Biodiesel production using blue-green cyanobacterium Synechococcus elongatus PCC 7942

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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

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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

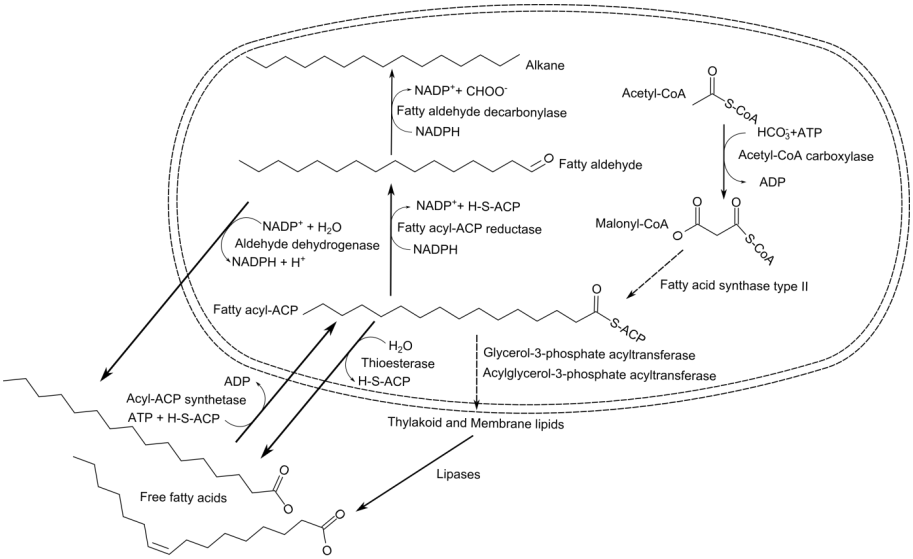

Fig 1 Overview of the lipid metabolism of PCC 7942 and its mutants. Fatty acid biosynthesis starts with the conversion of acetyl-CoA to malonyl-CoA catalyzed by the acetyl-CoA carboxylase (ACCase). The malonyl group is transferred to an acyl carrier protein (ACP) and subsequently elongated in a cyclic 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.

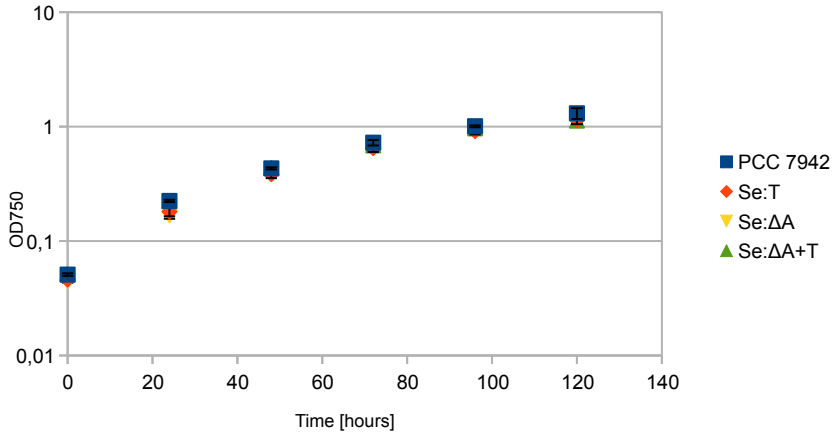

Fig 2 Cellular growth of wild type PCC 7942, strain Se:T, Se:ΔA and Se:ΔA+T. Growth was measured at 24 hour intervals using the optical density at 750 nm. Error bars represent the standard deviation of four replicates.

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)

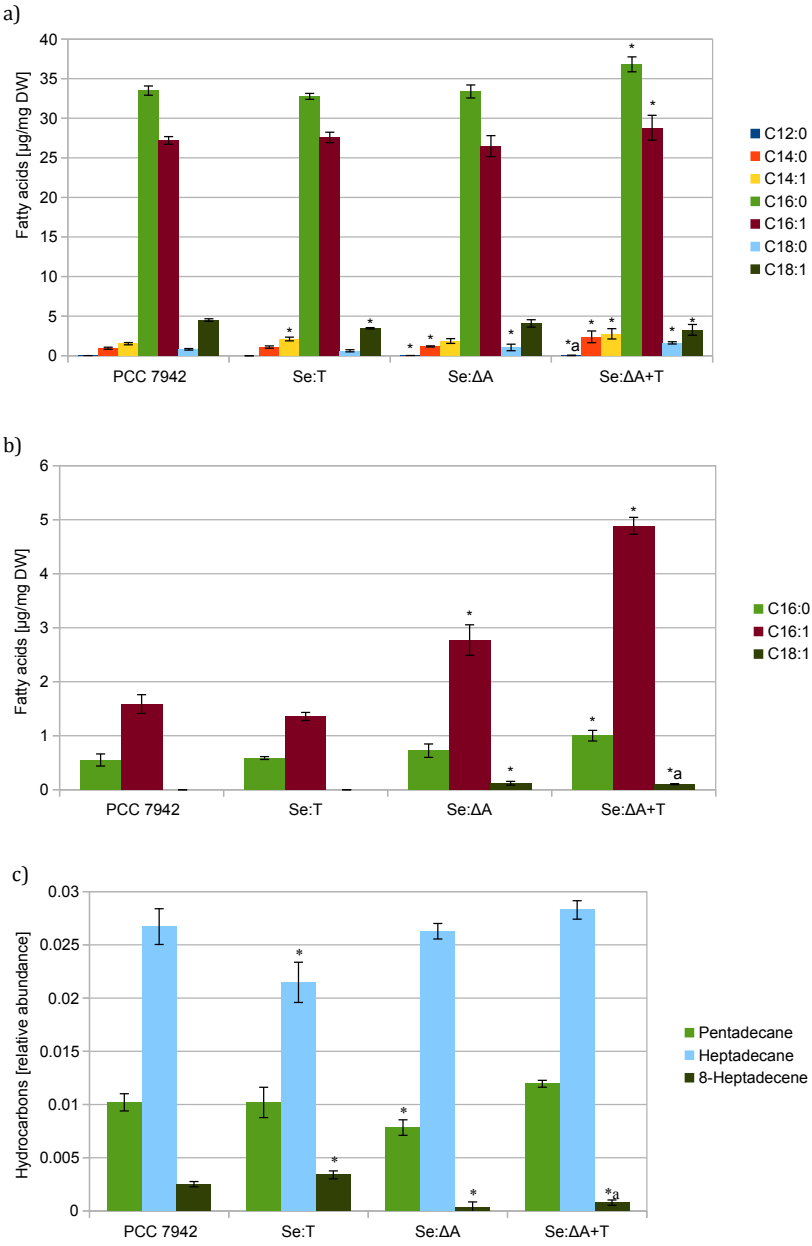

Fig 3 Quantitative measurement of fatty acids and hydrocarbons in PCC 7942, strain Se:T, Se:ΔA and Se:ΔA+T a) intracellular fatty acids, b) secreted fatty acids and c) intracellular hydrocarbons. Only those compounds are shown which are differentially produced among strains. Metabolites indicated with 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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