC 7942, strain Se:era, Se:Δera and Se:Δera+era. Metabolites indicated with an asterisk * are significantly different ($p < 0.05$) from those of strain PCC 7942. Error bars represent the standard deviation of four replicates.

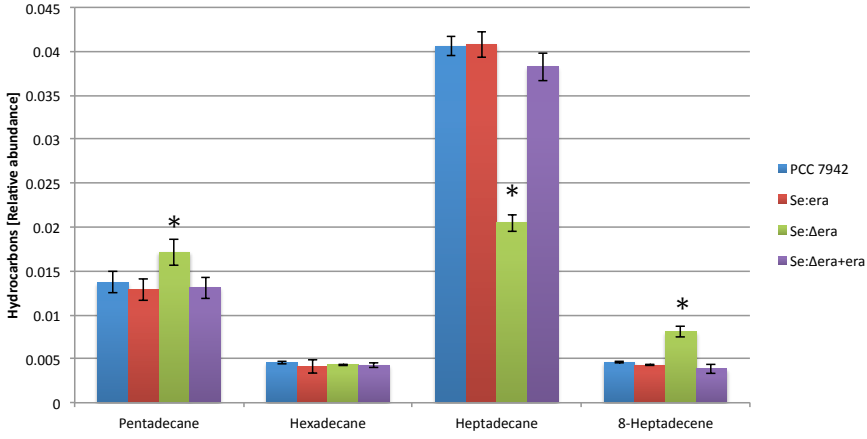

Figure 8 Quantitative measurement of intracellular hydrocarbons in PCC 7942, strain Se:era, Se:Δera and Se:Δera+era. Metabolites indicated with an asterisk * are significantly different ($p < 0.05$) from those of strain PCC 7942. Error bars represent the standard deviation of four replicates.

of fatty acids increased to 79. 6±1.9 µg/mg in Se:era versus 73.6±2.7µg/mg DW in PCC 7942 (an 8% increase). This increase in fatty acids is mostly due to a significant rise in the amount of C16:0 (7%), C18:1 (34%) and to a far lesser

extend C17:1 (26%) (Fig. 7). Together they account for 68% of the increase in total fatty acids, 42%, 26% and 1% for C16:0, C18:1 and C17:1 respectively.

No differences were observed in the amount of hydrocarbons compared to PCC 7942 (Fig 8.).

Fatty acid and Hydrocarbon profile Se: Δera

Strain Se:Δera, containing the inactivated transposon, contains a similar amount (75.2±1.0 µg/mg) of total fatty acids as PCC 7942 (73.6±2.7µg/mg DW) (Fig. 7). Nevertheless, there are some significant changes in the amount of individual fatty acids. This strain has an 162% increase in the amount of C14:0 and an 93% increase in C14:1 compared to PCC 7942. Furthermore, it has a 39% reduction in the amount of C18:0 and a 21% reduction in the amount of C18:1 versus PCC 7942.

This strain is the only strain with significant changes in the hydrocarbon profile (Fig. 8). It contains a higher amount of pentadecane and 8-heptadecene compared to PCC 7942, 25% and 76% respectively. Furthermore, it has a substantial reduction (50%) in the amount of heptadecane.

Fatty acid and Hydrocarbon profile Se: Δera+era

Strain Se:Δera+era, containing the inactivated transposon complemented ectopically by era, has an almost identical amount of total fatty acids (73.8±1.2µg/mg) compared to PCC 7942 (73.6±2.7 µg/mg DW) (Fig. 7). The only fatty acid, which showed a significant difference, is myristic acid (C14:0). This fatty acid was increased by 31% compared to PCC 7942.

The hydrocarbons were similar compared to PCC 7942 (Fig. 8).

Discussion

The era gene is essential for S. elongatus PCC 7942

In this study we generated a transposon library of *S. elongatus* PCC 7942 in order to isolate mutants with beneficial biodiesel production traits, including enhanced fatty acid and hydrocarbon content. The transposon mutant 2A01 was selected based on (i) increased staining with the lipophilic dye Nile red (indicating a higher lipid content), (ii) elongated cell morphology (might facilitate harvesting) and (iii) phenotypic stability. The transposon of mutant 2A01 was inserted into the 3 prime region of the Synpcc7942_0160 (GTP-binding protein Era) gene. Due to this insertion, a premature stop codon was

introduced into the KH-domain of this protein. The KH domain is involved in RNA binding in *E. coli* and the GTP binding domain regulates its activity (Britton 2009).

The *era* gene of PCC 7942 is the first gene of an operon consisting of 8 genes. To study whether the phenotype of the transposon insertion is (also) caused by a possible effect on the expression of downstream genes, we attempted to create a knockout of the *era* gene. We prevented transcription of all downstream genes by replacing the *era* gene with an antibiotic cassette flanked by two transcriptional terminators. Unfortunately, several transformation experiments with this cassette did not result in the isolation of the expected mutant, suggesting that one or several of the genes downstream of *era* are essential (data not shown). However, subsequent introduction of a knockout cassette lacking the transcriptional terminators resulted in the isolation of a transient knockout mutant of *era*. After subcultivation of this knockout mutant, cells either failed to grow or the wild type phenotype was restored. Restoration of the wild type phenotype was associated with the removal of the resistance cassette from the *era* gene. These results indicates that *era* might also be an essential gene in PCC 7942 as was previously shown for *E. coli* (March et al. 1988). Moreover, we found that the transient mutant also showed elongated cells and problems in septa formation. In addition, the deletion mutation was fully complemented by the introduction of an ectopic *era* gene (results not shown). We further verified the phenotype-genotype relationship by a) overexpressing era b) recreating the original transposon mutant, and c) complementing the recreated transposon mutant with an ectopic copy of era.

GTP-binding protein Era is involved in lipid and hydrocarbon production of PCC 7942

Analysis of an *era* overexpression strain, Se:era, showed a significantly higher amount of fatty acids compared to PCC 7942 (Fig. 7). These results demonstrate the importance of the GTP-binding protein Era for the production of fatty acids in *S. elongatus* PCC 7942.

To further verify the role of Era in biodiesel precursor production, we reintroduced the inactive transposon in PCC 7942 and complemented the resulted Se:Δera mutant with an ectopic copy of era. Strain Se:Δera does not show a significant difference in the total amount of fatty acids, compared to PCC 7942 (Fig. 7). However, because this strain has significantly longer cells (Fig. 5),

there are fewer cells per mg of dry weight compared to PCC 7942. These results indicate that there is a significantly higher amount of lipids per cell, supporting the original Nile red results. Moreover, several individual fatty acids are significantly different from PCC 7942 (Fig. 7), suggesting that era plays a role in fatty acid synthesis in this strain. Complementation of Se:Δera with the ectopically integrated *era* gene (se:Δera+era) fully restored the fatty acid profile to PCC 7942 (except for C14:0), supporting the previous observation that Era is responsible for the changes in fatty acid profile.

In Se:era we did not observe any significant changes in hydrocarbon production. However, we found significant changes in hydrocarbon production in Se:Δera, which were restored when complemented with era (Fig. 8), suggesting that Era is also involved in hydrocarbon metabolism.

Rational strain improvement for biodiesel precursor production of strain PCC 7942 has been attempted in the past by the introduction of thioesterase, acetyl-coA carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase. However, these modifications failed to increase the net FA production (Ruffing 2013b). Moreover, identified targets using RNA-seq to enhance the strain productivity of PCC 7942 led only to minor improvements in FA production (Ruffing 2013a). However, our results show a substantial increase in fatty acid production without causing negative effects on biomass accumulation, suggesting that the GTP-binding protein Era is a new promising gene target for further strain improvement for the production of fatty acids (and possibly hydrocarbons).

GTP-binding protein Era is important for cell size of PCC 7942

Several studies in *E. coli* showed that overexpression of *era* up to 5% of total cellular protein does not alter cellular growth, cAMP levels or protein production (Britton et al. 1998; Gollop and March 1991). This is possibly due to the fact that the activity of the GTP-binding protein Era is regulated by binding of GTP (Shimamoto and Inouye 1996). However, we observed a significant effect on cellular size during late exponential growth of PCC 7942 containing an additional *era* gene. Although the effect on cell size is subtle and can easily be missed during microscopic examination, cells of Se:era are significantly smaller than those of PCC 7942 (>11%; Fig. 5). To our knowledge, this is the first report demonstrating that overexpression of Era causes a change in cell size.

Depletion of Era in *E. coli* causes a decrease in growth, loss of viability, septum formation and elongated cells (Verstraeten et al. 2011). In the mutant

se:Δera, containing the inactivated transposon, we did observe an effect on septum formation and cell size. However, this mutant did not show a difference in growth rate nor viability compared to PCC 7942. Analysis of the phenotype of se:Δera suggests that the expression of *era* might be reduced to a level which is slightly lower than that required for normal cell division (Britton et al. 1998).

Introduction of an ectopic era in strain se:Δera resulted in restoring the formation of septa in the middle of a cell, but not entirely the cell length. While more than 96 % of all the cells were within the same length range as PCC 7942, some cells were up to 26% larger than WT. However, the median length of these cells was 31% smaller compared to strain Se:Δera. These results suggest the insertion in era itself and/or one or more downstream genes is responsible for the elongated cell morphology.

The fact that introduction of an additional *era* gene did not fully complement the Se:Δera mutant, might have several reasons. First of all, the expression of the era gene used for complementation is not regulated by its native promoter. For example, it has been shown that expression of *era* is coupled to cellular growth (fast growth, more Era) in *E. coli* (Britton et al. 1998). Another possibility is that downstream genes might be responsible for part of the phenotype and/or presence of a truncated era interferes with the complementation.

Concluding remarks and future prospects

It was previously shown that Era plays a role in many cellular processes including ribosome assembly and cell cycle regulation (Verstraeten et al. 2011). This protein has been well studied in diverse bacterial species, but never in cyanobacteria. In this report we showed that *era* is an essential gene for *S. elongatus* PCC 7942. In this strain, the GTP-binding protein Era is involved in hydrocarbon and fatty acid metabolism as well as the determination of cell size. We propose that the Era protein can be used as a novel target for improving biofuel production traits. It would be interesting to evaluate the effect of overexpression of *era* in combination with other genetic modifications, for example in a strain expressing a thioesterase and containing a knockout of the acyl-ACP synthetase. Furthermore, the question remains open whether overproduction of the GTP-binding protein Era also influences cellular size and fatty acid/hydrocarbon content in other bacteria.

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Chapter 6

General discussion

Rational approach for improving the production and secretion of biofuel precursors

Several studies describe how the authors have attempted to optimize the production of biodiesel precursors (i.e. fatty acids and hydrocarbons) in a number of cyanobacteria species such as *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002 and *Synechococcus elongatus* PCC 7942 (Ruffing 2014; Ruffing and Jones 2012; Wang et al. 2013). Many factors are known to influence the productivity of the engineered strains. These factors include the tolerance of the strain to metabolic fluctuations caused by the introduced changes e.g. product formation and toxicity; the genetic engineering applied to the strain and the specific culture conditions (e.g. temperature, light intensity). In **Chapter 3** we described how we achieved to get the same growth rate for wild strain *S. elongatus* PCC 7942 and its mutants Se:T, Se:ΔA and Se:ΔA+T using optimized precultivation conditions. This allowed us to evaluate the changes in productivity caused by the genetic engineering itself and not by the differences in growth and/or cultivation conditions. However, we were not able to find cultivation conditions that allowed strain Se:ACCase to grow with the same growth rate as that of wild type *S. elongatus* PCC 7942 (**Chapter 4**). Therefore, we can not rule out the possibility that the improved productivity of strain Se:ACCase is partially caused by differences in biomass accumulation and increased lag phase (leading to a possible overestimation in absolute and relative productivity).

A study by Ruffing and Jones (2012) indicated differences in the relative amounts of photosynthetic pigments in *S. elongatus* PCC 7942 strains engineered for free fatty acid production (*Δaas* and *Δaas+'tesA*) and suggested a link between free fatty acid production, photosynthesis and growth retardation (Ruffing 2013a; Ruffing and Jones 2012). More specifically, the authors found a reduction in the growth rate and photosynthetic pigmentation (chlorophyll a content) of strains Se:ΔA and Se:ΔA+T and suggested that this reduction is due to stress caused by free fatty acid toxicity.

In my opinion, it is unlikely that a slower growth rate and a change in pigmentation are caused by free fatty acid toxicity. Since Se:ΔA and Se:ΔA+T

produce similar amount of fatty acids (approximately 10mg/L after 12 and 8 days of growth, respectively), one would expect to see the "symptoms" of free fatty acid toxicity in both strains (Ruffing 2013a). However, in our experience, only strain Se:ΔA+T shows an extended lag phase and reduction of its specific growth rate (Fig 1), while strain Se:ΔA is not different from *S. elongatus* PCC 7942 in relation to its growth (**Chapter 2, Fig 1**) or photosynthetic pigmentation (Fig 2). Moreover, at the stage when both strains secrete 10 mg/L free fatty acids, approximately 40% of the cells from strain Se:ΔA+T stain reactive oxygen species (ROS) positive and ~10% of the cells become permeable, while strain Se:ΔA does not have a significant amount of ROS or permeabilized cells (Ruffing 2013a).

It is also noteworthy, that Se:ΔA and Se:ΔA+T did not show the characteristic decrease in phycobilin (Fig 2B), which is normally associated with free fatty acid toxicity in cyanobacteria (Wu et al. 2006). In case of Se:ΔA+T, the phycobilin content was even significantly increased compared to PCC 7942 (Fig 2B).

Exposing wild type *S. elongatus* PCC 7942 to palmitic (C16:0) and stearic (C18:0) acid up to 300 µM (similar to the concentration found in strain Se:ΔA+T) did not have an effect on photosynthetic yield, growth rate or photosynthetic pigmentation (Ruffing and Jones 2012).

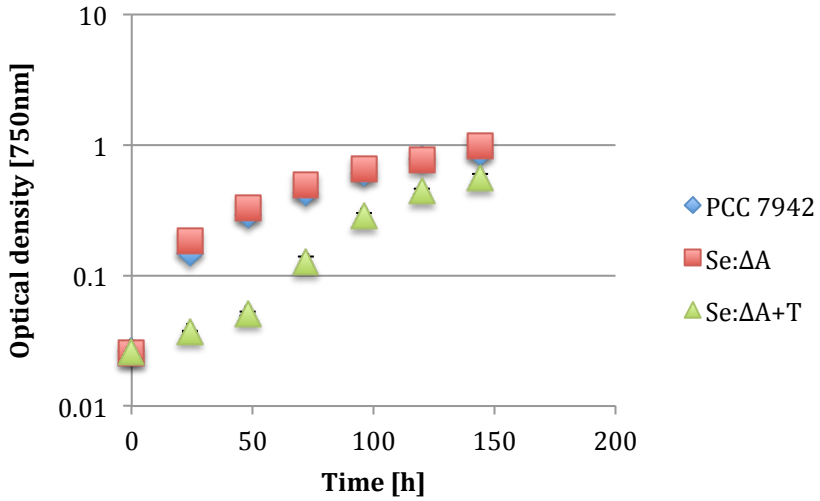

Figure 1 Cellular growth of wild type PCC 7942, strain Se:ΔA and Se:ΔA+T inoculated from stationary phase cultures at an initial OD750 of 0.025 (approximately 1/50 dilution). Growth was measured at 24 hour intervals using the optical density at 750 nm. Error bars represent the standard deviation of three replicates.

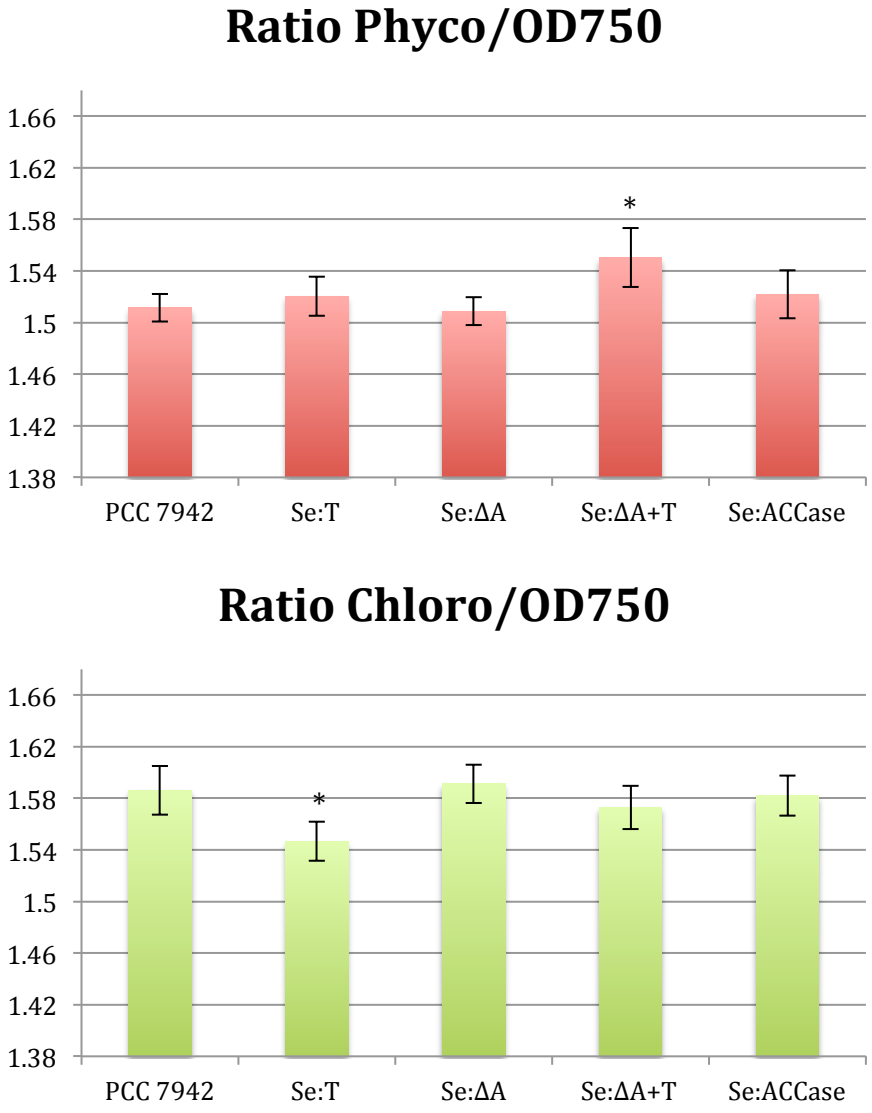

Figure 2 The effect of the genetic engineering on the amount of phycobilin (top) and chlorophyll a (bottom) of wild type *S. elongatus* PCC 7942, strain Se:T, Se:ΔA, Se:ΔA+T and Se:ACCase. The changes in photosynthetic pigments were determined by monitoring changes in the ratio of phycobilin (OD at 628nm/OD at 750 nm) and chlorophyll a (OD at 628nm/OD at 750 nm) as previously described **(Ruffing and Jones 2012; Schlebusch and Forchhammer 2010)**. Those indicated with an asterisk (*) are significantly different ($p < 0.05$) from wild type PCC 7942. Error bars represent the standard deviation of four replicates.

Furthermore, the negative physiological effects on growth rate, photosynthesis and pigmentation observed after the exogenous addition of linolenic acid

(C18:3; a fatty acid not normally synthesized by *S. elongatus* PCC 7942) were different from the effects observed in the free fatty acid producing strains *Δaas* and *Δaas+'tesA* (Ruffing 2013a). It has also been shown that a *Δaas* mutant strain of *Synechocystis* sp. PCC 6803 is unaffected by the addition of linolenic acid (von Berlepsch et al. 2012).

The most likely explanation for me is that the observed stress (changes in pigmentation, growth, ROS, permeability, etc.) in Se:ΔA+T is due to the removal of carbon from membrane biogenesis and synthesis of macromolecules for the production of free fatty acid. This explanation is supported by a recent finding that carbon metabolism is up regulated in free fatty acid overproducing cells, indicating carbon starvation (Ruffing 2013a). It would be interesting to get a deeper understanding of the carbon flow in *S. elongatus* PCC 7942 in order to further optimize the production of biodiesel precursors.

In **Chapter 4** we described how we aimed to increase the carbon flow towards malonyl-CoA and therefore fatty acid synthesis by overexpressing the native acetyl-CoA carboxylase (ACCase) of *S. elongatus* PCC 7942. This resulted in an increase in both the relative and absolute amount of extracellular free fatty acid production (uninduced; **Chapter 4**) and restoration of the level of photosynthetic pigmentation to that of *S. elongatus* PCC 7942 (Fig 2). However, strain Se:ACCase also showed an increased lag phase and a reduction in the specific growth rate (**Chapter 4**). These results are similar to those obtained in another study where an acetyl-CoA carboxylase was expressed in *S. elongatus* PCC 7942 from *Chlamydomonas reinhardtii* (Ruffing 2013b). Interestingly, one of the subunits of our ACCase construct, the biotin carboxyl carrier protein (Synpcc7942_2564), was also up regulated in cells of *S. elongatus* PCC 7942 which overproduce fatty acids (Ruffing 2013a). Expressing only the ACCase, might lead to a misbalance in the amount of acetyl-CoA and malonyl-CoA and, therefore, requires additional genetic engineering or alternative cultivation conditions (e.g. lower temperature, higher cellular density) to improve *S. elongatus* PCC 7942 for enhanced biodiesel production. Besides the suggestion to enhance the transcription and translation of the ACCase construct (**Chapter 4**), we could think of down regulating the phosphoenolpyruvate synthase (Synpcc7942_0781) (**Fig 2 of Chapter 2**). This enzyme can convert pyruvate to phosphoenolpyruvate and by reducing is abundance we can increase the carbon flow towards acetyl-CoA and membrane biosynthesis (Ruffing 2013a; Song et al. 2008). Moreover, overexpressing genes involved in carbon

metabolism such as carbonate dehydratase (Synpcc7942_1424) or the carbon dioxide concentrating mechanism protein (Synpcc7942_1423), should further improve the flow of carbon to primary cellular processes (Ruffing 2013a).

Another challenge is optimizing the efficient export of the produced free fatty acids out of the cell. In **Chapter 3** we proposed that reducing the polarity of the cell membrane, or introducing fatty acid exporters might enhance the secretion of fatty acid. We also showed that unsaturated fatty acids tend to accumulate outside the cell while saturated fatty acids tend to accumulate inside (**Chapter 3** and **4**) (Kaczmarzyk 2008; Kaczmarzyk and Fulda 2010). This observation suggests that the introduction of acyl-ACP desaturases might be exploited to optimize the export of free fatty acids. Also, because the *Δaas* mutant is not sensitive to unsaturated extracellular fatty acids, these fatty acids can be safely accumulated outside the cell (von Berlepsch et al. 2012) .

Inverse metabolic approach for improving the production of biofuel precursors

The rational approach (**Chapter 3** and **4**) is an effective way to improve the production of biofuel precursor, however this approach is limited by current knowledge about the cellular machinery and its metabolites. The inverse metabolic approach or random mutagenesis approach is a complementary method, in which one screens mutants for a desired phenotype and determines the genotype that caused it. In **Chapter 5**, we show the effectiveness of this approach with the *era* overexpression strain Se:era. This strain contains a significantly higher amount of fatty acids compared to *S. elongatus* PCC 7942 (79.59±1.85 µg/mg dry cell weight Se:era). Moreover, it has a similar amount of intracellular fatty acids as the rationally engineered double mutant Se:ΔA+T (79.04±2.11 µg/mg dry cell weight) (**Chapter 3** and **4**). Besides overexpressing the *era* gene in strain Se:ΔA+T, it would be interesting to apply the inverse metabolic approach of **Chapter 5** to strain Se:ACCase and Se:ΔA+T directly to isolate mutants with either enhance cell physiology to cope with free fatty acid production or increased free fatty acid secretion.

For mutant Se:era, it is easy to demonstrate that the phenotypic changes observed are caused by the introduced *era* gene by comparing it to strain *S. elongatus* PCC 7942. The comparison between Se:Δera, Se:Δera+era and PCC 7942 is however not that straight forward. The complementation by

introducing an additional *era* gene did not fully restore the wild type phenotype. In **Chapter 5**, we suggest several hypotheses why there is no full complementation, such as (i) the expression of the introduced *era* gene, (ii) possibility that one or several downstream genes are partly responsible for the observed phenotype and/or (iii) the presence of the truncated *era* gene. To discuss these hypotheses further it is important to understand what genes are downstream of the *era* gene and what other phenotypes do we expect because of these genes.

As mentioned in **Chapter 5**, transposon TN5-1063a (Wolk 1991) is inserted into the 3 prime of the *era* gene (Synpcc7942_0160). The *era* gene is the first gene of an operon containing a total of 8 genes. These include 5 genes encoding hypothetical proteins and 3 that encode a tRNA-Met, an Iojap-like protein and the GTP-binding protein Era itself (Table 1). Bretaudeau and colleagues (2013) recently elucidated the function of gene Synpcc7942_0161 and showed that this gene is involved in the assembly of the phycobilisomes (light-harvesting system used by cyanobacteria) (Bretaudeau et al. 2013). In *Synechococcus* sp. PCC 7002 disruption of this gene caused a slower growth rate, reduced phycobilin protein level and impaired assembly of the phycobilisomes (Shen et al. 2008).

Table 1 The original annotation of the eight genes of the era operon

Gene identifier	Original annotation	Comments	References
Synpcc7942_0160	GTP-binding protein Era	GTP-binding protein Era	(Ahnn et al. 1986)
Synpcc7942_0161	hypothetical protein	phycobilin lyase, CpcU subunit, involved in the assembly of the phycobilisomes	(Bretaudeau et al. 2013)
Synpcc7942_0162	hypothetical protein	hypothetical protein, has very few orthologs	
Synpcc7942_0163	hypothetical protein	contains a methyltransferase domain (PFAM PF08242)	(Finn et al. 2014)
Synpcc7942_0164	Iojap-like protein	ribosome-associated protein, possibly involved in impairing the small and large ribosome from joining in response to nutrient stress	(Häuser et al. 2012; Jiang et al. 2007)
Synpcc7942_0165	hypothetical protein	DUF1230, species distribution indicates a possible role in photosynthesis	(Batemann et al. 2010)
Synpcc7942_0166	hypothetical protein	L-asparaginase II, involved in asparagine catabolism and possibly the final steps of protein and cyanophycin (nitrogen storage compound) degradation	(Hejazi et al. 2002)
Synpcc7942_R0028	tRNA-Met	Involved in Aminoacyl-tRNA biosynthesis	

Based on these reports, we hypothesize that if the insertion of the transposon causes a disruption of the downstream genes, mutant Se:Δera would show a reduction in its phycobilin content compared to *S. elongatus* PCC 7942. This is exactly what we observe (Fig 3). Interestingly, complementation with the *era* gene expressed from neutral site 3 restored the phycobilin level to

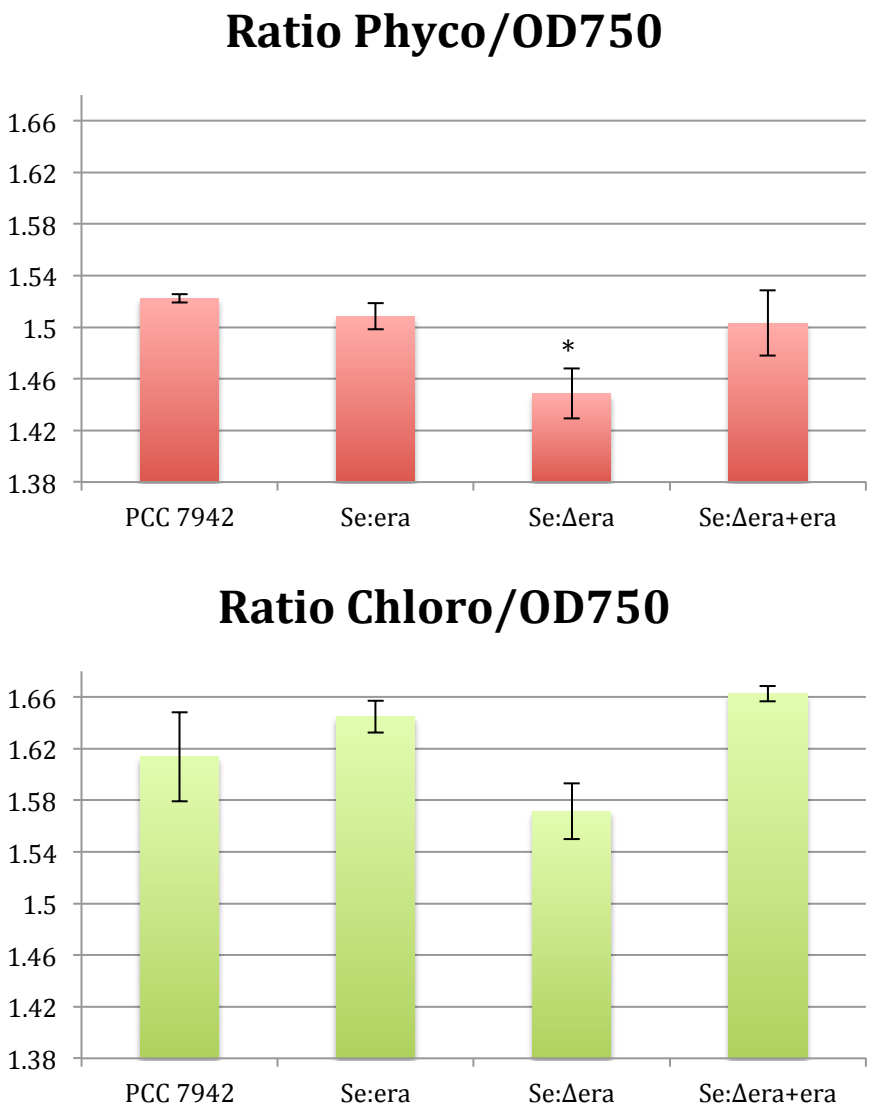

Figure 3 The effect of the genetic engineering on the amount of phycobilin (top) and chlorophyll a (bottom) of wild type *S. elongatus* PCC 7942, strain Se:era, Se:Δera and Se:Δera+era at 120 hours of cultivation. Those indicated with an asterisk (*) are significantly different ($p < 0.05$) from wild type PCC 7942. Error bars represent the standard deviation of four replicates.

that of *S. elongatus* PCC 7942. This result suggests that the transposon insertion into the *era* operon does not have a severe effect on the expression of downstream genes. It would be interesting to perform RNA sequencing on the era mutants described in **Chapter 5** and determine the direct effect of *era* overexpression on the transcription of its target genes as well as the effects of the transposon insertion on the transcription of the *era* operon.

Another interesting phenotypic trait of strain Se:Δera is its high sedimentation rate (Fig 4). At 96 hours, the sedimentation rate of strain Se:Δera was 18% higher than that of *S. elongatus* PCC 7942 and this difference increased to 27% at a 120 hours. A higher sedimentation rate might be beneficial, since it allows easier harvesting of cellular biomass without the need for industrial strength centrifuges. It would be interesting to determine whether the sedimentation rate is determined directly by the size of the cells (**Chapter 5**), or whether there are other unknown factors involved.

Phenotypic and genotypic stability of transposon mutants is an indication for the absence of stress and makes subsequent rational engineering easier because it makes it less likely that the genetic modification will be lost due to negative selection. Out of the initial transposon mutants, three strains

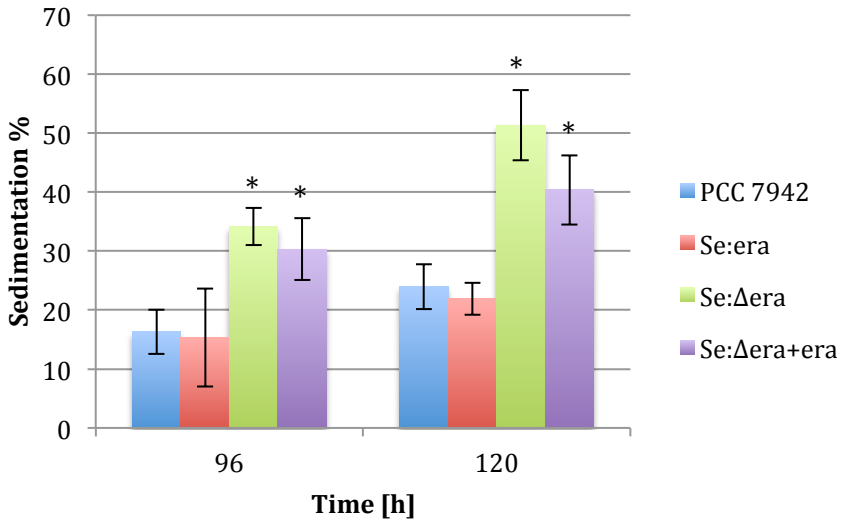

Figure 4 Sedimentation percentage after 96 and 120 hours of strain *S. elongatus* PCC 7942, Se:era, Se:Δera and Se:Δera+era. The Sedimentation percentages were determined by monitoring the reduction in OD750 in a 24-hour period. Those indicated with an asterisk (*) are significantly different ($p < 0.05$) from wild type PCC 7942. Error bars represent the standard deviation of four replicates.

were phenotypically stable for at least 3 months. Only in one mutant, 2A01, the transposon remained in exactly the same location within that period (**Chapter 5**). In all the other mutants the transposon jumped into other locations of the genome and this frequently caused a loss of the original phenotype. A possible explanation for this remarkable stability is that the transposon of 2A01 is trapped behind or inside an essential gene/operon and jumping away would be lethal. If this hypothesis is true, it could indicate that essential genes are natural transposon traps. It would also open a door for a new method to isolate essential genes instead of saturated mutagenesis (Moule et al. 2014). This novel method would involve performing transposon mutagenesis, isolating mutants and applying selection (e.g. resistance to sodium chlorate) to isolate mutants where the transposon jumped. If the transposon does not jump it can be near an essential gene.

In this thesis we successfully constructed several free fatty acid secreting strains with the best one synthesizing 25% more intracellular fatty and secreting 286% more free fatty acids compared to *S. elongatus* PCC 7942. We also revealed a gene, which is able to raise the intracellular fatty acid content by another 8-14%. This gene, encoding the GTP-binding protein Era, is present in both bacteria and eukaryotes and may link translational capacity with cell physiology, including stress, energy metabolism and cell cycle (Britton 2009; Verstraeten et al. 2011). The *era* gene is part of an operon and genes, which are grouped into operons together in multiple species, likely have similar functions even if transcribed individually in other species (Price et al. 2006). This suggests that Synpcc7942_0161, Synpcc7942_0164, Synpcc7942_0165, Synpcc7942_0166 and to a lesser extend Synpcc7942_0163 could also be promising novel targets for improved biodiesel production.

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Chapter 7

Summary

In **Chapter 1** I describe the main aims of my Ph.D. thesis and give a brief introduction. Due to concerns about global climate change, diminishing supplies of petroleum and political instabilities in oil producing countries, there is a need to develop a clean sustainable alternative. The two main alternative fuels are bioethanol and biodiesel. Production of these liquid biofuels using algae or cyanobacteria is a fast growing promising new technological development. Cyanobacteria have several advantageous traits for the production of these biofuels such as simple nutrient requirements, ability to produce a wide range of biofuel precursors (e.g. lipids, hydrocarbons) and they do not compete for arable land or fresh water. After cultivation, the lipids can be extracted and converted into biodiesel, while the remaining fraction of protein and carbohydrates can be converted to bioethanol.

In **Chapter 2** we give a detailed overview about the different types of bioenergy that can be produced using cyanobacteria including various organic (biodiesel, bioethanol and methane) and inorganic biofuels (hydrogen and electricity). Moreover, we describe the biochemical pathways involved in the production of these biofuels such as nitrogen fixation (hydrogen and methane), lipid (biodiesel) and carbohydrate metabolism (bioethanol). Furthermore, several possibilities to improve the production of these compounds are highlighted. Many of these strategies are still in the early stage of development and their energy yield is not sufficient enough to allow large-scale industrial production levels. To reach a level where commercial scale production is feasible, the metabolic networks need to be optimized further using rational and inverse metabolic engineering.

In **Chapter 3** I describe the initial rational metabolic engineering of *S. elongatus* PCC 7942 for the production of biodiesel precursors. Three strains were constructed, strain Se:T (expressing a cytosolic thioesterase from *E. coli* Dh5α), strain Se:ΔA (where the acyl-ACP synthetase was disrupted) and Se:ΔA+T (containing the modifications present in both Se:T and Se:ΔA). The soluble thioesterase, is responsible for the release of the fatty acid from its acyl carrier protein (ACP), and the acyl-ACP synthetase can reattach free fatty acids to an

ACP molecule. Strains Se:T, Se:ΔA and Se:ΔA+T were subsequently characterized with regard to their intracellular and extracellular fatty acid profile, intracellular hydrocarbon profile and effect on growth. Out of all three engineered strains only the strain Se:ΔA+T had a significant increase in the total amount of intracellular and extracellular free fatty acids. This is the first study, which provides a comprehensive picture of early effects caused by the introduced mutations on the fatty acid and hydrocarbon profile of PCC 7942.

In **Chapter 4** I describe further rational optimization of free fatty acid production by overexpressing the native acetyl-CoA carboxylase from *S. elongatus* PCC 7942. Introduction of the acetyl-Coa carboxylase in wild type *S. elongatus* PCC 7942 alone did not cause an increase in intracellular fatty acids. However, in strain Se:ΔA+T, engineered for fatty acid secretion, there was a significant increase in the amount of extracellular fatty acids (absolute and per dry cell weight basis). Inducing expression of the acetyl-Coa carboxylase and thioesterase with the addition of Isopropyl β-D-1-thiogalactopyranoside caused a further increase in the amount of secreted fatty acids per mg dry cell weight.

In **Chapter 5** I describe the initial inverse metabolic engineering of *S. elongatus* PCC 7942 for the production of biodiesel precursors. A transposon library was generated and subsequently screened for desirable traits. A single mutant, 2A01, was selected based on its elongated cell morphology, enhanced staining with the lipophilic dye Nile red and phenotypic and genotypic stability. The transposon of strain 2A01 is inserted in the 3 prime region of the era gene (Synpcc7942_0160; encoding the GTP-binding protein Era). Overexpression of the era gene showed that the GTP-binding protein Era is important for fatty acid metabolism as well as determination of the cell size in PCC 7942. Moreover, complementation of strain Se:Δera with era suggests that the GTP-binding protein Era is also involved in hydrocarbon metabolism. Our results suggest that the GTP-binding protein Era is a promising target to further rational engineering to improve the production of biofuel precursors.

In **Chapter 6** I discuss some of the findings reported in this thesis and included some unpublished results. For example, I indicate changes in the amount of phycobilin and chlorophyll a in *S. elongatus* PCC 7942 strains engineered for free fatty acid production and suggest a link between free fatty acid production and reduced ability to maintain membrane homeostasis. I also presented

several additional phenotypic traits of strain Se:Δera such as an increased sedimentation rate and decreased phycobilin content.

Samenvatting

In **hoofdstuk 1** beschrijf ik de belangrijkste doelstellingen van mijn proefschrift en geef ik een korte introductie. Als gevolg van de bezorgdheid over de wereldwijde klimaatverandering, afnemende voorraden van aardolie en de politieke instabiliteit in olieproducerende landen, is er behoefte om een schoon duurzaam alternatief te ontwikkelen. De twee belangrijkste alternatieve brandstoffen zijn bio-ethanol en biodiesel. De productie van deze vloeibare biobrandstoffen met algen of cyanobacteriën is een snel groeiende veelbelovende nieuwe technologische ontwikkeling. Cyanobacteriën hebben verschillende gunstige eigenschappen voor de productie van deze biobrandstoffen zoals eenvoudige behoeften aan nutriënten, de mogelijkheid om een breed scala van biobrandstof voorlopers te produceren (bijvoorbeeld lipiden en koolwaterstoffen) en ze concurreren niet voor landbouwgrond of zoet water. Na het kweken kunnen de lipiden geëxtraheerd worden en omgezet in biodiesel, terwijl de resterende fractie van eiwitten en koolhydraten kunnen worden omgezet naar bio-ethanol.

In **hoofdstuk 2** geven we een gedetailleerd overzicht over de verschillende soorten van bio-energie die kunnen worden geproduceerd met behulp van cyanobacteriën, waaronder diverse organische (biodiesel, bio-ethanol en methaan) en anorganische biobrandstoffen (waterstof en elektriciteit). Verder beschrijven we de biochemische routes betrokken bij de productie van brandstoffen zoals stikstoffixatie (waterstof en methaan), vetten (biodiesel) en koolhydraten (bio-ethanol). Bovendien zijn diverse mogelijkheden om verbetering van de productie van deze verbindingen gemarkeerd. Veel van deze strategieën zijn nog in een vroeg stadium van ontwikkeling en de energieopbrengst is niet voldoende voor grootschalige industriële productie niveaus mogelijk te maken. Om een niveau te bereiken waar de commerciële schaal haalbaar is, moeten de metabole netwerken verder worden geoptimaliseerd met behulp van rationele en invers metabolische engineering.

In **hoofdstuk 3** beschrijf ik de eerste rationele metabolische engineering van *S. elongatus* PCC 7942 voor de produktie van biodiesel precursoren. Drie

stammen werden geconstrueerd, stam Se:T (expressie van een cytosolische thioesterase uit *E. coli* DH5α), stam Se:ΔA (waarbij de acyl-ACP synthetase werd verstoord) en Se:ΔA+T (met de wijzigingen aanwezig in zowel Se:T als Se:ΔA). De oplosbare thioesterase, is verantwoordelijk voor de vrijmaken van het vetzuur van haar acyldragereiwit (ACP), en de acyl-ACP synthetase kan weer vrije vetzuren vast maken aan een ACP-molecuul. Stammen Se:T, Se:ΔA en Se:ΔA+T werden vervolgens gekarakteriseerd met betrekking tot hun intracellulaire en extracellulaire vetzuurprofiel, intracellulaire koolwaterstof profiel en effect op de groei. Van alle drie gemanipuleerde stammen heeft alleen stam Se:ΔA+T een significante verhoging van de totale hoeveelheid intracellulaire en extracellulaire vrije vetzuren. Dit is de eerste studie, die een volledig beeld van vroege effecten, veroorzaakt door de geïntroduceerde mutaties op het vetzuur en koolwaterstof profiel van PCC 7942 biedt.

In **hoofdstuk 4** beschrijf ik verdere rationele optimalisering van vrije vetzuur productie door overexpressie van de natieve acetyl-CoA carboxylase van *S. elongatus* PCC 7942. Introductie van de acetyl-CoA carboxylase in wild type *S. elongatus* PCC 7942 alleen leidde niet tot een verhoging van de intracellulaire vetzuren. Echter, in stam Se:ΔA+T, ontworpen voor vetzuur secretie, was er een significante toename van de hoeveelheid extracellulaire vetzuren (absoluut en per droog gewicht). Het induceren van expressie van de acetyl-CoA carboxylase en thioesterase met de toevoeging van isopropyl-β-D-1-thiogalactopyranoside veroorzaakte een verdere toename van de hoeveelheid uitgescheiden vetzuren per mg droog gewicht.

In **hoofdstuk 5** beschrijf ik de eerste invers metabolische engineering van *S. elongatus* PCC 7942 voor de produktie van biodiesel precursoren. Een transposon bibliotheek werd gegenereerd en vervolgens gescreend op wenselijke eigenschappen. Een enkele mutant, 2A01, werd geselecteerd gebaseerd op zijn langwerpige celmorfologie, verbeterde kleuring met de lipofiele kleurstof Nile red en fenotypische en genotypische stabiliteit. Het transposon van stam 2A01 is gelokaliseerd in het 3 accent gebied van het era gen (Synpcc7942_0160, codeert het GTP bindende eiwit Era). Overexpressie van het era gen toonde dat het GTP-bindend eiwit Era belangrijk is voor vetzuurmetabolisme en het bepalen van de celgrootte in PCC 7942. Bovendien suggereert complementatie van stam Se:Δera met era dat het GTP-bindend eiwit Era ook betrokken is bij het metabolisme van koolwaterstof. Onze

resultaten suggereren dat het GTP-bindende eiwit Era een veelbelovend doelwit is voor verdere rationele engineering om de productie van precursoren van biobrandstoffen te verbeteren.

In **hoofdstuk 6** bediscussieer ik een aantal van de bevindingen beschreven in dit proefschrift en beschrijf ik een aantal ongepubliceerde resultaten. Ik laat bijvoorbeeld zien dat er veranderingen in de hoeveelheid van phycobilin en chlorofyl a in *S. elongatus* PCC 7942 stammen ontworpen voor vrije vetzuren productie zijn en suggereer dat er een verband is tussen vrije vetzuren productie en een verminderd vermogen om membraan homeostase the behouden. Ik presenteerde ook verschillende aanvullende fenotypische kenmerken van stam Se:Δera zoals een verhoogde bezinkingssnelheid en een vermindering van de phycobilin inhoud.

Supplementary data

Chapter 3

Supplementary Table 1 Averages [µg/mg DCW] and Stdev of intracellular fatty acids determined from 4 biological replicates

	PCC 7942		Se:T		Se:ΔA		Se:ΔA+T	
Fatty Acid	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
C9:0	0,05	0,01	0,05	0,00	0,05	0,00	0,04	0,01
C12:0	0,00	0,01	[a]nd	nd	0,01	0,01	0,05	0,03
C14:0	0,94	0,13	1,09	0,15	1,21	0,07	2,75	0,74
C14:1	1,52	0,14	2,11	0,24	1,73	0,30	3,10	0,65
C15:0	0,05	0,02	0,08	0,03	0,08	0,02	0,10	0,03
C15:1	0,04	0,01	0,07	0,02	0,05	0,04	0,06	0,03
C16:0	33,49	0,59	32,76	0,38	34,04	0,82	37,67	0,94
C16:1	27,20	0,49	27,58	0,66	26,01	1,33	30,08	1,57
C17:0	0,19	0,04	0,18	0,05	0,21	0,03	0,21	0,03
C17:1	0,33	0,02	0,30	0,05	0,31	0,01	0,26	0,03
C18:0	0,81	0,10	0,62	0,13	1,30	0,42	1,71	0,14
C18:1	4,50	0,16	3,46	0,08	4,30	0,47	3,00	0,68
Total FA	69,12	1,11	68,31	1,17	69,29	1,92	79,04	2,11

[a]not detected

Supplementary Table 2 Averages [µg/mg DCW] and Stdev of Fatty acids found in the supernatant determined from 4 biological replicates

	PCC 7942		Se:T		Se:ΔA		Se:ΔA+T	
Fatty Acid	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
C14:0	0,78	0,40	0,61	0,46	1,32	0,94	0,94	0,31
C14:1	0,31	0,10	0,44	0,13	0,62	0,55	0,95	0,08
C16:0	0,55	0,11	0,59	0,03	0,72	0,12	1,00	0,10
C16:1	1,59	0,17	1,36	0,08	2,77	0,28	4,89	0,16
C18:0	0,13	0,04	0,08	0,05	0,15	0,02	0,14	0,03
C18:1	0,00	0,00	0,00	0,00	0,12	0,04	0,10	0,01
Total FA	3,36	0,71	3,08	0,54	5,71	1,85	8,02	0,32

Supplementary Table 3 Averages relative to C10:0 IS and Stdev of intracellular hydrocarbons determined from 4 biological replicates

	PCC 7942		Se:T		Se:ΔA		Se:ΔA+T	
hydrocarbon	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
n-Pentadecane	0,010	0,001	0,010	0,001	0,008	0,001	0,012	0,000
n-Hexadecane	0,005	0,001	0,004	0,000	0,005	0,001	0,004	0,001
n-Tetradecane	0,001	0,001	0,001	0,001	0,000	0,001	0,001	0,001
n-Heptadecane	0,027	0,002	0,021	0,002	0,026	0,001	0,028	0,001
8-Heptadecene	0,003	0,000	0,003	0,000	0,000	0,001	0,001	0,000
n-Octadecane	0,001	0,001	0,001	0,000	0,001	0,000	0,001	0,000
1-Octadecene	[a]tr	-	[b]nd	-	tr	-	nd	-

[a]trace amount

[b]not detected

Chapter 4

Supplementary Table 1 Averages [µg/mg DCW] and Stdev of Fatty acids found in the supernatant determined from 4 biological replicates

	Se:ΔA+T		Se:ACCase		Se:ΔA+T+		Se:ACCase+	
Fatty Acid	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
C14:0	0,94	0,31	1,50	0,67	1,32	0,77	2,11	0,40
C14:1	0,95	0,08	1,50	0,32	1,35	0,31	1,88	0,36
C16:0	1,00	0,10	1,51	0,14	1,18	0,17	1,80	0,21
C16:1	4,89	0,16	5,37	0,52	5,97	0,83	6,82	0,97
C18:0	0,14	0,03	0,22	0,02	0,17	0,04	0,25	0,03
C18:1	0,10	0,01	0,07	0,08	0,06	0,07	0,12	0,08
Total FA	8,02	0,32	10,16	1,02	10,04	2,00	12,98	1,68

Supplementary Table 2 Averages [µg/mg DCW] and Stdev of intracellular fatty acids determined from 4 biological replicates

	Se:ΔA+T		Se:ACCase		Se:ΔA+T+		Se:ACCase+	
Fatty Acid	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
C9:0	0,04	0,01	0,03	0,01	0,03	0,01	0,03	0,02
C12:0	0,05	0,03	0,03	0,01	0,06	0,03	0,04	0,02
C14:0	2,75	0,74	2,51	0,17	3,07	0,88	2,94	0,37
C14:1	3,10	0,65	2,37	0,41	3,50	0,71	2,56	0,53
C15:0	0,10	0,03	0,06	0,01	0,11	0,03	0,08	0,04
C15:1	0,06	0,03	[a]tr	0,02	0,08	0,02	[a]tr	0,05
C16:0	37,67	0,94	38,12	0,86	41,83	1,79	42,99	0,69
C16:1	30,08	1,57	28,92	1,64	33,80	0,75	32,86	1,71
C17:0	0,21	0,03	0,18	0,02	0,24	0,06	0,19	0,13
C17:1	0,26	0,03	0,20	0,05	0,31	0,06	0,19	0,11
C18:0	1,71	0,14	1,31	0,29	1,67	0,40	1,65	0,15
C18:1	3,00	0,68	2,49	0,39	2,72	0,97	2,93	0,27
Total FA	79,04	2,11	76,20	2,08	87,41	3,25	86,45	1,57

[a]trace amount

Supplementary Table 3 Averages relative to C10:0 IS and Stdev of intracellular hydrocarbons determined from 4 biological replicates

	Se:ΔA+T		Se:ACCase		Se:ΔA+T+		Se:ACCase+	
hydrocarbon	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
n-Pentadecane	0,012	0,000	0,012	0,001	0,014	0,002	0,010	0,001
n-Hexadecane	0,004	0,001	0,004	0,001	0,003	0,001	0,003	0,000
n-Tetradecane	0,001	0,001	[b]nd	-	[a]tr	-	[b]nd	-
n-Heptadecane	0,028	0,001	0,024	0,002	0,027	0,002	0,025	0,002
8-Heptadecene	0,001	0,000	0,001	0,000	0,001	0,000	0,001	0,000
n-Octadecane	0,001	0,000	[b]nd	-	[a]tr	-	[a]tr	-
1-Octadecene	[b]nd	-	[b]nd	-	[b]nd	-	[a]tr	-

[a]trace amount

[b]not detected

Chapter 5

Supplementary Table 1 Averages [µg/mg DCW] and Stdev of Fatty acids found in the supernatant determined from 4 biological replicates

	PCC 7942		Se:era		Se:Δera		Se:Δera+era	
Fatty acids	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
C14:0	0,57	0,04	0,64	0,02	1,50	0,04	0,75	0,03
C14:1	1,64	0,24	1,98	0,07	3,17	0,30	1,35	0,10
C15:0	[a]tr	-	[b]nd	-	[a]tr	-	[b]nd	-
C16:0	35,67	1,18	38,14	1,03	35,08	0,46	36,78	0,47
C16:1	30,08	1,24	31,58	0,66	31,02	0,40	28,76	0,75
C17:0	0,06	0,02	0,05	0,02	0,07	0,02	0,06	0,01
C17:1	0,20	0,02	0,26	0,01	0,22	0,02	0,23	0,01
C18:0	0,94	0,21	0,95	0,04	0,57	0,06	1,16	0,15
C18:1	4,47	0,29	6,00	0,04	3,54	0,17	4,69	0,16
Total FA	73,64	2,73	79,59	1,85	75,21	1,06	73,78	1,20

[a]trace amount

[b]not detected

Supplementary Table 2 Averages relative to C10:0 IS and Stdev of intracellular hydrocarbons determined from 4 biological replicates

	PCC 7942		Se:era		Se:Δera		Se:Δera+era	
hydrocarbon	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
Pentadecane	0,014	0,001	0,013	0,001	0,017	0,002	0,013	0,001
Hexadecane	0,005	0,000	0,004	0,001	0,004	0,000	0,004	0,000
Heptadecane	0,041	0,001	0,041	0,001	0,020	0,001	0,038	0,002
8-Heptadecene	0,005	0,000	0,004	0,000	0,008	0,001	0,004	0,001

Curriculum vitae

Gijsbert Petrus Voshol (Gerben) was born on the 2nd of Februari 1981 in Vlaardingen, (South-Holland, The Netherlands). After finishing his high school in 1999, he moved to Leiden where he started his biology study at the Institute of Biology Leiden in 2001. During his study he was a research analist and performed two internships. First internship was performed in the plant ecology group together with Dr. K. Vrieling about the genetics of invasive plant species. The other internship was done under the supervision of Dr. F. Constantinescu and Dr. G.V. Bloemberg, on plant beneficial bacteria for the biocontrol of tomato foot and root rot. In 2008 he obtained his Master degree in molecular microbiology and biotechnology from the University Leiden. After that he got the opportunity to start his PhD-research about Cyanobacteria within the former group of fungal genetics under the supervision of Prof. Dr. C.A.M.J.J. van den Hondel and Prof. Dr. V. Meyer. The research described in this thesis took place as part of the project “Groene diesel uit blauwwieren” and was financially supported by the Dutch Ministry of Economic Affairs (EOSLT 07039)

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