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Tapio Lehtinen

Solar Fuels and Chemicals

Engineering Bacterial Platform for the Production of Long-Chain Hydrocarbons from Carbon Dioxide and Electricity

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Abstract

Microbial synthesis of long-chain hydrocarbons is considered a promising method for the production of drop-in replacements for the currently utilized fossil fuels and chemicals. In a sustainable future, the carbon and energy required for the synthesis of these compounds should be ultimately derived from carbon dioxide (CO_2) and the sun. Additionally, the produced compounds should be of similar or higher quality than the fossil counterparts they aim to replace, enabling their utilization directly in the existing processes and infrastructure. Solar energy can be efficiently captured by man-made devices, and converted to electricity. Some microbes are capable of utilizing the electrical energy for the reduction of CO_2 into organic molecules, while other microbes hold the potential for the specific production of long-chain hydrocarbons. However, these two processes are difficult to achieve efficiently in any single organism, owing to their contrasting requirements for the host cells' metabolism.

In this study, a two-stage bacterial platform for the production of long-chain hydrocarbons from carbon dioxide was developed. In the first stage, acetogenic bacteria produce acetate from carbon dioxide and electricity as the sole sources of carbon and energy. In the second stage, the acetate is converted to long-chain hydrocarbons by a second bacterium, *Acinetobacter baylyi*. The platform is modular in nature, and different acetate-producing and -consuming processes can be combined. The final product can be determined and the production enhanced by genetically engineering *A. baylyi*.

Fatty aldehydes are specific intermediates in the biosynthesis of wax esters and alkanes. Fatty acyl-CoA reductases (FARs) are key enzymes in the production of fatty aldehydes. The natural fatty aldehyde production of *A. baylyi* was improved by overexpressing either native or heterologous FARs. The overexpression led to increased fatty aldehyde production, and enabled more efficient wax ester or alkane production, depending on the downstream pathway utilized. For the wax ester production, the native wax ester production pathway of *A. baylyi*, combined with the overexpression of the native FAR or a previously uncharacterized, putative reductase from *Nevskia ramosa*, was employed. The overexpression of either of these reductases led to increased wax ester production, resulting in the highest wax ester titers reported thus far for microbial production systems. *A. baylyi* does not naturally produce alkanes, but the expression of a two-step cyanobacterial pathway consisting of aldehyde- and alkane producing enzymes led to alkane synthesis. Furthermore, the natural ability of *A. baylyi* for alkane degradation was removed, thereby converting the natural alkane degrader into alkane producer.

The development of efficient production strains by metabolic engineering is restricted by the lack of sensitive and specific measurement methods for the target compounds. Intracellular biosensors may offer convenient means for high-throughput screening and optimization of the production strains. In order to study heterologous alkane biosynthesis in *A. baylyi*, an *in vivo* alkane biosensor working on two levels was developed. First, a gene encoding the green fluorescent protein (GFP) was placed under an alkane-inducible promoter, linking the alkane production to the expression of GFP. The second level is provided by a constantly expressed bacterial luciferase LuxAB, which utilizes the fatty aldehyde intermediate as a substrate in a reaction that produces visible light. Thus, the production of fatty aldehydes can be monitored with the luminescence signal. The two-level configuration of the sensor enabled the screening of different alkane-producing pathways, and the optimization of the expression levels of the pathway enzymes in order to improve the alkane biosynthesis in *A. baylyi*.

In summary, the production of wax esters from CO_2 and electricity, as well as long-chain alkanes from CO_2 and H_2 were demonstrated with the two-stage bacterial platform. In addition, heterologous alkane biosynthesis was demonstrated in *A. baylyi*, and the natural wax ester production of *A. baylyi* was improved by metabolic engineering. Even though the platform holds potential for the sustainable production of drop-in fuels and chemicals, significant improvements in the production efficiency are required before the potential can be realized.

Tiivistelmä

Mikrobien avulla toteutettavaa pitkäketjuisten hiilivety-yhdisteiden valmistusta pidetään lupaavana menetelmänä fossiilisten polttoaineiden ja kemikaalien korvaamiselle. Ollakseen kestävällä pohjalla, tämän menetelmän pitäisi saada käyttämänsä hiili hiilidioksidista ja energia auringosta. Lisäksi valmistettavien yhdisteiden tulisi vastata ominaisuuksiltaan niitä nykyisin käytössä olevia fossiilisia kemikaaleja, jotka on tarkoitus korvata, täten soveltuen käytettäväksi nykyisissä prosesseissa ja laitteissa. Auringon energiaa voidaan kerätä ja muuttaa sähköenergiaksi tehokkaasti keinotekoisilla laitteilla. Eräät mikrobit kykenevät käyttämään sähköä energianlähteenään ja pelkistämään hiilidioksidia orgaanisiksi yhdisteiksi. Toiset mikrobit puolestaan kykenevät tuottamaan tiettyjä pitkäketjuisia hiilivetyjä. Kuitenkaan näitä kahta prosessia ei voida tehokkaasti yhdistää samaan soluun, sillä ne edellyttävät erilaisia ominaisuuksia isäntäsolun aineenvaihdunnalta.

Tässä tutkimuksessa kehitettiin kaksivaiheinen bakteereihin perustuva tuottosysteemi, jota voidaan käyttää pitkäketjuisten hiilivetyjen tuottamiseen käyttäen hiilidioksidia ja sähköä lähtöaineina. Ensimmäisessä vaiheessa asetogeeniset bakteerit tuottavat asetaattia sähköstä ja hiilidioksidista. Seuraavassa vaiheessa toinen bakteeri, *Acinetobacter baylyi*, tuottaa pitkäketjuisia hiilivetyjä tästä asetaatista. Tuottosysteemi on rakenteeltaan modulaarinen, ja siihen voidaan yhdistää erilaisia asetaattia tuottavia ja kuluttavia prosesseja. Lopullinen hiilivety-tuote voidaan määrittää, ja sen tuottoa tehostaa, geneettisesti muokkaamalla *A. baylyi*-bakteeria.

Pitkäketjuiset aldehydit ovat keskeisiä välituotteita vahaestereiden ja alkaanien biosynteesissä. Pitkäketjuinen asyyli-KoA-reduktaasi (engl. fatty acyl-CoA reductase, FAR) on avainentsyymi pitkäketjuisten aldehydien tuotossa. Luonnollista vahaestereiden tuottoa parannettiin *A. baylyi*-bakteerissa yli-ilmentämällä joko natiivia tai heterologista FAR:ia. Yli-ilmentäminen johti lisääntyneeseen pitkäketjuisen aldehydin tuottoon, ja mahdollisti tehokkaamman vahaesterien tai alkaanien tuottamisen, riippuen käytetystä aineenvaihduntareitistä. Vahaestereiden tuottoon käytettiin luonnostaan *A. baylyi*-bakteerista löytyvää aineenvaihduntareittiä, yhdistettynä joko natiivin FAR:n, tai ennestään karakterisoimattoman, *Nevskia ramosa* -bakteerista peräisin olevan oletetun reduktaasin yli-ilmentämiseen. Kumman tahansa näistä reduktaaseista yli-ilmentäminen johti lisääntyneeseen vahaestereiden tuottoon, jonka seurauksena saavutettiin korkeammat vahaesteripitoisuudet verrattuna aiemmin raportoituihin mikrobeihin perustuviin tuottosysteemeihin. *A. baylyi* ei luonnostaan tuota alkaaneja, mutta kaksiosaisen, syanobakteereista peräisin olevan aineenvaihduntareitin, joka koostuu aldehydiä

ja alkaania tuottavista entsyymeistä, ilmentäminen johti alkaanien synteesiin. Lisäksi *A. baylyi* -bakteerin luontainen kyky hajottaa alkaaneja estettiin, jolloin tämä luotaisesti alkaaneja hajottava kanta muutettiin alkaaneja tuottavaksi.

Tehokkaiden tuottokantojen kehittämistä rajoittaa pula menetelmistä jotka mahdollistaisivat kohdemolekyylien mittaamisen spesifisesti ja sensitiivisesti. Solunsisäiset biosensorit voivat mahdollistaa tuottokantojen tehokkaan seulonnan ja optimoinnin. Alkaanien tuoton analysoimista varten kehitettiin solunsisäinen biosensori, joka toimii kahdella tasolla. Ensiksi, vihreää fluoresoivaa proteiinia (engl. green fluorescent protein, GFP) koodaava geeni asetettiin luonnollisen alkaaneilla indusoituvan promoottorin alle *A. baylyi*-kannassa, jolloin alkaanin tuotto linkittyi GFP:n ilmentämiseen. Toiseksi, jatkuvasti ilmennettävä bakteerilusiferaasi LuxAB käyttää pitkäketjuista aldehydiä substraattina reaktiossa, joka tuottaa näkyvää valoa. Täten pitkäketjuisten aldehydien tuottoa voidaan seurata mittaamalla luminesenssisignaalia. Kaksitasoinen rakenne mahdollisti alkaanituoton parantamisen eri alkaanituottoreittien seulonnan ja tuottoreitin entsyymien ilmentämistasojen optimoinnin avulla.

Yhteenvetona, käyttäen kaksivaiheista bakteereihin perustuvaa tuottosysteemiä, tässä tutkimuksessa osoitettiin vahaestereiden tuotto hiilidioksidista ja sähköstä, sekä pitkäketjuisten alkaanien tuotto hiilidioksidista ja vedystä. Lisäksi osoitettiin että *A. baylyi* -bakteeri voidaan muokata tuottamaan alkaaneja, ja niin ikään parannettiin luonnollista vahaestereiden tuottoa muokkaamalla kyseisen bakteerin aineenvaihduntaa. Vaikka tuottosysteemi vaikuttaa lupaavalta menetelmätä tuottaa kestävällä tavalla polttoaineita ja kemikaaleja, menetelmän käyttöönotto edellyttää merkittäviä parannuksia tuottotehokkuuteen.

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Tampere, September 2018

Tapio

Contents

List of publications

The thesis is based on the following original publications, referred as Roman numerals in the text (I-IV).

I. Lehtinen T., Santala V., Santala S. (2017) 'Twin-layer biosensor for real-time monitoring of alkane metabolism', *FEMS Microbiology Lett*ers, 364, pp. 1-7. doi:10.1093/femsle/fnx053.

II. Lehtinen T, Efimova E, Tremblay P-L, Santala S, Zhang T, Santala V. (2017) 'Production of long chain alkyl esters from carbon dioxide and electricity by a two-stage bacterial process', *Bioresource Technol*ogy, 243, pp. 30-36. doi:10.1016/j.biortech.2017.06.073.

III. Lehtinen T, Efimova E, Santala S, Santala V. (2018) 'Improved fatty aldehyde and wax ester production by overexpression of fatty acyl-CoA reductases', *Microbial Cell Fact*ories, 17. doi:10.1186/s12934-018-0869-z.

IV. Lehtinen T, Virtanen H, Santala S, Santala V. (2018) 'Production of alkanes from CO_2 by engineered bacteria', *Biotechnology for Biofuels,* 11. doi:10.1186/s13068-018-1229-2.

Author's contributions

I. Tapio Lehtinen wrote the paper and is the corresponding author. He planned and conducted the experimental work and interpreted the results. V. Santala and S. Santala participated in designing the experiments and interpretation of the results. All authors participated in manuscript drafting and accepted the final manuscript.

II. Tapio Lehtinen wrote the paper and is the corresponding author. He planned and conducted the experimental work and interpreted the results. P-L Tremblay, S. Santala, T. Zhang and V. Santala participated in designing the experiments and interpretation of the results. E. Efimova contributed to lipid analytics. All authors participated in manuscript drafting and accepted the final manuscript.

III. Tapio Lehtinen wrote the paper and is the corresponding author. He planned and conducted the experimental work and interpreted the results. S. Santala and V. Santala participated in designing the experiments and interpretation of the results. E. Efimova contributed to lipid analytics. All authors participated in manuscript drafting and accepted the final manuscript.

IV. Tapio Lehtinen wrote the paper and is the corresponding author. He planned and conducted the experimental work and interpreted the results. S. Santala and V. Santala participated in designing the experiments and interpretation of the results. H. Virtanen contributed to initial strain construction and characterization. All authors participated in manuscript drafting and accepted the final manuscript.

Abbreviations

AAR	Acyl-ACP reductase
ACP	Acyl carrier protein
AD	Aldehyde decarbonylase
ADO	Adehyde-deformylating oxygenase
AldR	Aldehyde reductase
ATP	Adenosine triphosphate
CAR	Carboxylic acid reductase
CoA	Coenzyme A
FA	Fatty acid
FAEE	Fatty acid ethyl ester
FAR	Fatty acyl-CoA reductase
$FMNH_2$	Flavinmononucleotide, reduced
GC-MS	Gas chromatography - mass spectrometry
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced
NMR	Nuclear magnetic resonance
RBS	Ribosome binding site
TLC	Thin layer chromatography
WE	Wax ester
WS/DGAT	Wax synthase/diacyl glycerol acyl transferase

1 Introduction

The life on earth is based on the circulation of carbon. Autotrophic organisms utilize solar or chemical energy to reduce carbon dioxide (CO_2) into organic molecules, such as sugars. Heterotrophic organisms can oxidize the carbon in these molecules back to CO_2 and utilize the energy that is released. Thus, the energy that is stored in the chemical bonds of the organic molecules is utilized for powering all forms of life.

Besides the biological carbon cycling, CO_2 is also released when the fossil fuels are burned. Due to the negative effects of this surplus CO_2 on the global climate, there is a strong need for the society to reduce the CO_2 emissions generated from fossil sources. Therefore, alternative solutions that allow the production of fuels and chemicals from non-fossil carbon sources are required. Carbon dioxide is the ultimate source of this carbon, whether fixed biologically by autotrophic organisms, or by chemical means. For example, methods for CO_2 capture and utilization are being developed (Markewitz et al. 2012). However, the reduction of CO_2 into multicarbon molecules requires energy, and in a sustainable future this energy must come from renewable sources.

An abundant and renewable energy source available for the carbon dioxide reduction is the sun. Indeed, the energy provided by the solar radiation is several orders of magnitude greater than the energy consumed by humankind (Armaroli & Balzani 2016). Photosynthesis is a natural process that utilizes the solar energy for the production of biomass from CO_2. Thus, biomass can be – and currently is being – used for the production of non-fossil fuels and chemicals (L. Wu et al. 2016). However, the efficiency of the natural photosynthesis is low; generally less than 1% of the solar energy is stored in the biomass (Blankenship et al. 2011). Moreover, when the biomass is utilized for fuel or chemical production, the yield decreases further. For example, the solar-to-product efficiency of ethanol production from sugarcane is estimated to be 0.2% (Claassens et al. 2016). The low efficiency translates into the requirement of large land areas for the crop cultivation, causing issues related to the biodiversity and land usage, as well as the competition of the energy crops cultivation with food production.

In addition to natural photosynthesis, the solar energy can be captured by man-made devices, such as photovoltaic cells, which convert the solar energy into electricity. Photovoltaic cells are more efficient than the natural photosynthesis at capturing the solar energy (Blankenship et al. 2011), and thus represent an attractive way of harvesting this renewable source of energy. Furthermore, solar panels may be positioned in non-arable land, thus alleviating the competition between energy and food production. However, the applicability of electricity is limited in many applications, including transportation. Batteries have lower energy density than

liquid fuels, limiting their use in transportation, especially in aviation (Liao et al. 2016). Furthermore, renewable electricity, such as wind or solar, is intermittent by nature, thus requiring the capacity to store the surplus electricity generated during production peaks.

A potential solution for the sustainable production of fuels and chemicals is the utilization of renewable electricity for the reduction of carbon dioxide into multicarbon products, a process referred to as artificial photosynthesis (Barber & Tran 2013). In artificial photosynthesis, the solar energy is stored in the chemical bonds of organic compounds, thus mimicking the natural photosynthesis. Due to the higher efficiency of light capturing by photovoltaic cells, artificial photosynthesis has the potential to produce organic molecules at greater efficiency than the natural counterpart. Photosynthesis – natural or artificial – involves two separate reactions; energy harvesting and CO_2 reduction. In natural photosynthesis, CO_2 is reduced to specific multicarbon molecules (e.g. sugars) with high specificity. In contrast, artificial systems typically generate simple reduced carbon compounds, such as CO, CH_4, or CH_3OH (Zhou et al. 2016). The challenges in controlling the specificity of carbon-carbon bond formation restricts the efficiency and specificity with which molecules with longer carbon chains can be generated in chemical systems (Yang et al. 2017). Thus, while inorganic systems are more efficient in the light harvesting, the advantage of biological systems is the high specificity of the CO_2 reduction (Lips et al. 2018).

Recently, hybrid systems combining the inorganic light harvesting and biological reduction of CO_2 to multicarbon compounds have been developed (Zhang & Tremblay 2017). In these systems, the solar energy is captured by inorganic devices, and used as an energy source for CO_2 reduction by living cells. For example, lithoautotrophic bacteria can utilize hydrogen gas (H_2) as the energy source for CO_2 reduction. The H_2 production can be powered by photovoltaic cells, thus linking the solar energy to CO_2 reduction. Other, more direct approaches for transmitting the solar energy to CO_2 reducing bacteria have also been developed (Zhang & Tremblay 2017).

Ideally, the compounds produced by artificial photosynthesis should be comparable in terms of physical and chemical properties to the fossil counterparts they aim to replace. For example, the currently utilized fossil fuels comprise mainly of branched or linear hydrocarbons. The fuel properties are largely determined by the length of the carbon chain; gasoline contains hydrocarbons with 4-10 carbon atoms, jet fuel with 10-14 carbon atoms, and diesel with 15-22 carbon atoms (Bergthorson & Thomson 2015). Thus, molecules with corresponding, long carbon chains, such as alcohols, alkanes and esters, are ideally suited for the replacement of these fuels (Liao et al. 2016). In addition to the CO_2 reduction, pathways for the production of these long-chain molecules can be found in nature. In particular, harnessing of microbial cells

for the production of these molecules is seen as a promising approach (Peralta-Yahya et al. 2012; Liao et al. 2016). However, typically the microbes capable of efficient CO_2 reduction are not efficient at the synthesis of long carbon chains, and *vice versa*. Thus, the aim of this study was to develop a microbial system capable of long-chain hydrocarbon synthesis using carbon dioxide and electricity as the substrates.

In Chapter 2, the potential of microbial production systems is reviewed, with the focus on CO_2 reduction and, on the other hand, long-chain hydrocarbon production. The experimental work of this thesis subsequently focuses on how to couple the two processes to achieve long-chain hydrocarbon production from CO_2 and electricity. Based on the background information, hypotheses and objectives of this study are described in Chapter 3. In Chapter 4, the methods used in the original publications are summarized. The main results of the original publications are presented and discussed in Chapter 5. In Chapter 6, conclusions and future outlook are presented.

2 Background

2.1 Metabolic engineering and synthetic biology

The humankind has a long history of utilizing microbes for the production of useful compounds. For example, the fermentative production of ethanol or organic acids for foodstuff dates back several millennia. More recently, the natural capabilities of different organisms have been exploited in the production of industrial chemicals (e.g. acetone-butanol-ethanol fermentation) or pharmaceuticals (e.g. penicillin) in the early 20th century. Traditionally, the production strain has been improved with random mutagenesis followed by the selection of the cells with improved characteristics (e.g. product titer, tolerance to toxic compounds, or utilization of desired substrate). The development of recombinant DNA technologies, together with the increasing understanding about the cellular functions, enabled more targeted genetic modification of organisms and led to the conception of the field of metabolic engineering in the early 1990's (Bailey 1991; Stephanopoulos & Vallino 1991).

Since that time, several enabling technologies have experienced significant development, supporting the development of the field of metabolic engineering into more and more comprehensive science of engineering cellular systems (Keasling 2010; Nielsen & Keasling 2016; Yadav et al. 2012). For example, the development of DNA sequencing technologies together with bioinformatics tools led to the development of extensive databases covering the genomes of different organisms, and linking the DNA sequences to enzymatic functions. Additionally, the development of novel tools for genetic manipulation and for controlling the gene expression enabled more precise and targeted modification of the cellular metabolism.

The inherent complexity and versatility of biological systems hamper metabolic engineering efforts by leading to lack of transferability between different systems. In an attempt to control the complexity, synthetic biology concepts have been developed and applied (Endy 2005; Heinemann & Panke 2006). The key aspect of synthetic biology is the idea of standardization and modularization of the biological parts, in an analogous way to electrical parts used in the manufacturing of electronic devices. The standardized parts (DNA sequences) could be utilized for the construction of biological devices that would work in a predictable manner regardless of the context. An early example of a biological device performing a specific task is the toggle switch, a bistable gene-regulatory network (Gardner et al. 2000). Synthetic biology as a research field emerged on the foundations of genetic and metabolic engineering, and was enabled by yet additional technology developments, such as the chemical DNA synthesis and

the availability of well-studied regulatory systems for the design of biological devices (Heinemann & Panke 2006).

Advanced DNA synthesis technologies allow the *de novo* synthesis of DNA elements, such as genes, promoters, regulatory elements, or even whole operons, with a reasonable price. This means that the DNA elements can be freely designed, irrespective of whether such sequences are found in nature, and for example genes can be codon-optimized for improved compatibility with the host organism's metabolism. An ultimate example of the application of the *de novo* DNA synthesis was the construction of the first synthetic genome (Gibson et al. 2008), followed by the first viable cell with an artificial genome (Gibson et al. 2010).

The vast diversity of the metabolic pathways found in different organisms throughout the nature, combined with the ability to analyze and synthesize DNA elements, provides metabolic engineers with a plentiful toolbox for the construction of strains with the desired characteristics. Thus, the spectrum of possible bioprocesses is not restricted by the natural capabilities of any single organism. Rather, metabolic pathways for the production or degradation of the target molecule can be designed and implemented in the host organism based on the genetic information and tools readily available. Indeed, metabolic pathways leading to the production of various target molecules can be constructed in relatively short time-frames, as demonstrated by a recent "pressure test" where researches succeeded in engineering micro-organisms to produce 6 (out of 10) small molecules previously unknown to them in just 90 days (Casini et al. 2018).

However, having the functional metabolic pathway in the organism is rarely enough for an efficient production of the target molecule. Biological systems have evolved to tightly regulate the allocation of the cellular resources in a way that enhances the survival of the organism, and usually the production of a single molecule in large quantities is not in line with that target. An exception are the products whose formation is linked to balancing the energy or cofactor metabolism of the organism. For example, fermentative production of organic acids or alcohols from sugars in anaerobic conditions allows the recycling of the reduced electron carriers and thus continued growth despite the lack of an electron acceptor (molecular oxygen). Another example is the acetate production by acetogenic bacteria, which is necessary for sufficient ATP production to support growth on CO_2 and H_2 (see Chapter 2.3.2). This kind of "evolutionary optimized production strains" can be utilized for the production of the specific compounds. Nevertheless, for other compounds, efficient production requires that the cellular resources are re-allocated by metabolic engineering.

Despite the increasing understanding about the cellular metabolism and the efforts of standardizing the biological research, our understanding about the regulation of the metabolism remains limited, and thus the metabolic engineering of an efficient production strain typically involves a considerable amount of trial and error. The typical workflow consists of several rounds of so called “design-build-test-learn” cycle (Nielsen & Keasling 2016). The cycle starts with the design of the metabolic pathway and the genetic changes required for its implementation based on previous knowledge (i.e. a model of the metabolism), followed by the construction of the required DNA parts and their introduction to the microbial host (build). The resulting strains are phenotypically characterized (test), and finally, the model is revised based on the results obtained (learn). The design phase benefits from the development of comprehensive metabolic models, as well as computer-aided design tools (bioCAD) (Nielsen & Keasling 2016). The build phase is arguably currently the most trivial of the phases, thanks to the development of DNA synthesis technologies, as well as the modern gene editing tools such as the CRISPR/Cas9 (Wang et al. 2016). In addition to the rational design, diverse genetic strains can be produced by random or targeted mutagenesis techniques, such as multiplex automated genome engineering (MAGE) (Wang et al. 2009). In contrast to the ease at which genetic variations can be introduced, the phenotypical characterization largely relies on laborious and low-throughput methods (Zhang et al. 2015). One option for speeding up the screening of the desired phenotypes is the application of metabolic biosensors (Chapter 2.1.3).

2.1.1 Bow tie structure of metabolism

The central carbon metabolism is highly conserved in nature, and is organized in the so-called bow tie structure (Figure 2.1) (Csete & Doyle 2004; Noor et al. 2010; Nielsen & Keasling 2016). In the bow tie, different substrates (energy and carbon sources) are funneled into a small set of central intermediates and energy carriers, such as acetyl-CoA, pyruvate, NAD(P)H, or ATP (fan in). The central metabolites are then used in the biosynthesis of the whole diversity of macromolecules found in cells, such as protein, nucleic acids, and lipids (fan out). The bow tie structure highlights the potential of microbial production systems for modularity; depending on the metabolic capabilities of the organism, very heterogeneous substrates can be converted to the standard central metabolites, which can subsequently be directed for the biosynthesis of the target compound. Thus, by combining different fan in- and fan out-modules by metabolic engineering, potentially any substrate can be converted to any product with microbial systems (within the metabolic landscape of the host organism). As an example, acetyl-CoA can be derived from substrates as diverse as sugars (Noor et al. 2010), aromatic compounds (Harwood & Parales 1996), and carbon dioxide (Drake et al. 2008).

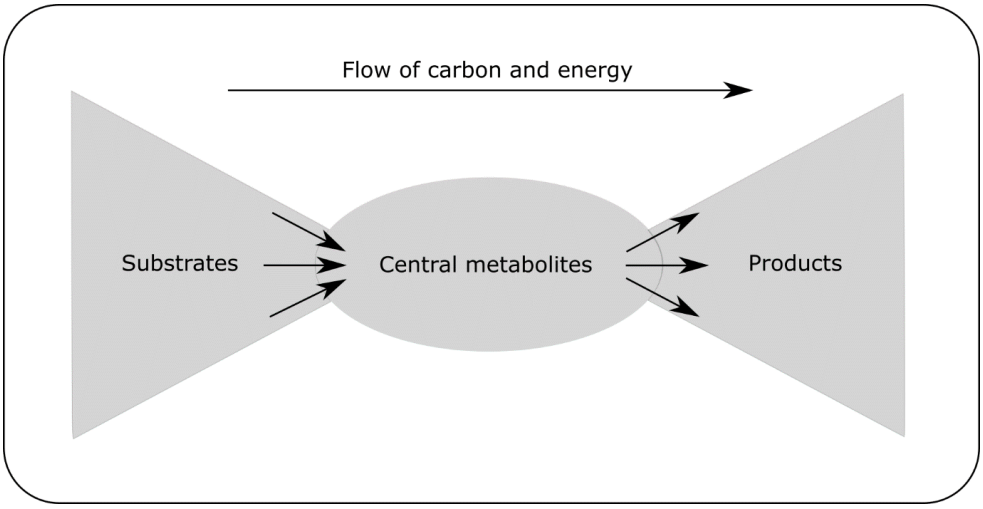

Figure 2.1. The bow tie structure of metabolism. A diversity of carbon and energy sources are converted to a handful of standard intermediates and energy carriers, which, in turn, are utilized for the production of the macromolecular constituents of the cell. Adapted from Backman et al. (2018) and Noor et al. (2010).

While the central metabolites and the cellular building blocks are well conserved, different microbial species have intrinsically different metabolic capabilities for the assimilation of various substrates, and for the biosynthesis of macromolecules (fan in and fan out). For example, some microbes can utilize recalcitrant substrates such as lignin or CO_2 as carbon sources. On the other hand, others have the tendency of naturally accumulating large amounts of high-energy storage compounds, such as polyhydroxyalkanoates (PHA), triacylglycerides, or wax esters. Although it might be possible to transfer the desired metabolic pathways to any host organism, synthetic pathways are typically less robust than the pathways operating in their natural cellular context (Czajka et al. 2017). This is pronounced with long and complex pathways; generally it is easier to implement a synthetic pathway that requires only a couple of enzymatic steps (Wu et al. 2015). Thus, it is becoming more and more recognized that the selection of a suitable microbial host is a crucial part in the construction of microbial production systems (Czajka et al. 2017; Kim et al. 2016). An organism with the natural capability for the desired metabolic functions may serve as a convenient chassis, in which further metabolic engineering can be applied in order to construct high-performing microbial strains.

The choice of the host organism depends on multiple factors; first, sufficient knowledge about the metabolism and efficient tools for genetic editing are required. The long-studied model organisms, such as the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, are

ideal in this respect; the cumulative knowledge about their metabolism and the array of available tools for the genetic engineering exceed those available for any other organism. Consequently, these two organisms have been widely applied in metabolic engineering studies. Second, the metabolic capability of the organism with regard to the targeted process must be considered. This is where alternative hosts may have an advantage over the traditional model organisms. For example, cyanobacteria or acetogenic bacteria can utilize carbon dioxide as the carbon source (Chapter 2.3). On the other hand, soil bacteria such as *Pseudomonas putida* or *Acinetobacter baylyi* can tolerate and utilize lignin-derived compounds, which are toxic to many other organisms (Salvachúa et al. 2015). In processes where such carbon sources are preferred, these organisms may be more suitable chassis than other, more traditional hosts. This applies also to the target product; for example, organisms that naturally accumulate storage lipids are seen as promising hosts for the microbial production of lipids (see Chapter 2.2.2).

2.1.2 Division of labor

The capability of a single cell for harboring complex engineered pathways is limited for several reasons. First, the host cell's resources (energy carriers, cofactors, and carbon building blocks) are utilized for the construction and operation of the engineered pathway, causing metabolic burden (G. Wu et al. 2016). The increasing metabolic burden associated with increasingly long and complex pathways leads to decreased cell viability and robustness (S. G. Wu et al. 2015; G. Wu et al. 2016). Second, different enzymes in the pathway may require different cellular environments for maximal functionality. For example, oxygen-sensitive enzymes cannot be utilized in aerobic hosts or processes. Third, imbalances in the metabolic network can lead to the accumulation of unwanted by-products or intermediates, compromising cell viability and leading to lower yields of the target product. In order to alleviate these issues and to utilize metabolic capacity exceeding that of any single cell, co-culture strategies, comprising the cultivation of multiple genetically different strains or organisms in the same system, have been developed (Goers et al. 2014; Jones & Wang 2018; Zhang & Wang 2016).

The utilization of multiple strains in the production system allows the distribution of the tasks between the organisms, relieving the metabolic burden experienced by each strain. Furthermore, it increases the modularity of the system, because each production strain can be optimized independently of each other. For example, Zhou *et al.* developed a co-culture of *E. coli* and *S. cerevisiae* for the production of oxygenated taxanes (Zhou et al. 2015). The production of these complex molecules requires multiple steps, and has been difficult to achieve in either *E. coli* or *S. cerevisiae* alone. The pathway was divided between the organanisms; *E. coli* produced taxadiene, which was oxygenated to the final products by *S. cerevisiae* (Zhou et al. 2015). The study is an example of how microbes' different strengths can be combined; *E. coli* is more efficient in the production of terpenes, whereas *S. cerevisiae* can

better express the eukaryotic enzymes required for the oxygenation reactions. Thus, in addition of relieving the metabolic burden, this kind of approach enables the efficient execution of all the pathway steps, in cases where all the pathway components cannot be efficiently expressed in the same host organism.

Co-culture strategies can also be utilized to alleviate issues caused by toxic compounds or by-products. For example, *E. coli* cells typically produce acetate as an overflow metabolite during rapid growth on glucose (Wolfe 2005). The acetate accumulation inhibits growth and represent a significant carbon loss from the production pathway. Santala *et al.* developed a co-culture where *A. baylyi* ADP1 was utilized for the removal of the acetate produced by *E. coli*, and thus relived the inhibition and enhanced the growth of *E. coli* cells (Santala, Karp, et al. 2014). Furthermore, when both of the strains were harnessed for the product formation, the carbon lost as acetate could be recovered for the product synthesis (Santala, Karp, et al. 2014).

In the aforementioned examples, the cells in the co-culture grow simultaneously in the same vessel. In addition, methods where different strains grow sequentially can be seen as an extension of the co-culture strategy. For example, a specific micro-organism can be utilized for the selective removal of growth inhibitors from the medium in order enable the growth of the actual production strain. The inhibitory compound to be removed can be, for example, oxygen (Yokoi et al. 1998) or organic acids (Kannisto et al. 2015).

2.1.3 Biosensors in metabolic engineering

As discussed earlier, metabolic engineering involves the generation of variations in the population, and the testing and selection of the desired phenotypes among the variants. With the modern DNA synthesis and editing techniques, genetic variants can be efficiently produced either by rational or random design. However, the capacity to screen and select for the desired phenotypes relies largely on laborious and expensive low-throughput methods, such as chromatographic analysis. Exceptions are the tolerance towards toxic compounds, for which the natural selection pressure can be applied for the selection, and the production of colorful compounds, such as lycopene, for which colorimetric screening can be applied. However, for the production of most small molecules, no direct selection or screening method exists. Furthermore, the dynamics and possible bottlenecks of the biosynthesis pathway are not revealed by the analysis of the end product concentration alone. For improvements of the production by rational means, information is needed also about the intermediates of the pathway and/or other metabolites of the cell. Thus, biosensors that are able to produce a measurable signal in response to the concentration of a specific molecule are valuable tools in metabolic engineering.

In nature, a common solution for the biosensing of small molecules are transcription factor-promoter pairs. The transcription factor (TF) recognizes and binds the target molecule, and the binding influences the transcription factor's ability to induce expression from the promoter. Thus, the expression of the gene(s) under the promoter is altered in response to the concentration of the target molecule. The natural TF-promoter pairs can be employed in the construction of biosensors. In addition, the properties of the sensor, including specificity, sensitivity, and dynamic range, can be altered to produce optimal response. A significant research effort has been dedicated for the development of biosensors for the detection of exogenous molecules, such as environmental pollutants (Su et al. 2011). In the metabolic engineering context, however, biosensors are utilized for the detection of metabolites produced by the cell itself. The output of the sensor is determined by placing genes encoding the output function under the promoter. Depending on the targeted application, the output can be related to monitoring, selection, or dynamic regulation.

For monitoring purposes, the biosensing element is coupled to a reporter; for example, a fluorescent protein is expressed under the inducible promoter. When the biosensor is introduced to a strain producing the target molecule, the production is reflected in a measurable signal (e.g. fluorescence). This kind of biosensors can aid in screening of the production strains by allowing considerably higher throughput than conventional analysis methods, such as chromatography. Furthermore, depending on the biosensor, they may allow real-time monitoring during cell growth, which would not be possible with other methods. In addition, biosensors can readily be developed for molecules that are present in such a small concentrations or have short life times *in vivo* that analysis by other means would be difficult (D. Liu et al. 2015). Typically, the monitoring biosensors are utilized in screening a library of cells with mutations (either directed or random) that affect the production of the target molecule. For example, Tang & Cirino (Tang & Cirino 2011) developed a mevalonate biosensor and applied it for improving the non-native mevalonate biosynthesis in *E. coli*. The sensor was based on the AraC transcription factor modified to specifically recognize mevalonate instead of arabinose, and the output was meditated by *lacZ* placed under the cognate promoter P_{BAD}. With the sensor, the authors screened a library of 10^6 variants with varying expression levels of the mevalonate biosynthesis genes.

Regarding hydrocarbon production, a TF-promoter based biosensor recognizing alkanes has been developed for the detection of endogenously produced alkanes in *E. coli* (W. Wu et al. 2015), albeit it was not applied for the improvement of the production. The authors utilized the alkane-inducible AlkR-P_{alkM} pair from *Acinetobacter baylyi*. AlkR binds n-alkanes of various chain lengths, and the binding induces expression from the P_{alkM} promoter (A Ratajczak et al. 1998).

Wu *et al*. placed a *gfp* gene under the P_{alkM} promoter, and demonstrated correlation between endogenous alkane production and fluorescence signal in *E. coli*.

The biosensors utilized for monitoring are usually designed to interfere as little as possible with the host metabolism, as their function is merely to monitor and report. On the other hand, biosensors designed for selection or dynamic regulation target the output towards the host metabolism. For example, the biosensor can be used for the elimination of selected cells, or to dynamically control the expression of certain genes in response to the concentration of a target metabolite.

When the biosensor output is coupled to the fitness of the host, the sensor can be used for the selection of cells with the desired phenotype. For example, Dietrich *et al*. (Dietrich et al. 2013) utilized a TF-promoter pair that is activated by alcohols. By placing a tetracycline resistance gene under the promoter, the authors were able to couple the growth rate of the cells to the butanol concentration in the medium. Further, they demonstrated that the sensor could be used in the screening of the most efficient butanol-producing strains amongst a library of 960 strains (Dietrich et al. 2013). A step further is the utilization of the sensors in directed evolution, where mutations are introduced at high rates, and the sensor output is used for the selection. These steps can be repeated in cyclical manner, allowing the enrichment of more and more efficient production strains. A potential issue with the cyclic selection is that cells can develop mutations that activate the sensor in the absence of the inducer, leading to the enrichment of these "cheater" cells. Raman *et al.* provided an elegant solution for the issue of the cheater cells in a study where TF-based sensors were used to control the expression of TolC (Raman et al. 2014). Notably, TolC can be used in both positive and negative selection, which enabled the elimination of the cheater cells. In the study, the production of a target molecule induced the production of TolC via TF-based sensor, and the cells expressing TolC were selected for by using sodium dodecyl sulfate. On the other hand, the cheater cells expressing TolC in the absence of the target molecule were eliminated by colicin E1. By combining the toggled positive and negative selection to targeted mutagenesis, the authors produced and enriched mutants efficient in the production of naringenin or glucaric acid.

Another application of biosensors is the dynamic regulation of the host metabolism. A common issue with synthetic metabolic pathways is the lack of regulatory mechanisms that would adjust the flux through different nodes of the pathway in changing conditions. In natural pathways, various control and feedback mechanisms balance the activities of the pathway in order to avoid the accumulation of potentially harmful intermediates or wasteful utilization of cellular resources. Introduction of such regulatory systems to the synthetic pathways can greatly improve the performance of the host and the production of the target molecule. In a study by

Zhang *et al.*, the natural fatty acyl-CoA responsive transcription factor FadR from *E. coli* was employed for the dynamic regulation of a synthetic fatty acyl ethyl ester (FAEE) production pathway (F. Zhang et al. 2012). Binding of FadR to the cognate promoters prevents translation, and the binding in antagonized by fatty acyl-CoA or free fatty acids. First, the authors developed synthetic promoters with improved dynamic range. The sensor was then utilized in a FAEE-producing *E. coli* strain. The genes for ethanol biosynthesis, as well as for the wax ester synthase, were placed under the modified promoter. Thus, these genes were only expressed when fatty acyl-CoA was present in sufficiently high concentrations, avoiding the accumulation of ethanol in the early stages of cultivation, and leading to improved strain fitness and FAEE production.

In addition to TF-promoter based detection systems, alternative biosensors employed in metabolic engineering include riboswitches (Zhang et al. 2015) and catalytic biosensors (Dietrich et al. 2010). Although less easily generalizable than TF-based sensors, catalytic biosensors utilizing an enzymatic reaction for the production of the output can offer interesting biosensing possibilities in certain cases. Catalytic biosensors comprise of one or multiple enzymatic steps that turn inconspicuous metabolites (inputs) into measurable outputs (Dietrich et al. 2010). An early example was a sensor for L-tyrosine developed by Santos and Stephanopoulos (Santos & Stephanopoulos 2008). The authors utilized tyrosinase, an enzyme that catalyzes the conversion of L-tyrosine to dopaquinone, which polymerizes nonenzymatically to form melanin. The heterologous expression of the tyrosinase in *E. coli* linked the L-tyrosine production to the formation of the easily detectable pigment melanin, and allowed the authors to screen a random knockout library of 21 000 variants for improved L-tyrosine production.

An interesting catalytic biosensor is the bacterial luciferase (LuxAB), catalyzing the oxidation of long-chain aliphatic aldehyde (fatty aldehyde) to the corresponding fatty acid in a reaction that produces visible light. Other substrates required for the reaction are reduced riboflavin phosphate ($FMNH_2$) and molecular oxygen (Meighen 1991). In nature, the fatty aldehyde substrate is provided by a multienzyme fatty acid reductase complex (LuxCDE), encoded in the same operon with the LuxAB (Meighen 1991). The bacterial luciferase has served as a convenient reporter in various biosensing applications due to the possibility to detect the luminescence *in vivo*, in real time, and with high specificity and sensitivity (Close et al. 2012). Usually the fatty aldehyde substrate is provided by expressing the LuxCDE complex, or by external addition of the fatty aldehyde to the medium prior the measurement. In typical TF-based sensor applications, the expression of the luciferase genes is controlled at transcriptional level, and the output is relative to the expression level of the luciferase, thus functioning with the same principle as other TF-based sensors. In contrast, because the bacterial luciferase

catalyzes a reaction with easily detectable output (light), it can be used as a catalytic biosensor for detecting its substrates (fatty aldehyde, $FMNH_2$, O_2), as recently demonstrated for the fatty aldehyde (Santala et al. 2012; Santala, Efimova, Karp, et al. 2011). In this case, the luciferase is constantly expressed, and the luminescence signal is relative to the availability of the fatty aldehydes. This application thus provides metabolic engineers with a convenient means of studying the fatty aldehyde metabolism in real time and with high specificity (Santala, Efimova, Karp, et al. 2011).

2.2 Microbial lipid production

Metabolic pathways capable of the synthesis of long carbon chains from the common intermediates, such as acetyl-CoA, include the fatty acid pathway, the reverse β-oxidation pathway, and the isoprenoid pathway (Liao et al. 2016). The following section introduces the most important enzymes and pathways that have been utilized in the production of various fatty acid-derived molecules. Most of the research on the area has been done with the conventional microbial hosts such as *E. coli* or *S. cerevisiae*. It should be noted, however, that the metabolic engineering strategies are likely not directly transformable between different hosts due to their differing metabolic capabilities.

2.2.1 Fatty acyl-derived molecules

The long alkyl chains produced by fatty acid biosynthesis are ubiquitously found in nature as components of the membrane lipids or storage compounds. Free fatty acids are toxic due to their amphiphilic nature, and thus are not naturally present in high concentrations. Rather, fatty acids are commonly found as ester or ether linked lipid compounds, such as phospholipids, in the cell membrane. Additionally, some organisms produce storage lipids, such as triacylglycerol or wax esters, where the fatty acids are in the ester form as well. During the FA synthesis, the acyl chain undergoing elongation remains bound to acyl carrier protein (ACP) via thioester bond.

The biosynthesis of the highly reduced long acyl chains of FAs requires energy in the form of ATP and NADPH. Thus, it is not surprising that the fatty acid synthesis is tightly regulated. For example, several enzymes of the fatty acid biosynthesis pathway have been shown to be inhibited by the end product of the pathway, fatty acyl-ACP, in *E. coli* (Heath & Rock 1996; Davis & Cronan 2001). Thioesterases are enzymes that cleave the free fatty acid from the ACP. The cytosolic (over)expression of a thioesterase increases the FA production in *E. coli* by lowering the concentration of acyl-ACP and thus relieving the feedback inhibition (Cho & Cronan 1995). Thioesterase expression leads to accumulation of free fatty acids, and the chain length distribution can be modulated by expressing thioesterases with different substrate specificities (Voelker & Davies 1994; Yuan et al. 1995). Thus, thioesterase expression is almost routinely

included in *E. coli* strains engineered for the production of fatty acid-derived compounds. A commonly used thioesterase in metabolic engineering applications is the leaderless TesA (designated 'TesA), an *E. coli* thioesterase from which the leader sequence targeting periplasmic expression has been removed (Cho & Cronan 1995).

Extensive amount of research has focused on overproduction of fatty acids or their derivatives. In addition to their direct uses in chemical industry, fatty acids and their activated versions represent common intermediates in the biosynthesis of various lipid molecules, such as alkanes, fatty alcohols, wax esters, and fatty acid ethyl esters. Examples of potential pathways for the production of fatty acid-derived molecules and enzymes catalyzing the conversions are presented in Figure 2.2.

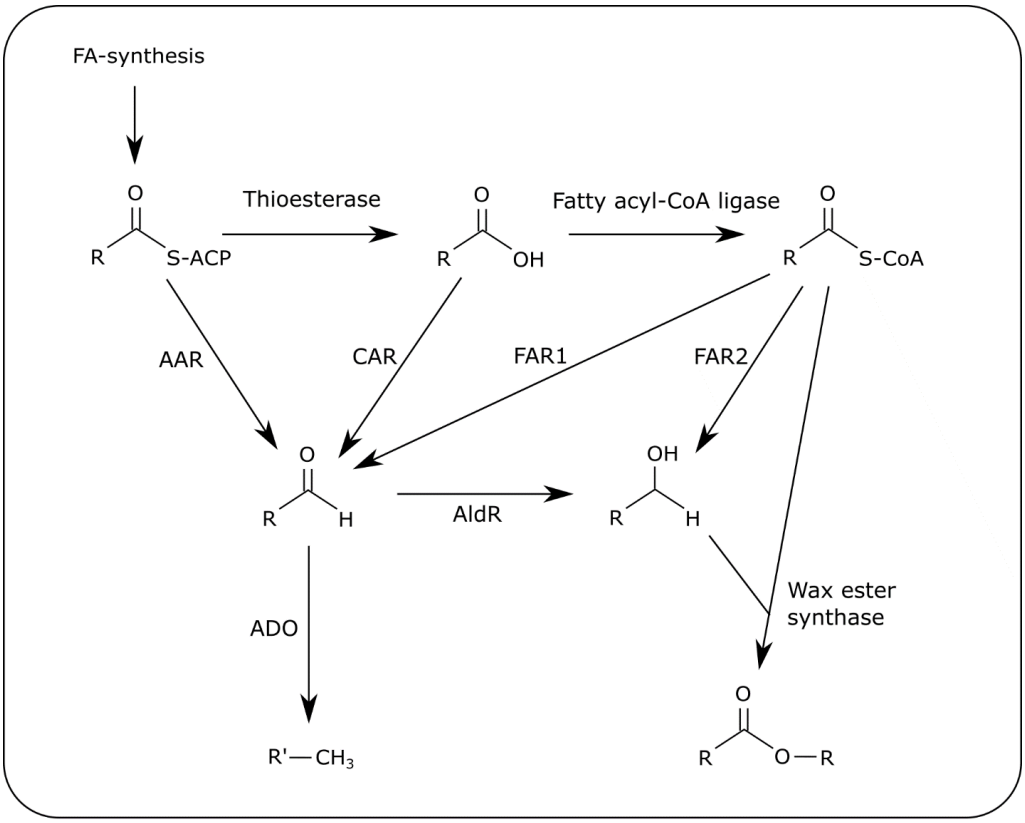

Figure 2.2. Examples of enzymatic reactions and pathways for the production of fatty acid-derived molecules. Examples of each enzyme class are given in the text. FA - Fatty acid; ACP – Acyl carrier protein; CoA - Coenzyme A; AAR – Acyl-ACP reductase; CAR – Carboxylic acid reductase; FAR1 – Fatty acyl-CoA reductase (aldehyde-forming); FAR2 – Fatty acyl-reductase (alcohol-forming); AldR – Aldehyde reductase; ADO – Aldehyde-deformylating oxygenase; R – acyl chain with n carbon atoms (typically n = 15/17); R' – acyl chain with n-1 carbon atoms.

2.2.1.1 Fatty aldehydes

Fatty aldehydes are key intermediates in the biosynthesis of various fatty acid derived molecules such as fatty alcohols, alkanes and wax esters (Figure 2.2). Fatty aldehydes can be produced by fatty acyl-ACP/CoA reductases (AAR/FAR). AAR from cyanobacteria can utilize either fatty acyl-ACP or –CoA as substrate, but the activity is higher towards the former (Schirmer et al. 2010). Acr1 from *Acinetobacter baylyi* ADP1, on the other hand, only accepts

acyl-CoA as substrate (Reiser & Somerville 1997). Additionally, carboxylic acid reductases (CAR) can produce aldehyde from fatty acid (Akhtar et al. 2013). Aldehydes do not generally accumulate in the cells, but are rapidly metabolized by endogenous enzymes, such as aldehyde reductases (Schirmer et al. 2010; Rodriguez & Atsumi 2014). Even though *E. coli* does not naturally produce fatty aldehydes, there are several native aldehyde reductases that are active also against the fatty aldehydes (Rodriguez & Atsumi 2014). Fatty aldehydes can also be oxidized back to fatty acids by aldehyde dehydrogenases (Bertram et al. 2017; Ishige et al. 2000).

2.2.1.2 Fatty alcohols

In addition to the aldehyde-producing FARs, some FARs found in marine bacteria or eukaryotes reduce the activated fatty acyl directly to fatty alcohol. Examples of this kind of alcohol-producing FARs include Maqu_2507 and Maqu_2220 from *Marinobacter,* and CER4 from *A. thaliana* (Rowland et al. 2006; Willis et al. 2011; Hofvander et al. 2011).

Various studies have been focused on the overproduction of fatty alcohols (reviewed by (Fillet & Adrio 2016)). In many studies, the expression of a heterologous FAR in *E. coli* has been accompanied with the overexpression of the thioesterase and the fatty acyl-CoA synthase FadD. Interestingly, some studies have employed aldehyde-producing FARs, thus relying on the native aldehyde reductases of *E. coli* for the final conversion of aldehydes to alcohols (Steen et al. 2010; Fatma et al. 2016). The highest fatty alcohol production has been achieved by Fatma *et al.* (2018). In the study, the authors applied-constraint based metabolic modeling in order to identify knockout and overexpression targets that would increase the fatty alcohol and alkane production. The modifications predicted by the model and validated *in vivo* increased flux towards NADPH synthesis, and decreased by-product and biomass formation, leading to more efficient long-chain hydrocarbon production. The engineered strain produced fatty alcohols with a remarkable yield of 84% of the theoretical maximum, and titer of 12.5 g/l, in fed-batch cultivation (Fatma et al. 2018).

2.2.1.3 Fatty acyl ethyl esters

Fatty acid methyl esters and ethyl esters (FAMEs and FAEEs, respectively) represent modified FAs with more suitable properties for uses as fuels in diesel engines. An enzyme class originally found from *Acinetobacter* (Kalscheuer & Steinbüchel 2003), called wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) can catalyze the transesterification between activated acyl chain (acyl-CoA) and alcohol. In the natural context, the accepting alcohol is usually long-chain primary alcohol or diacylglycerol, and the product is therefore either wax ester, or triacylglycerol, respectively. However, the enzyme is remarkably unspecific with regard to the chain length of the substrates, and accepts primary alcohols from ethanol to triacontanol

(C30), as well as cyclic, aromatic, and linear secondary alcohols (Stöveken et al. 2005). Thus, WS/DGAT has been successfully used for the production of FAEEs in heterologous hosts. Kalscheuer *et al.* expressed WS/DGAT from *A. baylyi* in *E. coli* engineered for ethanol production (Kalscheuer, Stölting, et al. 2006). In their production system, the cells produced ethanol from glucose, whereas the fatty acids required for fatty acyl-CoA and eventually FAEE production were provided exogenously. Using this strategy, the authors reported FAEE production of 1.2 g/l. Steen *et al*. went further, and engineered *E. coli* to synthetize both the ethanol and fatty acyl components *de novo* from glucose, thus alleviating the need for the exogenous fatty acid supplementation (Steen et al. 2010). Increased fatty acyl-CoA production was achieved by the expression of thioesterase ('TesA) and fatty acyl-CoA ligase (FadD), combined with the blockage of the β-oxidation pathway (by deletion of FadE) to prevent FA degradation. In addition, the strain expressed heterologous pyruvate decarboxylase and alcohol dehydrogenase for ethanol production, and WS/DGAT for the final esterification step. This production system was further improved by designing a dynamic sensor-regulator system, which enabled the automatic tuning of the ethanol production rate in response to the FFA concentration (F. Zhang et al. 2012). This resulted in reduced ethanol production and increased FAEE production, along with increased strain fitness. The study presents an example of the importance of pathway balancing and the prevention of the accumulation of a toxic intermediate in microbial production systems.

2.2.1.4 Wax esters

Wax esters are esters between a fatty acid and a fatty alcohol. Compared to FAMEs or FAEEs, the alcohol moiety of WEs is considerably longer, leading to different properties and potential applications. WEs are widespread in nature and are produced in small quantities for example in plant leaves and animal skin. In larger quantities, WEs are employed for the buoyancy control by spermaceti whales or as carbon and energy storage by certain plants and bacteria. For example, the desert plant jojoba (*Simmondsia chinensis)* accumulates high amounts of WEs in its seeds (Miwa 1971). Additionally, some bacteria, including *Acinetobacter* and *Marinobacter* strains, produce high amounts of WEs for energy and carbon storage (Fixter et al. 1986; Alvarez 2016). In *A. baylyi*, the proposed WE synthesis pathway involves the aldehyde-producing fatty acyl-CoA reductase Acr1, an unidentified fatty aldehyde reductase, and the aforementioned WS/DGAT (Alvarez 2016; Santala, Efimova, et al. 2014). Different versions of the wax ester synthesis pathway have been utilized for WE production in heterologous hosts. As *E. coli* does not naturally produce fatty alcohols, the expression of *A. baylyi* WS/DGAT alone does not lead to WE synthesis (Kalscheuer, Stöveken, et al. 2006). However, the combination of WS/DGAT expression with FAR from jojoba led to WE production in *E. coli* (Kalscheuer, Stöveken, et al. 2006). Similarly, *E. coli* expressing 'TesA, FadD, FAR from mouse from *Mus musculus*, and WS/DGAT from *A. baylyi*, produced wax esters (Steen et al. 2010). However, the evaluation of

the efficiency of these strategies is complicated by the lack of reported titers. Recently, the production of very long-chain WEs in the yeast *S. cerevisiae* was reported (Wenning et al. 2016). The authors expressed and alcohol-forming FAR from *Marinobacter* and a wax ester synthase from jojoba in *S. cerevisiae*, and reported a WE yield of 12 mg/g CDW (cell dry weight).

2.2.1.5 Alkanes

Long-chain alkanes are produced in small quantities by many organisms, including plants, insects, algae and bacteria (Schirmer et al. 2010; Wang & Lu 2013). Due to their ideal properties as liquid fuels, alkanes represent an appealing target for microbial production. Naturally produced alkanes typically comprise an odd number of carbons, reflecting the fact that they are derived from even-number fatty acyl chains via decarbonylation (Marsh & Waugh 2013). Early studies with plants, animals, and algae indicated a pathway in which fatty acid is reduced to fatty aldehyde, following its decarbonylation to produce alkane and carbon monoxide (CO) (Cheesbrough & Kolattukudy 1984; Cheesbrough & Kolattukudy 1988; Dennis & Kolattukudy 1991; Yoder et al. 1992). The enzymes responsible for the decarbonylation were termed aldehyde decarbonylases (AD), and characterized to function in the absence of added cofactors or oxygen. However, the genetic and biochemical details of alkane biosynthesis remained elusive for a long time. Recently, a cyanobacterial alkane-producing pathway was identified with a comparative genomics approach (Schirmer et al. 2010). Genomes of alkane-producing cyanobacteria were compared to one species that was deficient of alkane synthesis. A biosynthetic pathway that produces alkanes via an aldehyde intermediate was identified; acyl-ACP reductase (AAR) catalyzes the reduction of acyl-ACP to fatty aldehyde, and aldehyde deformylating oxygenase (ADO) converts aldehydes to alkanes (Schirmer et al. 2010; Warui et al. 2011).

Following their discovery, the cyanobacterial ADOs have been the subject of intensive characterization. Initially, the enzyme was assumed to catalyze a cleavage of the aldehyde to alkane and CO (Schirmer et al. 2010). However, later studies identified the C1 product as formate rather than CO (Warui et al. 2011). Additionally, the reaction requires molecular oxygen as a cosubstrate, as well as electrons supplied by an auxiliary reducing system (Li et al. 2012). Thus, the enzyme was named aldehyde deformylating oxygenase (ADO).

In contrast to the membrane-bound plant and animal decarbonylases, the cyanobacterial ADOs are soluble enzymes, which has facilitated their *in vitro* characterization (Marsh & Waugh 2013). A prominent feature of the ADOs is their apparent low activity, reflected by the extremely low turnover values (k_{cat} < 1/min) measured *in vitro* (Marsh & Waugh 2013; Wang et al. 2017; J. Zhang et al. 2013). Furthermore, it appears that uncoupled electrons at the active site result in the formation of hydrogen peroxide (H_2O_2), which inhibits the activity of ADO in a

reversible manner (Andre et al. 2013). Interestingly, it was found that an electron transfer system originating from cyanobacteria resulted in higher alkane production and less H_2O_2 formation compared to heterologous or chemical electron transfer systems commonly utilized in the *in vitro* assays (J. Zhang et al. 2013). Later, the most efficient reducing partner for ADO among the studied ferredoxins was found to be PetF, an abundant and essential ferredoxin from *Synechocystis sp.* PCC6803 (Rajakovich et al. 2015). Thus, efficient and precise electron delivery appear to be highly important for the alkane production by ADO. Furthermore, high O_2 concentrations reduce the alkane production by ADO irrespective of the reducing system utilized, potentially due to off-pathway free radical formation (Rajakovich et al. 2015; Kallio et al. 2014).

In addition to the cyanobacterial ADOs, another known enzyme that converts fatty aldehydes to alkanes is CER1 from the plant *Arabidopsis thaliana* (Bernard et al. 2012). By combining the expression of ADO or CER1 to fatty aldehyde production, heterologous alkane production has been achieved in non-native alkane producers, such as the model organisms *E. coli* and *S. cerevisiae*. Examples of heterologous alkane production strategies are presented in Table 2.1. Without further modifications affecting the chain length, the majority of the alkanes produced with this two-step pathway consist of 15 or 17 carbons, reflecting the fact that they are derived from the naturally most abundant C16 and C18 fatty acyl chains. Usually both unsaturated and saturated hydrocarbons are produced, also reflecting the availability of the corresponding precursor acyl chains in the cells.

Extensive metabolic engineering has been applied in the attempt to increase the titer of alkane production and/or achieve the production of alkanes with modified chain length (Table 2.1). Various approaches include the testing of different pathway designs and enzymes from different organisms, as well as optimization of the expression levels and deletion of competing pathways.

Commonly, the aldehyde production is achieved with the cyanobacterial AAR, but other aldehyde-producing enzymes have also been utilized. For example, Howard *et al.* utilized the LuxCDE complex for the conversion of free fatty acids to fatty aldehydes, followed by their deformylation to alkanes by the cyanobacterial ADO (Howard et al. 2013). This approach enabled the authors to modify the carbon chain of the aldehydes, and correspondingly of the alkanes, by modifying the upstream free fatty acid pool, in order to produce alkanes with specific length or branched chain iso-alkanes (Howard et al. 2013).

Expression of CER1 in *E. coli* with other modifications that increased the fatty aldehyde production, led to the production of 580 mg/l alkanes (Choi & Lee 2013). In that study, a

modified thioesterase with a specificity for shorter-chain acyl-ACPs was used to promote the production of medium-chain alkanes such as nonane and tridecane. The free fatty acids produced by the thioesterase were activated to fatty acyl-CoAs by the endogenous fatty acyl-CoA ligase FadD, and further reduced to fatty aldehydes by the fatty acyl-CoA reductase Acr from *Clostridium acetobutylicum*.

In contrast to the relatively long-chain alkanes produced by most studies, Kallio *et al*. engineered *E. coli* for the production of propane (C3) (Kallio et al. 2014). The modulation of the chain length was achieved by utilizing a short-chain specific thioesterase Tes4 from *Bacterioides fragilis.* The expression of Tes4 led to the production of butyrate, which was converted to butyraldehyde by carboxylic acid reductase (CAR) from *Mycobacterium marinum*, and finally to propane by ADO. The propane production was enhanced by the deletion some of the endogenous aldehyde reductases and the co-expression of a ferredoxin-based electron transfer system.

The fatty aldehyde intermediate is problematic because it is toxic in large quantities and is rapidly consumed by endogenous enzymes such as aldehyde reductases (Rodriguez & Atsumi 2014). Due to these endogenous activities, cells expressing the heterologous alkane biosynthesis pathway typically produce more fatty alcohols than alkanes (Schirmer et al. 2010; Fatma et al. 2018). The issue has been addressed by deleting some of the aldehyde dehydrogenase genes (Rodriguez & Atsumi 2014; Song et al. 2016) or by scaffolding the enzymes of the pathway (AAR and ADO) to improve the flux of the aldehyde intermediate between the enzymes (Sachdeva et al. 2014). Interestingly, recent studies found that the deletion of certain endogenous fatty aldehyde reductases is not beneficial for alkane production in *E. coli* (Wang et al. 2018; Cao et al. 2016); rather, a moderate aldehyde reductase activity seems to boost the alkane biosynthesis. The mechanisms behind this phenomenon are not currently understood.

The highest alkane titers have been reported by Cao *et al.* (2016) and Fatma *et al.* (2018) (Table 2.1). Cao *et al.* optimized the expression levels of AAR, ADO, and AdhP (an endogenous aldehyde reductase), and found out that moderate expression of AdhP was beneficial for the alkane production. Further improvements on the alkane production were achieved by the overexpression of genes related to fatty acid biosynthesis, the expression of an electron transfer system, and the redirection of acyl chains from membrane lipids to alkane synthesis (Cao et al. 2016). Fatma *et al*. utilized the same metabolic engineering approach as with the fatty alcohol production mentioned earlier, including increased flux towards NADPH formation and reduced flux to byproducts and biomass formation.

Heterologous alkane synthesis has also been studied in yeast. The expression of either CER3 and CER1 from *A. thaliana*, or AAR and ADO from *S. elongatus*, led to the production of alkanes in *S. cerevisiae*. Additionally, alkanes have been produced in the oleaginous yeast *Yarrowia lipolytica* (Xu et al. 2016). *Y. lipolytica* is capable of accumulating large amounts of lipids, and is considered a potential chassis for the production of a range of fatty acyl-derived products (Beopoulos et al. 2009; Xu et al. 2016; Blazeck et al. 2014; Qiao et al. 2015).

Table 2.1. Examples of heterologous alkane production. Please refer to the text for more details.

Organism	Enzymes	Main products	Titer	Reference
E. coli	SeAAR-NpADO	C15 - C17	300 mg/l	Schirmer et al. (2010)
	LuxCDE - NpADO	C13 - C17, iso-alkanes	5 mg/l	Howard et al. (2013)
	'TesA(L109P) - FadD - Acr - CER1	C9 - C13	580 mg/l	Choi & Lee (2013)
	Tes4 - CAR - PmADO	C3	32 mg/l	Kallio et al. (2014)
	SeAAR - SeADO	C15 - C17	1.3 g/l	Cao et al. (2016)
	SeAAR - NpADO	C15 - C17	2.5 g/l	Fatma et al. (2018)
	SeAAR - NpADO	C17	1 g/l	Wang et al. (2018)
S. cerevisiae	CER3 - CER1	C28 - C31	Not reported	Bernard et al. (2012)
	SeAAR - SeADO	C13 - C17	Not reported	Buijs et al. (2015)
Y. lipolytica	CAR - PmADO	C15 - C17	23.3 mg/l	Xu et al. (2016)

ADO – Aldehyde-deformylating oxygenase; AAR – Acyl-ACP reductase; Se – *Synechococcus elongatus*; Np – *Nostoc punctiforme*; Pm – *Prochlorococcus marinus*; Tes – Thioesterase; FadD – fatty acyl-CoA ligase; Acr – Fatty acyl-CoA reductase; CER1 – Eceriferum 1 (*Arabidopsis thaliana*); CER3 – Eceriferum 3 (*A. thaliana*); CAR – Carboxylic acid reductase

2.2.2 Acinetobacter baylyi ADP1: potential for lipid production from simple precursors

Acinetobacter spp. are gram-negative and strictly aerobic bacteria widespread in nature. Their natural habitat includes soil and animal skin. The laboratory strain *Acinetobacter baylyi* ADP1 (here ADP1, formerly known as *Acinetobacter calcoaceticus* BD413) was derived from *A. baylyi* BD4 with ultraviolet mutagenesis (Taylor & Juni 1961; Barbe et al. 2004). Both strains produce extracellular polysaccharides (EPS) that form a protective capsule around the cells, but in ADP1 the capsule is considerably smaller than in the parental strain (Kaplan & Rosenberg 1982).

Following the sequencing of its genome (Barbe et al. 2004), ADP1 has been increasingly used in genetic and metabolic engineering studies (Elliott & Neidle 2011; Young et al. 2005; Elbahloul & Steinbüchel 2006; Metzgar et al. 2004; Jezequel et al. 2013; Santala, Efimova, Kivinen, et al. 2011; Kannisto et al. 2014). The strain is naturally competent for transformation and has a tendency for homologous recombination, making genetic manipulations straightforward and simple to produce (Palmen & Hellingwerf 1997; Elliott & Neidle 2011). The transformation efficiency is not affected by the DNA sequence, and both linear DNA fragments and plasmids can be transformed (Palmen et al. 1993). Following transformation, the DNA may be incorporated to the genome by homologous recombination, if homologous sequences of < 200-400 bp are present. This feature has been utilized in the construction of a complete single gene knockout library, where each target gene was replaced by a synthetic deletion cassette (De Berardinis et al. 2008). The deletion cassette contains a selection marker, flanked on both sides by homologous sequences targeting the integration to a specific chromosomal *locus*. The knockout library was utilized in the construction of a global metabolic model (Durot et al. 2008), as well as in the "multiomics" level characterization of *A. baylyi* grown in different conditions (Stuani et al. 2014). Other tools available for the genetic modification of ADP1 include plasmid-based and genome-integrative expression cassettes for controlled gene expression (Murin et al. 2012).

A. baylyi is nutritionally versatile and is able to utilize a wide variety of compounds, including dicarboxylic acids, aromatic compounds and aliphatic alkanes. *A. baylyi* ADP1 is also able to grow on glucose, although this trait is not common among *Acinetobacter* strains (Young et al. 2005). ADP1 lacks several genes related to carbohydrate catabolism, such as pyruvate kinase, glucokinase, hexokinase and glucose transporter system, and glucose is utilized with a complex pathway involving its oxidation in the periplasm, transportation of gluconate to the cells and further processing via a modified Entner-Doudoroff pathway (Young et al. 2005; Barbe et al. 2004). The complexity of glucose catabolism highlights the fact that glycolytic carbon sources are not the preferred substrate for ADP1 and it grows better with gluconeogenic carbon sources, such as succinate and acetate (Young et al. 2005; Barbe et al. 2004).

ADP1 naturally produces storage lipids, namely wax esters and triacylglycerides (Fixter et al. 1986; Stöveken et al. 2005; Kalscheuer & Steinbüchel 2003), which makes it an appealing host for microbial hydrocarbon production. These energy-rich molecules serve as carbon and energy storage, and are mostly accumulated in the conditions where an excess of carbon and energy source is available (Fixter et al. 1986; Wältermann et al. 2005). Wax esters are more prevalent, and are synthesized by esterification of fatty acyl-CoA and fatty alcohol. The fatty alcohol is also derived from fatty acyl-CoA, via an aldehyde intermediate (Fig. 2). The wax esters typically compose of acyl chains with 16 or 18 carbons (Fixter et al. 1986), but the chain lengths can be modified by replacing the native pathway components with heterologous enzymes (Santala, Efimova, et al. 2014). Besides the availability of carbon, WE production in ADP1 is affected by temperature. While the optimal growth temperature of *A. baylyi* is 30 °C, the wax esters are more efficiently produced in lower temperatures (Santala, Efimova, et al. 2014).

2.3 Microbial CO_2 fixation

2.3.1 CO_2 reduction pathways

The reduction of carbon dioxide to organic compounds, performed by autotrophic organisms, is fundamental for the life on earth. There are currently six known carbon fixation pathways that support autotrophic growth (Fuchs 2011; Bar-Even, Flamholz, et al. 2012). Each pathway utilizes energy in the form of reduced electron carriers and ATP to assimilate two or three CO_2 molecules into a multicarbon product, such as acetyl-CoA or pyruvate (Bar-Even, Noor, et al. 2012). The pathways differ from each other with respect to oxygen sensitivity, and to the amount of energy required. A summary of the currently known six pathways is presented in Table 2.2. In the following section, two of these pathways, namely Calvin-Benson-Bassham cycle and Wood-Ljungdahl pathway, are briefly introduced.

Table 2.2. Properties of carbon fixation pathways. Modified from Bar-Even, Noor, et al. (2012)

Pathway	**Oxygen sensitivity**	**Number of ATP equivalents invested for the production of pyruvate**
Reductive pentose phosphate pathway	Aerobic	7
Wood-Ljungdahl pathway	Anaerobic	< 1*
Reductive TCA cycle	Anaerobic	2
Dicarboxylate-4-hydroxybutyrate cycle	Anaerobic	5
3-Hydroxypropionate-4- hydroxybutyrate cycle	Aerobic	9
3-Hydroxypropionate bicycle	Aerobic	7

* One molecule of ATP is consumed in the WL pathway, but some energy is conserved in a membrane gradient, and utilized for ATP production.

The most widespread pathway is the reductive pentose phosphate cycle (rPP, also called Calvin-Benson-Bassham cycle), which is employed by all plants and some prokaryotes, such as cyanobacteria. Most of the organisms utilizing the rPP cycle employ the light reactions of photosynthesis to power the CO_2 reduction. However, some bacteria, such as *Cupriavidus necator*, utilize the rPP cycle for chemoautotrophic growth, where hydrogen gas is used as the energy source (Pohlmann et al. 2006). The rPP cycle is relatively inefficient in terms of energy, requiring 7 molecules of ATP for the production of one acyl-CoA (Fast & Papoutsakis 2012). The key enzyme of the pathway is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is the only carboxylase in this pathway (Claassens et al. 2016). Due to the abundancy of the rPP cycle in nature, RuBisCO is responsible for the majority of CO_2 fixation of earth.

However, it is notoriously slow and inefficient, forcing the organisms utilizing rPP to express it in large quantities (Spreitzer & Salvucci 2002). Consequently, RuBisCO has been estimated to be the most abundant protein on earth (Ellis 1979; Spreitzer & Salvucci 2002). The inefficiency of RuBisCO is attributed to its catalytic mechanism, and to the utilization of O_2 as an alternative substrate. In the side-reaction involving molecular oxygen, the 1,5-bisphosphate is oxidized instead of carboxylated, leading to loss of CO_2 and waste of energy in the form of ATP and reducing equivalents (Erb 2011; Spreitzer & Salvucci 2002; Ducat et al. 2011), further increasing the ATP cost of the rPP pathway. There has been considerable interest towards enhancing the efficiency of RuBisCO by protein engineering. However, the specificity of Rubisco for CO_2 over O_2 is inversely correlated with the enzyme's catalytic activity; variants of RuBisCO that exhibit higher selectivity towards CO_2, are slower (Ducat et al. 2011; Tcherkez et al. 2006).

The most efficient pathway in terms of ATP requirements is the Wood-Ljungdahl pathway (WL pathway, also called reductive acetyl-CoA pathway), which requires less than one molecule of ATP for every acetyl-CoA produced (Fast & Papoutsakis 2012). In contrast to the rPP cycle, the WL pathway operates in anaerobic conditions, and many of the enzymes are extremely sensitive for oxygen (Ragsdale & Pierce 2008). In the Wood-Ljungdahl pathway, 2 molecules of CO_2 are reduced to form acetyl-CoA, with electrons derived from H_2 as the source of energy (Figure 2.3). The pathway consists of two branches, so called methyl branch and carbonyl branch (Drake et al. 2008). In the methyl branch, one molecule of CO_2 is reduced to methyl group, while in the carbonyl branch, the other molecule of CO_2 is reduced to carbon monoxide. The carbon monoxide is condensed with CoA and the methyl group to form acetyl-CoA. The acetyl-CoA can be further carboxylated to form pyruvate, and utilized for biomass formation. Alternatively, the acetyl-CoA can be converted to acetate, accompanied with substrate level phosphorylation of ADP to yield ATP.

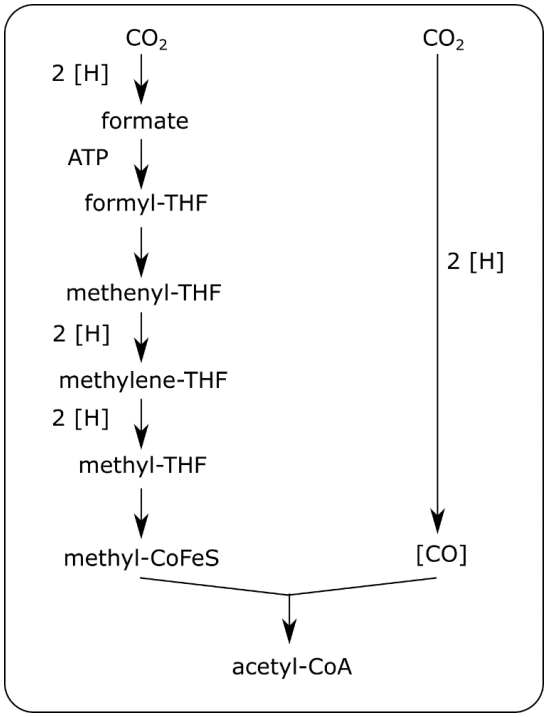

Figure 2.3. Simplified representation of the WL pathway. The energy investments required for each step are shown. If H_2 is the energy source, the reduction of electron carriers is accompanied with the formation of a chemiosmotic gradient across the cell membrane, which can be used for ATP synthesis. [H] – reducing equivalent; ATP – adenosine triphosphate; THF – tetrahydrofolate; [CO] – enzyme-bound carbon monoxide; CoFeS – corrinoid-iron-sulfur protein; CoA – coenzyme A. Modified from Bertsch & Müller (2015).

2.3.2 Acetogenic bacteria: potential for CO_2 reduction to organic molecules

Acetogenic bacteria (acetogens) are defined as anaerobes that can utilize the Wood-Ljungdahl pathway for (i) the synthesis of the acetyl moiety of acetyl-CoA from CO_2, (ii) the conservation of energy and (iii) the assimilation of CO_2 into biomass (Drake et al. 2008). The production of acetate is not included in the definition, for many non-acetogenic organisms are capable of acetate production by other mechanisms. Acetogenesis is not a phylogenetic trait; acetogens have been found in 22 different bacterial genera (Drake et al. 2008). Some genera contain both acetogenic and non-acetogenic species, whereas other genera contain only acetogens. Despite of the unifying feature of acetogenesis, acetogens are a very diverse group of bacteria with varying genetic and metabolic properties.

The WL pathway is different from all the other carbon assimilation pathways in that it can be coupled to the conservation of energy in the form of ATP (Schuchmann & Müller 2014; Bar-Even, Noor, et al. 2012). Acetogens can grow with CO_2 as the final electron acceptor, which means that the ATP required by the cells is produced by the WL pathway (Müller 2003). In the methyl branch of the WL pathway, one ATP molecule is consumed in the activation of formate. The ATP can be regained by the conversion of acetyl-CoA to acetate, but an additional mechanism for energy conservation is required in order to obtain positive ATP yield. However, the acetogens have very limited energy available for the synthesis of the extra ATP; the overall free energy change of acetyl-CoA production from H_2 and CO_2 allows the production of only less than one ATP molecule per acetyl-CoA produced (Poehlein et al. 2012). Thus, ATP cannot be produced by substrate level phosphorylation. Rather, the acetogens use either Na^+ or H^+ for the establishment of a chemiosmotic gradient across the cell membrane, and the gradient is used to produce ATP with a membrane-bound ATP synthase (Schuchmann & Müller 2014). Depending on the organism, the net ATP gain per molecule of acetate (including the WL pathway, the chemiosmotic gradient, and the conversion of acetyl-CoA to acetate) is estimated to vary from 0.13 to 0.6 (Schuchmann & Müller 2014). Thus, without the conversion of acetyl-CoA to acetate, the ATP yield would be negative. Consequently, the acetogens convert most of the acetyl-CoA to acetate during lithoautotrophic growth on CO_2 and H_2, and only a small fraction of the reduced CO_2 is used for biomass formation (Bertsch & Müller 2015). Therefore, acetogens present a robust and efficient, "evolutionarily optimized" chassis for acetate production from CO_2.

The reducing power for acetogens can be provided by oxidation of organic substrates or H_2 (Schuchmann & Müller 2014). Additionally, some bacteria are also capable of acquiring electrons directly from solid surfaces (Tremblay et al. 2017). Microbial electrosynthesis (MES) is a process where microbial catalyst reduces CO_2 into multicarbon compounds with electrons derived from the cathode of a bioelectrochemical system (Tremblay & Zhang 2015). MES was first demonstrated with the acetogen *Sporomusa ovata* in a study by Nevin et al (Nevin et al. 2010). *S. ovata* cells were cultivated in the cathode chamber of an H-cell with graphite electrodes, and a potentiostat was used to generate a potential difference between the anode and cathode. The cells formed a biofilm on the surface of the cathode, and utilized electrons generated from water at anode and provided to the cells through the cathode as the sole source of energy. In later studies, several other acetogenic bacteria, including *Clostridium ljungdahlii, Clostridium aceticum,* and *Moorella thermoacetica* have been reported to be capable of MES (Nevin et al. 2011; Tremblay & Zhang 2015). In addition, undefined mixed microbial populations have been utilized in MES (Jourdin et al. 2015; Bajracharya et al. 2017).

In MES, the electrons can potentially be transferred from the cathode to the cells by direct physical contact, or via various shuttle molecules, such as H_2, formate, or Fe^{2+} (Tremblay et al. 2017; Blanchet et al. 2015). Increasing the rate of the electron transfer from the cathode to the microbes has been the focus of intensive study, as this is the most straightforward way to increase the productivity of MES systems (May et al. 2016). One efficient strategy has been the development of novel cathode materials that support the interaction between the cells and the cathode, facilitating efficient electron transfer (Aryal et al. 2017; T. Zhang et al. 2013; Nie et al. 2013). In addition, the cathode potential has an effect on the production rate. Initially, the MES studies were conducted with the cathode poised at -400 mV vs standard hydrogen electrode (SHE) (Nevin et al. 2010; Nevin et al. 2011). This potential is above the potential required for abiotic H_2 production, and thus the electrons were presumably transferred by direct physical contact (Nevin et al. 2010). However, it was later noticed that the application of lower potentials, and thus intentional co-production of H_2, facilitated higher acetate production rates (Bajracharya et al. 2015; Marshall et al. 2013). However, the generation of H_2 lowers the efficiency of MES, because some of the electrons are lost, even though potentially some of the H_2 generated is utilized by the acetogens.

Because of the energetic limitations of the Wood-Ljungdahl pathway discussed earlier, acetogens are highly efficient in acetate production when grown with H_2 or with electricity as reducing power. In MES, ca. 80-95% of electrons is diverted to other products than cell biomass (mostly acetate) (Nevin et al. 2010; Tremblay & Zhang 2015). Thus, MES could serve as an efficient way of storing electricity. If coupled to solar electricity, MES can be thought as a form of artificial photosynthesis, converting solar energy, water and CO_2 into multicarbon molecules and O_2, but with significantly greater efficiency compared to the natural photosynthesis (Tremblay & Zhang 2015). As the renewable electricity production methods, such as solar or wind energy, are intermittent by nature, their large-scale application as part of the global energy production requires that the fluctuations are balanced by storing the surplus of electricity generated during production peaks. The surplus could potentially be utilized to convert CO_2 into acetate, which can be further utilized in the production of chemicals and fuels.

With regard to the utilization of solar energy for the powering of the WL pathway, a couple of interesting, even more direct approaches have been developed. Liu *et al.* developed a hybrid semiconductor nanowire-bacteria system for the direct application of solar energy for powering the acetogenesis (C. Liu et al. 2015). In their system, the light-harvesting nanowires directly delivered the electrons to *S. ovata* cells, which utilized them for CO_2 reduction. In another study, cadmium sulfide nanoparticles that were associated with *M. thermoacetica* cells harvested energy from light and delivered electrons to the cells, which utilized them for the CO_2 reduction (Sakimoto et al. 2016). These examples demonstrate the possibility to combine

inorganic light harvesting directly to non-photosynthetic bacteria in order to achieve photosynthetic production of organic molecules.

In addition to CO_2 and H_2, many acetogens can also grow on CO as the sole source of carbon and energy (FM Liew et al. 2016). In that case, part of the CO is oxidized to CO_2 in order to obtain the electrons for the reduction of CO to acetyl-CoA (Schiel-Bengelsdorf & Dürre 2012). Thus, acetogens can potentially utilize a wide range of feedstocks as carbon sources, including industrial waste gases, and gasified organic matter (Cheon et al. 2016; FungMin Liew et al. 2016). For example, synthesis gas (syngas) is a mixture of mostly CO_2, CO and H_2, and can be produced by gasification of heterogeneous raw materials such as municipal waste, biomass, or agricultural wastes (Daniell et al. 2012; Köpke et al. 2010). The gasification of biomass followed by gas fermentation with acetogens could potentially circumvent the issues associated with the utilization of recalcitrant biomass. Due to the possibility to utilize wastes or otherwise low-value carbon feedstock, gas fermentation is seen as a commercially attractive possibility to produce substitutes for fossil-derived chemicals and fuels (FM Liew et al. 2016).

While acetogens are naturally efficient at acetate production, the productivity has been increased further with metabolic engineering. For example, the heterologous expression of four WL pathway genes from *C. ljungdahlii* in *A. woodii* increased the acetate production rate compared to the wild type *A. woodii*, and enabled the production of 51 g/l acetate in less than 4 days (Straub et al. 2014). In another study, adaptive laboratory evolution was used in order to improve the acetate production rate of *S. ovata* in MES (Tremblay et al. 2015). In addition, improved reactor design involving full cell retention enabled the acetate production from H_2 and CO_2 with a maximum titer of 59 g/l and a space-time yield of 6.2 g/(l*h), in a study utilizing the wild type *A. woodii* (Kantzow et al. 2015). In MES systems, the highest reported acetate titer is 13.5 g/l, achieved with improved reactor design using a mixed microbial community (Gildemyn et al. 2015).

Even though acetate is the main product of acetogens, some strains produce also other compounds, such as ethanol and 2,3-butanediol (Bertsch & Müller 2015; Molitor et al. 2017). Furthermore, metabolic engineering has been applied in order to increase the production of non-acetate products, and to enable the production of non-natural compounds (reviewed in (FM Liew et al. 2016)). For example, *C. ljungdahlii* has been engineered for the butanol and butyrate production (Ueki et al. 2014; Köpke et al. 2010). The metabolic engineering tools for acetogens are not as well-established as for more conventional microbial hosts (Nybo et al. 2015). However, the development of new tools have been reported, including an inducible expression system (Banerjee et al. 2014) and a CRISPR/Cas9 based genome editing system for *C. ljungdahlii* (Huang et al. 2016). Despite the increasing understanding about the acetogenic

metabolism and the development of novel tools, the energy metabolism of acetogens poses fundamental limits for their metabolic engineering (Molitor et al. 2017; Bertsch & Müller 2015). Specifically, as the acetogens have limited capacity for ATP production during growth on CO_2 and H_2, relatively large fraction of the carbon has to be directed to acetate to obtain positive ATP balance, even if metabolic engineering is applied to enable the production of other compounds (Bertsch & Müller 2015).

3 Objectives and hypotheses of the study

Based on the background information presented in Chapter 2, the **objectives** and hypotheses of the study are detailed as:

1) **To establish a two-stage bacterial platform for long-chain hydrocarbon production from carbon dioxide and electricity (II, IV)**
   According to the hypotheses, acetogenic bacteria can be used to produce acetate and/or other organic molecules, which can be used for growth and lipid production by *A. baylyi* ADP1. The acetogens can utilize CO_2 as the sole carbon source and H_2 or electricity as the sole energy source. *A. baylyi* can utilize the liquid end products of the acetogenic cultivation as the sole sources of carbon and energy. (An) acetogenic strain(s) can be found that support efficient *A. baylyi* growth in the used medium. A two-stage production system can be established, where the acetogenic strain is first grown in anaerobic conditions with CO_2 and H_2/electricity, and in the second phase, *A. baylyi* is grown in aerobic conditions in the medium used by the acetogen. The final product can be determined by genetically engineering *A. baylyi*.

2) **To improve the fatty aldehyde production in A. baylyi (III)**
   According to the hypotheses, the production of fatty aldehydes can be improved in *A. baylyi* by the overexpression of one or several key enzymes of the pathway. The enzymes may be either native or heterologous. The production of the fatty aldehydes can be monitored with a previously developed in vivo monitoring tool, which will give valuable information about the function and dynamics of long-chain hydrocarbon production in *A. baylyi*. The overexpression will lead to relaxation of the native regulation of the fatty aldehyde biosynthesis, allowing efficient production in more diverse conditions, such as different temperatures. The improved flux towards the fatty aldehydes will enhance the production of downstream products by either native or heterologous pathways (e.g. wax ester or alkane biosynthesis).

3) **To construct an alkane biosensor that can be utilized to study and improve alkane biosynthesis (I)**

   According to the hypotheses, an alkane biosensor that will respond to exogenous and endogenous aliphatic alkanes can be constructed in *A. baylyi*. The sensor will work on two layers, recognizing both long-chain alkane and the central intermediate in alkane biosynthesis, fatty aldehyde. The sensor will respond to alkanes and fatty aldehydes independently of each other, and produce output signals that can be distinguished from each other. The signals can be measured *in vivo*, in real time, during the cell growth. Because of the two-layer configuration, the sensor will give valuable information about the dynamics of the pathway, which can be used in the construction and optimization of a heterologous alkane biosynthesis pathway in *A. baylyi*.

4) **To convert the natural alkane degrader *A. baylyi* ADP1 into alkane producer (I, IV)**

   According to the hypotheses, the native alkane-degradation pathway can be eliminated from *A. baylyi*, leading to a strain that is incapable of growth on aliphatic long-chain alkanes. Furthermore, this strain can be conferred with the capability of *de novo* alkane biosynthesis by expressing one or more heterologous enzymes, such as the aldehyde-deformylating oxygenase.

5) **To demonstrate the production of long-chain alkanes and wax esters from acetate in *A. baylyi* and to connect the acetate production by the acetogens to the hydrocarbon production by *A. baylyi* (II, IV)**

   According to the hypotheses, acetate can be used as the sole carbon and energy source for the biosynthesis of long chain hydrocarbon products by *A. baylyi*. These products can be either wax esters, produced by the wild type *A. baylyi*, or alkanes, produced by genetically engineered *A. baylyi*. Furthermore, the acetate can be produced by the acetogens from CO_2 either with H_2 or electricity as the energy source. The acetogenic culture medium, containing the acetate, can be used for the *A. baylyi* cultivation as such. By connecting the two modules, the production of long-chain hydrocarbons from CO_2 can be demonstrated, and targets for future development of the platform identified.

4 Summary of materials and methods

This chapter summarizes the materials and methods used in the original studies (I-IV). More detailed descriptions can be found in each original publication.

4.1 Bacterial strains and genetic engineering

Acinetobacter baylyi ADP1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, accession number DSM 24193), *Acetobacterium woodii* (DSM 1030), *Clostridium aceticum* (DSM 1496), *Clostridium ljungdahlii* (DSM 13528), and *Sporomusa ovata* (DSM 2662) were used as the wild type strains in the studies. For microbial electrosynthesis, a *S. ovata* strain previously developed by adaptive laboratory evolution (Tremblay et al. 2015) was used. For cloning and plasmid maintenance, *Escherichia coli* XL1-Blue (Stratagene, USA) was used.

The molecular work was carried out using established methods. Primers were ordered from ThermoFischer Scientific (USA), and synthetic genes from GenScript (USA).

An integrative cassette used for inducible gene expression in *A. baylyi* (I, III, IV) was derived from the cassette described by Murin et al (Murin et al. 2012). The cassette contains the *lacI* gene and a Biobrick RFC10- accepting cloning site (EcoRI-XbaI--SpeI-PstI) under an IPTG-inducible promoter *lac*/T5, as well as a spectinomycin resistance marker under its own promoter (Figure 4.1). The BioBrick RCF10 cloning cite allows the sequential cloning of multiple genes to the same cloning site (Shetty et al. 2008). The construct is flanked by sequences targeting the homologous recombination of the cassette to a prophage region of *A. baylyi*.

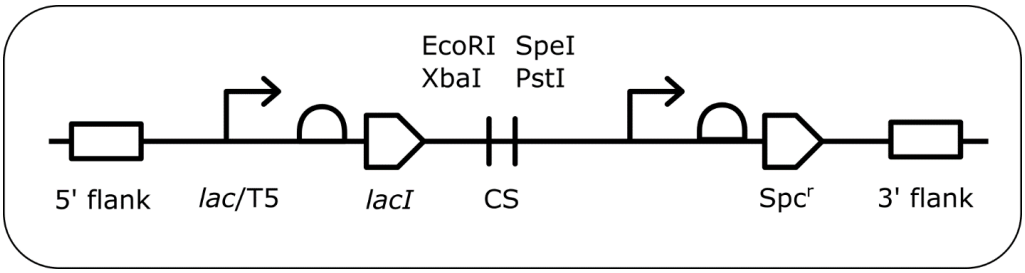

Figure 4.1. The expression cassette used for inducible gene expression. The target gene(s) were cloned in the cloning site (CS) under the lac/T5 promoter using the restriction enzymes indicated in the figure. The flanking sites contain homologous sequences targeting the homologous recombination of the cassette to a prophage region of *A. baylyi* genome. CS – cloning site; Spc^r – spectinomycin resistance marker. Modified from (III).

Genomic deletions in *A. baylyi* were performed by utilizing a previously developed knockout cassette (Santala, Efimova, Kivinen, et al. 2011) as a scaffold. The cassette was modified by changing the flanking sites to facilitate the replacement of the target gene(s) with the cassette by homologous recombination. In addition, the cassette contained a selection marker (either chloramphenicol or kanamycin resistance). In the biosensor applications, the cassette was further equipped with reporter genes (either *luxA* and *luxB* (I, III, IV), or *gfp* (I, IV)). The key knockouts generated and utilized in the study were *acr1* and *alkM*. *Acr1* encodes for the native fatty aldehyde reductase (FAR) in *A. baylyi*, and it was removed to produce a FAR-negative strain where the performance of heterologous FARs could be studied. *AlkM* is induced by aliphatic alkanes (A Ratajczak et al. 1998) and was replaced with a *gfp* gene, resulting in a biosensor strain where the expression of GFP is induced by alkanes (II, IV). In addition, *alkM* is essential for growth on alkanes (A Ratajczak et al. 1998), and thus the knockout strain was utilized in alkane production experiments. Properties of the most relevant genetically engineered strains utilized in the study are summarized in Table 4.1, and the enzymes related to hydrocarbon production that were utilized in the study are listed in Table 4.2.

Table 4.1 Properties of genetically engineered *A. baylyi* strains used in the study.

Strain	Relevant knockouts	Biosensor elements	Overexpressed genes	Reference
S1	*acr1*	*luxAB*	Fatty acyl reductases[1]	III
W1	*acr1*		Fatty acyl reductases[1]	III
ASI	*acr1, alkM*	*luxAB, gfp*	Fatty aldehyde- and alkane-producing genes[2]	I, IV

[1] Three different FARs expressed separately (see Table 4.2)

[2] Three different FARs and three different alkane-producing genes expressed in pair-wise combinations (see Table 4.2)

Table 4.2. Enzymes utilized for hydrocarbon production in the study.

Name	Origin	Function	Reference
Acr1	*Acinetobacter baylyi*	Fatty acyl-CoA reductase (FAR)	III-IV
Ramo	*Nevskia ramosa*	Putative FAR	III-IV
AAR	*Synechococcus elongatus*	Fatty acyl-ACP reductase	I, III-IV
SeADO	*Synechococcus elongatus*	Aldehyde-deformylating oxygenase	I, IV
PmADO	*Prochlorococcus marinus*	Aldehyde-deformylating oxygenase	IV
CER1	*Arabidopsis thaliana*	Aldehyde decarbonylase	IV

A. baylyi was transformed with natural transformation as described previously (Metzgar et al. 2004). The cells were cultivated in LB medium and the purified plasmid containing the integrative cassette was added to the cultivations in exponential growth phase. The cells were cultivated for an additional 2-3 hours and plated on a selective LA plates. The concentrations of antibiotics were 25 µg/ml for chloramphenicol and 50 µg/ml for spectinomycin and kanamycin.

4.2 Cultivations

For cloning and routine maintenance, *E. coli* and *A. baylyi* were grown in Lysogeny Broth (LB), containing (per liter): 10 g tryptone, 5 g yeast extract, and 5 g NaCl. For growth on solid medium, 15 g/l agar was added to LB. For growth and hydrocarbon production experiments, *A. baylyi* was grown in modified minimal salts medium MA/9 (I-IV), containing (per liter): 5.52 g Na_2HPO_4 * 2 H_2O, 3.4 g KH_2PO_4, 1 g NH_4Cl, 0.008 g nitrilotriacetic acid, 0.5 mg $FeCl_3$, 11.1 mg $CaCl_2$ and 240 mg $MgSO_4$. The medium was supplemented with casein amino acids, carbon source (acetate or glucose), antibiotics, and an inducer, as appropriate. The acetogens were cultivated in DSM 311 (II), or DSM 135 (IV) media without organic carbon substrate. The acetogenic cultures were sparged with a gas mixture of H_2/CO_2 (gas fermentation) or N_2/CO_2 (microbial electrosynthesis). All cultures were carried out at 20-37 °C.

For microbial electrosynthesis (II), two graphite stick electrodes (36 cm^2 each) were suspended in 250 ml of the DSM 311 medium in two chambers separated by an ion exchange membrane. A potentiostat was used to poise the cathode at -690 mV (vs. SHE). The cells were grown in the cathode chamber at room temperature, and both chambers were continuously flushed with N_2/CO_2 gas.

4.3 Analytical methods

The analysis methods of used in the study are summarized in Table 4.3.

Table 4.3. Summary of the analytical methods used in the study.

Analysis	Method/Instrument/Parameters	Reference
Biomass		
Optical density	Spectrophotometer, 600 nm	I-IV
	Microplate reader (Tecan Spark), 600 nm	IV
Cell dry weight	Freeze-drying, gravimetric	II-III
Luminescence	Xenogen IVIS Lumina	I, III
	Microplate reader (Tecan Spark)	IV
Fluorescence	Xenogen IVIS Lumina, ex: 445-490 nm, em: 515-575 nm	I
	Microplate reader (Tecan Spark), ex: 475-495, em: 505-515 nm	IV
Acetate, glucose	HPLC	II-IV
Wax esters	Solvent extraction + TLC or NMR	II-III
Alkanes, fatty alcohols	Solvent extraction + GC-MS	I, III-IV

TLC – thin layer chromatography; NMR – nuclear magnetic resonance spectroscopy, GC-MS – gas chromatography-mass spectrometry

5 Summary of results and discussion

This chapter presents and discusses the most important results obtained in the study, and their implications with respect to the objectives and hypotheses described in Chapter 3, and to the background information presented in Chapter 2. The specific findings are discussed in more detail in the original publications (I-IV).

5.1 Developing a two-stage bacterial platform for long-chain hydrocarbon production from carbon dioxide and electricity (II, IV)

As described in Chapter 2, the Wood-Ljungdahl pathway utilized by the acetogenic bacteria is an efficient way of producing organic molecules from carbon dioxide. The majority of the carbon fixed through this pathway during lithoautotrophic growth is converted to acetate and secreted out from the cells. As man-made devices (e.g. photovoltaic cells) are more efficient at capturing the solar energy than the natural photosynthesis (Blankenship et al. 2011), the acetogenesis powered with solar energy is seen as a promising technology for the production of solar fuels and chemicals.

Even though the acetogens are efficient at acetate production, due to their restrained capacity for energy conservation they have very limited energy available for the production of more energy-intensive molecules (Schuchmann & Müller 2014; Molitor et al. 2017). Furthermore, acetogens are not as well studied as the conventional microbial hosts, and thus the limited knowledge about their genetics and the lack of established gene editing tools hinders their metabolic engineering. However, energy-intensive long-chain hydrocarbon products, such as alkanes, fatty alcohols, and wax esters, would be the desired products for many industrial applications, such as the production of liquid fuels. In contrast to acetogens, microbes that are more amenable for genetic engineering and have a more relaxed energy metabolism have the potential to produce the long carbon chains from simple precursors.

In this study, a two-stage bacterial platform for the production of long-chain hydrocarbons from carbon dioxide and electricity was developed. The system consists of two modules. In the first module, acetate is produced by acetogenic bacteria from CO_2 as the carbon source, and electricity or H_2 as the energy source (Figure 5.1, A). In the second module, the acetate is used as a carbon and energy source for *A. baylyi* ADP1 for growth and hydrocarbon production (Figure 5.1, B). Acetate is the central connection between the modules. Consequently, the platform can be utilized for the construction of different processes by utilizing different

acetate-producing and -consuming modules. The modular nature of the platform enabled the development of both parts independently of each other. In paper II, the focus was on the establishment of the platform, and the intercompatibility of the two modules, whereas papers I, III, and IV focused more on the improvement of the second (*A. baylyi*) module.

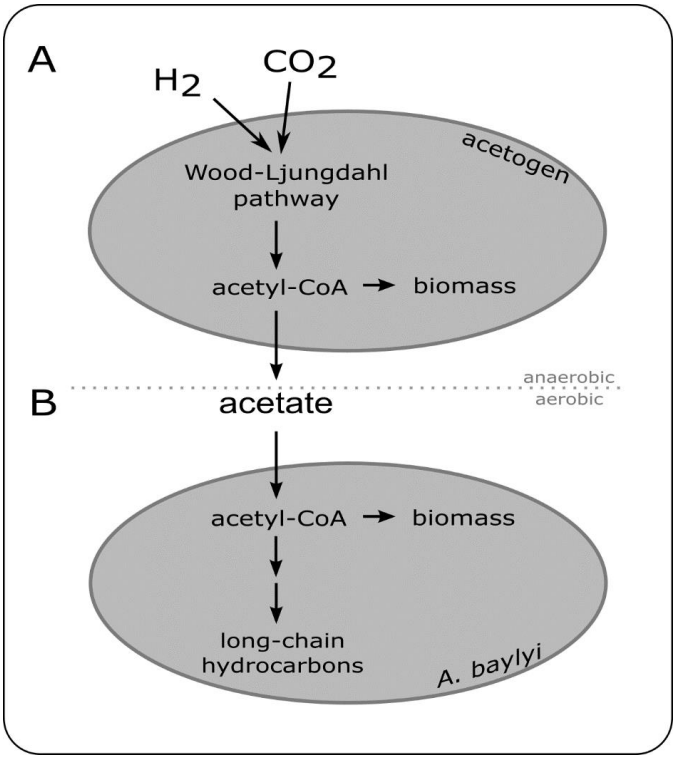

Figure 5.1. Two-stage bacterial platform for long-chain hydrocarbon production from carbon dioxide. Simplified representation of the distribution of the metabolic pathways between the two bacteria; Acetogens produce acetate from CO_2 in anaerobic conditions (A), whereas *A. baylyi* utilizes acetate for the production of long-chain hydrocarbons in aerobic conditions (B). CoA – Coenzyme A

A prerequisite for the establishment of the two-stage production system is that the two species do not inhibit each other's growth. The platform utilized here relies on sequential growth of the microbes, first in anaerobic and then in aerobic conditions. In this respect it is different from many co-culture strategies (discussed in Chapter 2), where the microbes are grown simultaneously. However, after the first phase, the medium should ideally be able to support *A. baylyi* growth as such, without the requirement for addition or removal of any components.

Thus, different acetogenic strains were studied for their ability to grow and produce acetate in a defined medium that had a suitable composition to support *A. baylyi* growth (II). The acetogens were grown with CO_2/H_2 in sealed bottles, and their growth and acetate production were measured. Two of the tested acetogens (*A. woodii* and *S. ovata*) performed markedly better than the other two (*C. aceticum* and *C. ljungdahlii*), in terms of growth and acetate production. In these small-scale batch cultivations, *A. woodii* and *S. ovata* produced approximately 20-25 mM acetate.

In the second phase, the acetogenic cells were removed from the medium by centrifugation, and the ability of the supernatant to support the growth and lipid production of wild type *A. baylyi* was studied. It was found that the L-cysteine, used in the anaerobic medium as a reducing agent, inhibited *A. baylyi* growth, inducing a long lag phase. In *E. coli*, cysteine has been noticed to specifically inhibit the biosynthesis of several amino acids (Kari et al. 1971), which might explain the observed results. Indeed, the addition of casein amino acids relieved the growth inhibition and enabled *A. baylyi* growth in the acetogenic medium. When provided with the casein amino acids, *A. baylyi* utilized the acetate produced by the acetogens for growth and lipid production. The hydrocarbon production from acetate is discussed in more detail in Chapters 5.2 and 5.3.

Based on the good performance of *A. woodii* and *S. ovata* in the first test, they were utilized for the acetate production throughout the rest of the study. In paper II, the possibility to utilize microbial electrosynthesis as the acetate production module was studied. *S. ovata* grown in a MES reactor with electricity and carbon dioxide as the sole sources of energy and carbon, respectively, produced approximately 25 mM acetate. The medium from the cathode chamber of MES was demonstrated to support *A. baylyi* growth and lipid production equally well as the medium from the bottle cultivations. In paper IV, *A. woodii* was utilized for acetate production in gas fermentation. The *A. woodii* cells were cultivated in a bioreactor with H_2 and CO_2 as the sole sources of energy and carbon, respectively. Potentially due to the pH control during the cultivation, the cells produced markedly more acetate (~190 mM) than in bottle cultivations. The medium from *A. woodii* cultivation was utilized as the feed in a fed-batch cultivation of *A. baylyi*, and served as the carbon and energy source for *A. baylyi* growth and lipid production.

The two-stage production system enables the growth of *A. baylyi* with carbon derived from CO_2 and energy derived from H_2 or electricity. Three different modes of acetate production were applied (bottle, MES and bioreactor), highlighting the possibility to utilize different acetate-producing strategies. Potentially also other acetate-producing processes, such as wastewater treatment (Pan et al. 2017), could be coupled to the system. The modification of the second module by genetically engineering *A. baylyi* is discussed in the following sections.

5.2 Engineering *A. baylyi* for improved lipid production (I, III-IV)

A. baylyi naturally produces wax esters with approximately 32-36 carbons through the fatty aldehyde intermediate (Reiser & Somerville 1997; Fixter et al. 1986). This natural ability for long-chain hydrocarbon synthesis was utilized in the study. In paper II, the native WE synthesis pathway was used as a proof-of-concept for the production of long carbon chains from CO_2. In paper III, the production of fatty aldehydes and wax esters through the native pathway was improved by overexpressing a key enzyme, fatty acyl-CoA reductase (Figure 5.2). In papers I and IV, heterologous alkane-producing pathway was introduced and studied, in order to enable the production of long-chain alkanes (Figure 5.2).

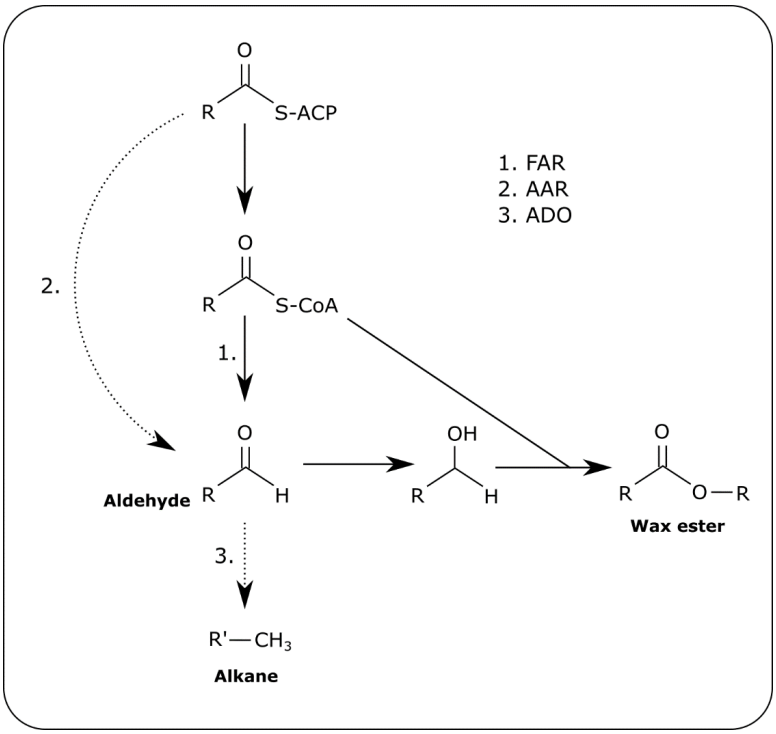

Figure 5.2. Engineering the hydrocarbon metabolism of *A. baylyi*. The natural wax ester (WE) production pathway (proposed pathway, black arrows) was utilized for WE production (II). Additionally, aldehyde and WE production was improved by overexpression of native of heterologous fatty acyl-CoA/ACP reductases (FAR/AAR; III). Heterologous alkane production was achieved in *A. baylyi* by the expression of cyanobacterial AAR and ADO (I, IV). ACP – Acyl carrier protein; CoA - Coenzyme A; AAR – Acyl-ACP reductase; FAR – Fatty acyl-CoA reductase; ADO – Aldehyde-deformylating oxygenase; R – acyl chain with n carbon atoms; R' – acyl chain with n-1 carbon atoms.

5.2.1 Improved fatty aldehyde and wax ester production (III)

Fatty aldehydes are central intermediates in the biosynthesis of long-chain n-alkanes and wax esters (Figure 2.2). Thus, improving the fatty aldehyde production was selected as the first target in the engineering of *A. baylyi*. In *A. baylyi*, fatty aldehydes are naturally produced by the fatty acyl-CoA reductase (FAR) Acr1 (Figure 2.2). In order to increase the fatty aldehyde production, the natural expression of the Acr1 was overridden by overexpressing either the native Acr1 or two heterologous FARs (**III**). The two heterologous FARs selected for study were the fatty acyl-CoA/ACP reductase AAR from *Synechococcus elongatus* and a putative short chain dehydrogenase WP_022976613.1 (designated Ramo) from *Nevskia ramosa*. As discussed in Chapter 2.2, AAR has been widely applied in alkane biosynthesis in native and non-native hosts. In an attempt to increase the pool of available reductases for metabolic engineering purposes, a previously uncharacterized, putative reductase was included in the study. Ramo was selected based on its sequence homology with Acr1. In addition, a putative WS/DGAT homolog was identified from the genomic sequence of *N. ramosa*, indicating that Ramo could be part of a functional wax ester producing pathway. Importantly for aldehyde production, Acr1 and Aar have been characterized not to reduce aldehydes to alcohols, unlike many other reductases characterized to date (Chapter 2.2). The three FAR enzymes were separately overexpressed in a FAR-negative *A. baylyi* strain, and the effect of the overexpression on the fatty aldehyde and WE production was studied. The aldehyde production was measured with a previously developed luciferase-based *in vivo* biosensor (Santala, Efimova, Karp, et al. 2011). In the biosensor, the bacterial luciferase LuxAB utilizes the fatty aldehyde as a substrate in a reaction that produces visible light, allowing real-time analysis during cell growth.

The cells were grown at 30°C or room temperature (ca. 24°C), and the aldehyde production of the overexpression strains was compared to the strain with the native Acr1 expression (III). At 30°C, the overexpression of any of the three genes led to increased fatty aldehyde production, as measured by the biosensor. Furthermore, higher induction of the FAR expression led to higher aldehyde production. Thus, the possibility to increase the fatty aldehyde production in *A. baylyi* in a controllable manner was demonstrated. The highest luminescence signals were obtained with AAR. In contrast, at room temperature, the overexpression did not significantly increase the aldehyde production compared to the native Acr1 expression.

In order to study the fate of the overproduced aldehydes, wax ester production of the overexpression strains was studied. It was expected that at least part of the increased fatty aldehydes would be directed to the WE production pathway, and thus increase the WE production. Indeed, the overexpression of Acr1 or Ramo at 30°C led to two-fold improvement in the WE production compared to the control cells with native Acr1 expression. However, the

strain overexpressing AAR produced only a small amount of WEs, despite the high luminescent signal obtained. At 20°C, the overexpression of Acr1 or Ramo did not significantly increase the WE production compared to the wild type ADP1.

The optimal growth temperature of *A. baylyi* is 30 °C, but wax ester accumulation is more efficient at lower temperatures (Santala, Efimova, et al. 2014). However, the mechanisms regulating WE production are currently not known. The overexpression of FARs at 30 °C increased the WE production to a comparable level to that of the wild type cells at 20 °C. Thus, based on the luminescence and WE production data, it can be concluded that fatty aldehyde production is an important regulatory step in the WE production. The overexpression of Acr1 or Ramo overrides the natural temperature dependency and enables simultaneous efficient growth and wax ester production at the optimal growth temperature of *A. baylyi*. The cultivation time in all experiments was 48 hours, which allowed also the cells grown at lower temperatures enough time to reach the stationary phase. However, as the overexpression enables efficient WE production also at the optimal growth temperature, the cultivation time could potentially be shortened, thus improving the productivity.

As the FAR overexpression did not increase the aldehyde or WE production at lower temperatures (room temperature or 20 °C, respectively), is seems that the FAR activity is not limiting the activity of this pathway at these temperatures. Acr1 expression levels were not measured, but it can be speculated that the natural expression of Acr1 might be higher at lower temperatures, thus leading to more efficient aldehyde production.

Even though the expression of AAR led to high fatty aldehyde production (as measured by the biosensor), the cells produced little WEs. In order to elucidate the fate of the aldehydes, fatty alcohol production of the overexpression strains were also determined. Fatty alcohol is the next step in the WE production pathway (Figure 2.2). In *E. coli*, aldehydes are rapidly converted to corresponding alcohols by endogenous aldehyde reductases (Rodriguez & Atsumi 2014). Presumably, the case is similar in *A. baylyi*, although the gene(s) associated with fatty aldehyde reduction have not been identified. Some FARs from other organisms also possess aldehyde reductase activity; however, the native *A. baylyi* FAR, Acr1, has been characterized not to reduce aldehydes (Reiser & Somerville 1997). Nevertheless, there must be at least one unidentified enzyme in *A. baylyi* performing this conversion, because it is essential in the WE production pathway. When compared to *A. baylyi* with the native Acr1 expression, cells overexpressing AAR produced less fatty alcohols, suggesting that the reason for the poor WE production might be inefficient reduction of fatty aldehydes to fatty alcohols. As discussed in paper III, one possible explanation for this inefficiency could be the potential differences in the subcellular location of the aldehydes produced by AAR as compared to those produced by Acr1.

Interestingly, in an earlier study, the expression of LuxCDE complex in the place of Acr1 did not increase the wax ester production either (Santala, Efimova, et al. 2014). In that study, the aldehyde production was not compared to the wild type ADP1, and thus it is not clear if the case is similar to that of AAR (high aldehyde production and low WE production). Nevertheless, the increased WE production associated with the expression of Ramo demonstrates that the aldehydes produced by some heterologous enzymes can be directed to the WE synthesis.

In contrast to AAR, the Acr1 overexpression led to increased fatty alcohol production, suggesting that the overproduced aldehydes were efficiently converted to alcohols by the endogenous enzyme(s). The accumulation of fatty alcohols suggests that the final enzyme, WS/DGAT may be limiting the WE production when Acr1 is overexpressed. Thus, in future studies, the WE production could potentially be further increased by combining WS/DGAT overexpression with the FAR overexpression.

The luminescence signal produced by the biosensor is dependent on multiple factors, such as the cofactor and energy status of the host cell, and thus the signal must be considered only semi-quantitative. Consequently, the luminescence signals cannot be compared between different cultivations or growth conditions. In the studied conditions, it was assumed that the fatty aldehyde concentration was the limiting factor, and thus that the luminescence signal would be proportional to the fatty aldehyde concentration. This is supported by the observation that increasing FAR expression led to increasing luminescence signals. However, for example the subcellular location and diffusion of the fatty aldehydes might affect their availability to the LuxAB, thus potentially affecting the results. Fatty aldehydes were not detected using GC-MS in cells or in the culture medium, indicating that they were rapidly converted to other compounds by endogenous enzymes. Similar observation has been done in *E. coli*; fatty aldehydes were not detected by GC-MS from cells expressing cyanobacterial AARs (Kudo et al. 2016). Kudo *et al.* estimated the aldehyde-producing activity by converting the aldehydes to alkanes *in vivo* and measuring the alkanes with GC-MS. Compared with that strategy, the luminescence biosensor enables more straightforward detection, which can be performed in real time during cell growth.

5.2.2 Construction of a twin-layer alkane biosensor and heterologous alkane production in *A. baylyi* (I, IV)

The metabolic engineering of microbial alkane production is hampered by the lack of sensitive and specific tools for high-throughput analysis of the produced alkanes. As discussed in Chapter 2, intracellular biosensors may enable straightforward and sensitive analysis of small molecules. In this study, an *in vivo* biosensor was developed and utilized for the construction and optimization of the alkane-producing pathway in *A. baylyi*. The sensor, named twin-layer alkane

biosensor, consists of two parts that monitor different parts of the alkane metabolism (Figure 5.3). First, the bacterial luciferase LuxAB was employed for the detection of the fatty aldehyde intermediate. Secondly, a *gfp* gene under an alkane responsive promoter responds to alkanes. The *luxA* and *luxB* genes are constitutively expressed, and the signal is determined by the concentration of the fatty aldehyde substrate. On the other hand, the expression of GFP is dependent on the alkane concentration. The two different signals can be measured with microplate readers in real time during cell growth.

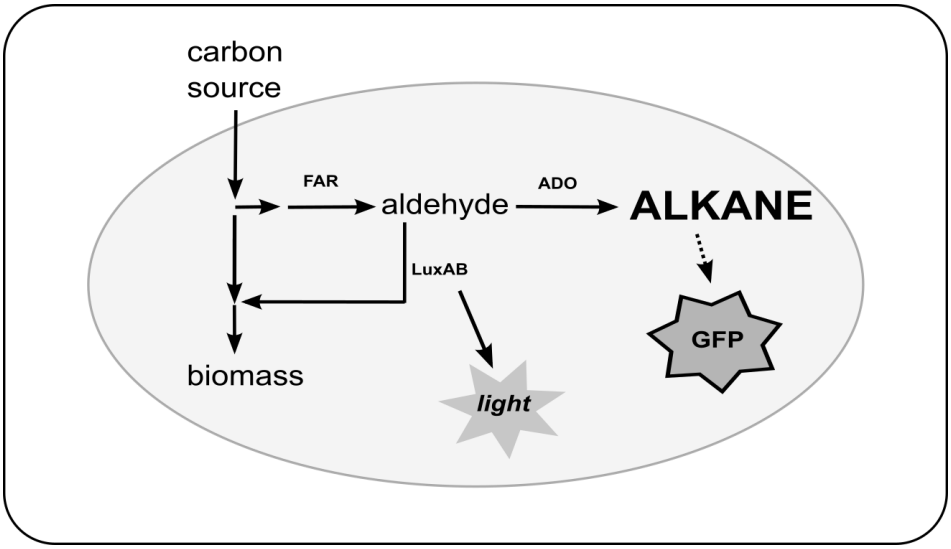

Figure 5.3. Twin-layer biosensor for monitoring of alkane biosynthesis. The biosensor is constructed in *A. baylyi* and consists of two parts. In the first part, the bacterial luciferase LuxAB utilizes fatty aldehyde as a substrate in a reaction that produces visible light. In the second part, the expression of GFP is controlled by a native alkane-responsible promoter of *A. baylyi*. Thus, the aldehyde concentration is reflected in the visible light, and alkane concentration in the fluorescence signal. The heterologous expression of the alkane-producing pathway leads to the production of alkanes via the aldehyde intermediate. FAR – fatty acyl-ACP reductase; ADO – aldehyde-deformylating oxygenase. Adapted from **I**.

The combination of two different levels (intermediate and the final product) yields information about the pathway function and dynamics, and allows rational design for improved pathway performance. Specifically, in the alkane biosynthesis pathway, the fatty aldehyde intermediate is problematic for two main reasons. Firstly, it has toxic effects to the cells and is usually rapidly metabolized by the endogenous enzymes in the cells, such as aldehyde reductases or dehydrogenases (Rodriguez & Atsumi 2014; Ishige et al. 2000). The competing enzymes divert

the long carbon chains from the alkane-producing pathway, lowering the yield. Secondly, the alkane-producing enzyme (ADO) is notoriously inefficient, and is considered to be the bottleneck in the heterologous alkane biosynthesis (Marsh & Waugh 2013). Thus, the aldehyde production must be carefully balanced in order to avoid the negative effects of fatty aldehyde accumulation, while simultaneously providing ADO with sufficient substrate to enable efficient alkane production. Because of the twin-layer configuration, the biosensor developed in this study holds potential for more thorough optimization of the aldehyde production than a sensor working on a single level.

A. baylyi can naturally degrade aliphatic alkanes and utilize them for growth. Thus, as the first step towards the heterologous alkane production in *A. baylyi*, the *alkM* gene that encodes for alkane hydroxylase and is essential for alkane degradation (Andreas Ratajczak et al. 1998; A Ratajczak et al. 1998), was deleted. In the alkane biosensor strain, *alkM* is replaced with *gfp*, leading to a strain that is incapable of growth on alkanes, and where the expression of GFP is regulated by the alkane-inducible promoter of *alkM* (A Ratajczak et al. 1998).

In paper I, the suitability of the sensor for monitoring the alkane biosynthesis and degradation was demonstrated. For the alkane biosynthesis, the cyanobacterial two-step pathway consisting of AAR and ADO was expressed in the sensor strain. The biosensor revealed the functionality of the heterologous pathway. Namely, both luminescence and fluorescence signals were associated with the expression of the pathway, indicating fatty aldehyde and alkane production, respectively. The production of alkanes was also confirmed with GC-MS. This was the first report of alkane production in the non-native producer *A. baylyi*.

In paper IV, the sensor was used for the optimization of the alkane biosynthetic pathway. Although various aldehyde- and alkane-producing enzymes have been utilized in heterologous alkane production (Chapter 2.2), the performance of synthetic pathways consisting of enzymes originating from different organisms has not been compared. It has been previously speculated that ADO and AAR might interact with each other in order to ensure the efficient delivery of the intermediate fatty aldehyde between the enzymes (Warui et al. 2015). Furthermore, as shown earlier (Chapter 5.2.1, III), the aldehydes produced by different enzymes might be channeled to different downstream pathways in *A. baylyi*. Specifically, as the aldehydes produced by AAR were not efficiently converted to alcohols in *A. baylyi*, AAR might be an optimal reductase for heterologous alkane production pathway.

In order to screen for the most efficient alkane-producing pathway, nine synthetic, two-step pathways were constructed. The pathways consisted of aldehyde- and alkane-producing genes.

The alkane-producing enzymes selected in this study were ADOs from *S. elongatus* (seADO) and *P. marinus* (pmADO), as well as CER1 from the plant *A. thaliana*. All of these enzymes have been previously utilized in heterologous alkane production in *E. coli* or *S. cerevisiae* (Chapter 2.2). For the aldehyde production, the same reductases studied previously (Acr1, Ramo, and AAR), were utilized. The expression of any of these reductases was shown in paper III to increase the fatty aldehyde production in ADP1. The aldehyde- and alkane-producing genes were sequentially cloned into the expression cassette in pair-wise combinations, resulting in nine different synthetic operons. These synthetic operons were expressed in the twin-layer biosensor strain, and the aldehyde and alkane production was compared with the sensor. The highest aldehyde production was associated with the expression of CER1-AAR, whereas the expression of SeADO-AAR led to the highest alkane production.

In order to avoid the potentially harmful accumulation of pathway intermediates, as well as the unnecessary burden caused to the host cells by high-level protein expression, the enzyme activities of synthetic pathways need to be balanced (Jones et al. 2015). In the cyanobacterial alkane synthesis pathway, the relatively low activity of ADO compared to AAR may lead to the accumulation of fatty aldehydes, potentially causing toxic effects or loss of carbon to other pathways. Thus, in paper IV, the biosensor was utilized for balancing the relative expression levels of SeADO and AAR in order to further increase the alkane production. The ribosome binding site (RBS) of AAR was altered, and the effects on aldehyde and alkane production were measured. As expected, the highest aldehyde production was obtained with the strongest RBS. In contrast, an RBS with intermediate strength resulted in the highest alkane production, suggesting that lowering the relative expression of AAR leads to more balanced metabolism and higher alkane production. This result is supported by the observation that accumulation of fatty aldehydes was not beneficial for alkane production in *E. coli* (Cao et al. 2016).

Despite the optimization of the alkane-producing pathway, the alkane yields remained very low (IV). It has been noticed that ADO works more efficiently *in vitro* when a cyanobacterial electron transfer system is used to provide the electrons instead of a chemical electron transfer system (J. Zhang et al. 2013). Although the physiological electron transfer system of ADO has not been confirmed, a system comprising a ferredoxin (Fd) and a ferredoxin-$NADP^+$ reductase (FNR) is present at cyanobacteria and has been shown to support ADO activity efficiently (Rajakovich et al. 2015). In addition, co-expression of the cyanobacterial electron transfer system improves the heterologous alkane production in *E. coli* (Kallio et al. 2014; Cao et al. 2016). Thus, the heterologous expression of the Fd-FNR system in *A. baylyi* could support higher ADO activity and improve the alkane production.

In addition to the electron transfer system, and efficient production of the reducing equivalents is required for the long-chain hydrocarbon synthesis. Fatma *et al.* (2018) used metabolic modeling to identify potential modifications to increase long-chain hydrocarbon production from glucose in *E. coli*. The metabolic model implied that increasing the flux through the pentose phosphate pathway would increase the long-chain hydrocarbon production, potentially due to increased NADPH production. This prediction was also confirmed *in vivo*; the alkane and fatty alcohol production was increased by overexpressing the first enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (Fatma et al. 2018). In my study, acetate was used instead of glucose as the carbon source and thus the same strategy cannot be directly adopted. However, other modifications that ensure efficient NADPH generation might improve alkane production.

Other strategies that have been used to increase heterologous alkane production include modifications that increase the availability of fatty acyl precursors by increasing fatty acid biosynthesis or directing cellular lipids to alkane biosynthesis (Cao et al. 2016; Fatma et al. 2018; Choi & Lee 2013). For example, overexpression of FabI, the last enzyme in fatty acyl-ACP synthesis, increased the alkane production in *E. coli* (Cao et al. 2016). In the same study, it was reported that enhancing the phospholipid degradation also increased the alkane production. Both of these modifications are expected to increase the availability of the fatty acyl-ACP substrates, and thus the improvements in the alkane production indicate that the availability of the fatty acyl-ACP substrate is limiting the alkane production in *E. coli* under the studied conditions. Biomass and product synthesis typically compete with the same cellular precursors, and thus achieving a proper distribution between cell growth and product formation is crucial for efficient biosynthesis. For example, Fatma *et al*. (2018) reported that limiting the phospholipid synthesis increased alkane production. As membrane lipid synthesis is essential for cell growth, this strategy limited biomass formation, and directed a higher fraction of the carbon flux towards alkanes (Fatma et al. 2018).

In this study, heptadecane (C17:0) was the major hydrocarbon product, while 8-heptadecene (C17:1), and pentadecane (C15:0) were also detected (II, IV). The product profile is similar to that reported in *E. coli* (e.g. (Schirmer et al. 2010)), reflecting the derivation of alkanes from the most prevalent acyl groups (C18 and C16) in the cells. ADO does not exhibit strong substrate preference with regard to the chain length (Andre et al. 2013), whereas AAR prefers substrates with 16 or 18 carbons (Lin et al. 2013; Kudo et al. 2016). In *A. baylyi*, C18:1 acyl chains are the most abundant, followed by C16:0 and C16:1 (Santala, Efimova, et al. 2014). The relatively higher proportion of the saturated alkanes compared to the acyl precursors might be explained by the higher activity of AAR towards the substrates with saturated acyl chain (Lin et al. 2013). The alkanes or alkenes with 15-17 carbons are in the range of hydrocarbons used in the diesel

oil, and thus could be used as drop-in replacements for the fossil-derived fuel. Alternatively, if modified carbon chain lengths are preferred, the chain length could potentially be altered by metabolic engineering. For example, a thioesterase with specificity towards shorter-chain acyl-ACPs can be used to shift the distribution of the hydrocarbons to shorter chains (Kallio et al. 2014; Choi & Lee 2013). In ADP1, replacing the natural fatty acyl-CoA reductase Acr1 with LuxCDE shifted the distribution of the lipids towards shorter and more saturated carbon chains (Santala, Efimova, et al. 2014).

The low activity of ADO is considered a major bottleneck for efficient alkane biosynthesis (Patrikainen et al. 2017; Marsh & Waugh 2013). Consequently, the structure and catalytic mechanism of ADO have been the subject of intensive study, and the catalytic mechanism is relatively well-understood (Rajakovich et al. 2015). Numerous studies have also focused on the identification of amino acids important for substrate specificity and/or catalytic activity (Bao et al. 2016; Zhang et al. 2016; Khara et al. 2013; Wang et al. 2017; Hayashi et al. 2015). Despite these efforts, limited improvements to the overall catalytic activity have been achieved. For example, even though the activity of ADO towards short-chain alkanes was improved by changing one amino acid at the substrate binding pocket, the total alkane titer was reduced when the mutant ADO was expressed as part of alkane synthetic pathway in *E. coli* (Bao et al. 2016).

Due to the difficulties in enhancing the performance of cyanobacterial ADOs, a more fruitful option could be to employ enzymes from other organisms. For example, CER1 from *A. thaliana* has been used in heterologous alkane production, even though it has not been characterized in as much detail as the ADOs. CER1 was also included in my study, as the alkane production by CER1 was compared to the cyanobacterial ADOs using the biosensor. However, the expression of CER1 together with the aldehyde-producing enzymes led to poor alkane production, as compared to the ADOs (IV). The substrate specificity and cofactor requirements of CER1 remain to be determined; however, it has been used both in the production of very long-chain (>29) (Bernard et al. 2012) and short-chain (<13) (Choi & Lee 2013) alkanes. Despite these successful examples, another study reported that the expression of CER1 did not lead to alkane production in *S. cerevisiae* (Kang et al. 2017). As CER1 originates from a plant, the microbial cells might not be optimal for the folding and function of the enzyme. Clearly, a more detailed characterization of this enzyme is required to enable its reliable utilization in heterologous alkane biosynthesis.

In addition to ADOs and CER1, P450 oxidative decarbonylases found in insects also catalyze the conversion of aldehydes to alkanes (Herman & Zhang 2016; Qiu et al. 2012). Furthermore, as alkanes are widespread in nature (Herman & Zhang 2016), currently unknown enzymes may be

found that are more efficient than the enzymes utilized thus far. Apart from the alkanes and alkenes produced from aldehydes, terminal alkenes can be produced from medium-chain (~C12) carboxylic acid by decarboxylation (Herman & Zhang 2016; Rude et al. 2011; Rui et al. 2014). Enzymes catalyzing the decarboxylation of fatty acid to the corresponding n-1 terminal alkene include $OleT_{JE}$, another P450 protein from the bacterium *Jeotgalicoccus* sp. (Rude et al. 2011), as well as UndA and UndB found in *Pseudomonas* species (Rui et al. 2014; Rui et al. 2015). Of these, UndB was shown to be the most efficient in heterologous 1-alkene production in *E. coli* (Rui et al. 2015). These recently discovered enzymes expand the toolbox available for the engineering of microbial hydrocarbon production. The medium-chain 1-alkenes can potentially be utilized as drop-in fuel. More importantly, however, due to the reactivity of the terminal double bond, they can be used as precursors in the chemical industry in the synthesis of more valuable compounds, such as lubricants, detergents, and cosmetics (Ray et al. 2012).

The *in vivo* alkane biosensor developed in this study could serve as a convenient tool for the screening of novel enzymes, either found from nature or generated by engineering the known enzymes. Even though various whole-cell alkane biosensors have been developed (D. Zhang et al. 2012; W. Wu et al. 2015), they have not previously been utilized for the evaluation of different alkane-producing enzymes. The successful application of the biosensor for the screening of alkane-producing pathways in this study demonstrates the utility of the sensor and encourages further efforts aiming at identifying efficient alkane-producing pathways. Even though the utilization of the biosensor revealed differences in the performance of the various synthetic pathways, only less than three-fold inductions in the fluorescence signal were detected with the endogenously produced alkanes. While the sensor exhibited stronger induction with externally added alkanes (data not shown and I), thereby demonstrating a relatively wide dynamic range, improving the sensitivity of the sensor would help in screening of the low-producing synthetic pathways.

The role of the fatty aldehyde intermediate in the alkane production is intriguing. Typically, the *E. coli* strains engineered for alkane production produce a large amount of fatty alcohols as a side-product (e.g. (Schirmer et al. 2010; Cao et al. 2016)). In *A. baylyi*, fatty alcohols were also detected when the alkane-producing pathway was expressed (data not shown). The alcohol production has been attributed to the activity of endogenous aldehyde reductases (Rodriguez & Atsumi 2014; Schirmer et al. 2010). Even though long-chain aldehydes are not part of the natural metabolism of *E. coli*, several of the reductases accept them as substrates (Fatma et al. 2016; Rodriguez & Atsumi 2014). Consequently, the rational approach has been to try and eliminate these reductases, in order to enable higher production of alkanes and/or aldehydes (Rodriguez & Atsumi 2014; Cao et al. 2016; Rodriguez & Atsumi 2012; Wang et al. 2018; Song et al. 2016). However, in recent studies it has been found that the deletion of the aldehyde

reductases is not necessarily beneficial for alkane production (Cao et al. 2016; Wang et al. 2018). Rather, it was suggested that a certain level of fatty aldehyde reductase activity improved alkane production, even though the possible mechanism behind this was not investigated (Cao et al. 2016). Aldehyde accumulation is potentially toxic to the cells (Kunjapur & Prather 2015), and thus a potential benefit from aldehyde reductase expression is to prevent the accumulation of the aldehyde intermediate. This is supported by the observation that lowering the aldehyde production increased alkane production (IV). Due to the central and controversial role of fatty aldehyde in the alkane biosynthesis, the twin-layer biosensor developed in this study is a valuable tool in rational engineering of the pathway.

5.3 Evaluation of the platform

5.3.1 Long-chain hydrocarbon production (I-IV)

In this study, the long-chain hydrocarbon production of *A. baylyi* was studied using acetate (either pure or produced by the acetogens; II, IV) or glucose (I, III) as the carbon sources. The ability to utilize acetate for growth and lipid production is crucial for the production platform. However, acetate is a challenging substrate due to its toxic effects. The undissociated acetic acid is lipophilic and can readily cross the cell membrane by diffusion. At the higher intracellular pH, a larger fraction of the acetic acid molecules dissociates, leading to the release of proton and the acetate anion (Trček et al. 2015). The accumulation of protons in the cytosol leads to dissipation of the proton gradient and, thus, wasting of energy. Furthermore, as the acetate anion cannot freely diffuse through the membrane, it accumulates in the cells in high concentrations, interfering with the metabolism of the cells (Trček et al. 2015).

A. baylyi can grow on acetate (Du Preez et al. 1981), but lipid production from acetate has not been studied. In paper II, wax ester production of the wild type *A. baylyi* ADP1 from acetate was studied. The cells were cultivated in minimal salts medium with 100 or 200 mM acetate. *A. baylyi* tolerated both concentrations, but the growth was slow at 200 mM concentration. Wax esters were produced in both conditions. The highest wax ester titer was 91 mg/l, measured in the 100 mM cultivation after 24 hours (Table 5.1).

In *A. baylyi*, wax esters serve as carbon and energy storage. They are accumulated in growth conditions with plentiful carbon source, and degraded in carbon-limiting conditions (Fixter et al. 1986; Santala, Efimova, Karp, et al. 2011). This trend was also noticed in my study; the wax esters were degraded when all the acetate had been consumed. In order to avoid the WE degradation, the acetate consumption was followed during the experiments, and the cultivations were stopped before the complete depletion of acetate (II). The acetate toxicity limits the maximum concentration that can be utilized, and thus the maximum cell density that can be achieved in batch cultivations. In order to enable growth to higher cell densities, a fed-

batch cultivation strategy was established in paper IV. *A. baylyi* was grown in a bioreactor and either pure acetic acid or the acetate-containing medium from the acetogenic cultivation was used as the feed. The feed rate was manually controlled to maintain the acetate concentration in the reactor between 30 and 70 mM. With this strategy, higher cell density was obtained than in the batch cultivations, in relatively short time (IV).

Despite the higher biomass obtained with the fed-batch cultivations relative to the batch cultivations, both the biomass yield (biomass formed/substrate consumed) and the cell density remained relatively low (IV). Due to the effect of uncoupling the proton gradient, growth on acetate requires high maintenance energy. This is pronounced at acetate-limited growth, when a large proportion of the consumed substrate is utilized for the generation of the maintenance energy, and a small proportion is used for growth (Du Preez et al. 1981). On the other hand, the growth rate of *A. baylyi* on acetate is inversely proportional to the acetate concentration (Du Preez et al. 1981). Thus, the strategy to maintain the acetate concentration around 50 mM in the fed-batch cultivation represented a compromise between the biomass yield and growth rate. A more detailed optimization of growth parameters might enable more efficient growth of *A. baylyi* on acetate. For example, Xu *et al*. established a "carbon-restrained" growth method for the cultivation of yeast *Y. lipolytica* on dilute acetate (Xu et al. 2017). In the "carbon-restrained" cultivation, the feed is adjusted automatically in order to provide the cells with exactly the amount of acetate they can assimilate. Thus, the acetate concentration in the medium is maintained very close to zero, while avoiding the starvation of the cells (Xu et al. 2017).

The cultivation of microbes to high cell densities is a prerequisite for high volumetric productivity, especially in the case of intracellularly accumulating products, such as lipids. While high cell-density cultivation methods have been developed for industrially utilized hosts, including *E. coli, Bacillus subtilis,* and *Pichia pastoris* (Riesenberg & Guthke 1999), the suitability of *A. baylyi* for high cell-density cultivation remains largely unexplored. In my studies, the cell dry weight concentrations were in the range of 1-5 g/l, being almost two orders of magnitude lower than the maximum cell densities achieved with some organisms in optimized cultivations (approximately 200 g/l, (Riesenberg & Guthke 1999)). Thus, developing culture methods for high cell-density cultivation of *A. baylyi* is crucial for its potential application in industrial scale production. One option for increasing the cell density, especially when grown on dilute substrate, is continuous culture mode with cell retention. For example, Hu *et al.* produced 46 g/l lipids with *Y. lipolytica* grown on dilute acetate in a continuous cultivation involving the removal of medium and recycling of the cells back to the reactor (Hu et al. 2016).

Lipid accumulation typically occurs in conditions where the growth is limited by the availability of an essential nutrient (commonly nitrogen), while an excess of carbon source is available (Wältermann et al. 2005; Garay et al. 2014). In order to avoid carbon limitation, glucose was used in some of the experiments (I, IV) as the carbon source instead of acetate. *A. baylyi* tolerates high concentrations of glucose, enabling the evaluation of different genetically engineered strains in simple batch cultivations. In these experiments, 50 g/l of glucose was used, which ensured that there was an excess of carbon source available throughout the cultivation.

The results of the wax ester and alkane production are summarized in Table 5.1. Interestingly, it can be seen that with the wild type *A. baylyi*, the yield of WE production was higher with acetate than with glucose, despite the fact that acetate was used in much smaller concentrations. The initial acetate concentration utilized in those experiments varied from approximately 20 to 100 mM (corresponding to 1.2-6 g/l; II), while glucose was used in the initial concentration of 50 g/l (III). As noted earlier, the timing of the sample collection strongly affected the results; in a carbon-depleted medium, the wax esters were degraded. Thus, these data were collected while there was still acetate left in the culture medium (II). The composition of the medium was otherwise similar (II, III), and thus the carbon-to-nitrogen ratio (C/N) was at approximately ten-fold higher with glucose. The complex glucose catabolism of *A. baylyi* may explain this result to some degree; indeed, acetate is preferred over glucose as the carbon source by *A. baylyi* ADP1 (Kannisto et al. 2015). With the overexpression of FAR (either Acr1 or Ramo), the yield from glucose increased significantly (Table 5.1, III). It could be expected that the FAR overexpression would increase the yield also from acetate, potentially leading to higher yields than the ones achieved in this study. The WE titers achieved with FAR overexpression are the highest reported for microbial production systems involving the *de novo* synthesis of the fatty acyl chains (i.e. without hydrocarbon supplementation; III).

One potential strategy for improving the WE accumulation could be the prevention of their degradation; however, the mechanisms and genetic basis of WE degradation in *A. baylyi* are not known. In contrast, the ability of *A. baylyi* for growth on alkanes was removed with a single gene knockout (I). Thus, alkane production with this knockout strain should be less sensitive to the amount of available carbon source.

Both the alkane yield and titer were significantly lower than those for WEs (Table 5.1). As fatty aldehyde is a precursor for both alkane and wax ester synthesis, it is unlikely that its concentration would limit the alkane production. Thus, the low alkane production can be most likely attributed to the low activity of ADO. The alkane production titers reported in *E. coli* (Table 2.1) are significantly higher than the titers obtained in my study with *A. baylyi*. Thus, it

might be speculated that the activity of ADO is lower in *A. baylyi* than in *E. coli*. As ADO requires efficient electron transfer system for optimal functionality (Chapter 2.2), a potential explanation could be that the native electron transfer systems of *A. baylyi* are poorly compatible with ADO. In addition, the expression levels of ADO were not determined, and thus the efficiency of the protein expression and folding are not known.

Table 5.1. A summary of the long-chain hydrocarbon production. All data are from experiments performed at 30 °C.

Strain	Product	Method	Carbon source	Titer[a] (mg/l)	Yield[a] (c/c)[b]	Reference
A. baylyi ADP1	Wax ester	batch, flask	Acetate Acetate[c]	91 18	4.6% 4.8%	II
A. baylyi ADP1	Wax ester	batch, flask	Glucose	200	2.7%	III
ADP1 + Ramo ADP1 + Acr1	Wax ester	batch, flask	Glucose	410 400	5.1% 6.4%	III
ADP1 + AAR-ADO	Alkane[d]	fed-batch, bioreactor	Acetate Acetate[c]	0.54 0.074	0.005% 0.003%	IV

[a] Calculated using and average molar mass of 506 g/l (ref: III) for WEs

[b] carbon atoms in products compared to carbon atoms in consumed substrate

[c] Acetate produced by acetogens

[d] Heptadecane (C17:0)

5.3.2 Potential for solar-to-chemicals conversion (II, IV)

Solar energy and carbon dioxide are seen as the ideal sources of energy and carbon for the production of fuels and chemicals (Li & Liao 2013; Zhang & Tremblay 2017). In recent years, hybrid production systems have been developed, combining the light harvesting by inorganic devices to the formation of select carbon-carbon bonds by living cells (Lips et al. 2018; Zhang & Tremblay 2017). These systems have the potential to be substantially more efficient than the natural photosynthesis. It has been calculated that microbial electrosynthesis powered with commercially available photovoltaic cell could enable acetate production with the solar energy-to-acetate conversion efficiency of ca. 10% (Zhang & Tremblay 2017). As an example, a recently developed hybrid water splitting-biosynthetic system was reported to reduce CO_2 to bacterial biomass and short-chain alcohols with an energetic efficiency of ca. 50% (Liu et al. 2016). In the system, H_2 is produced by the electrolysis of water, and is utilized by *C. necator* for CO_2 reduction. Powering this system with a standard photovoltaic cell with an efficiency of ca. 20%

would enable CO_2 reduction with 10% efficiency (Liu et al. 2016). While this is already considerably higher than the solar-to-biomass conversion efficiency of natural photosynthesis (at maximum 1-3%; (Blankenship et al. 2011)), the development of novel photovoltaic technologies may allow significantly higher efficiencies (Green et al. 2018).

The energy and carbon required for the synthesis and maintenance of the cellular components limits the efficiency of the product synthesis. In my study, the coulombic efficiency (electrons in the product/electrons in the substrate) of WE production from acetate was ca. 7% (II), whereas the c/c yields were in the range of 3-6% (Table 5.1), indicating that a large proportion of the carbon was utilized for biomass and energy production. Further metabolic engineering could potentially improve the yields; for example, a single gene knockout impairing the synthesis of exopolysaccharides improved the WE yield from glucose in *A. baylyi* (Kannisto et al. 2017).

In terms of metabolic pathways, the long-chain hydrocarbon production from carbon dioxide consists of two different parts; first, the reduction of CO_2 to organic molecules, and second, the utilization of these molecules for the synthesis of the long carbon chains. As discussed earlier, these two processes impose different requirements for the metabolism of the host cell, and are thus regarded difficult to achieve efficiently in any single host. For example, the products of microbial electrosynthesis systems are typically short-chain carboxylic acids or alcohols (Zhang & Tremblay 2017). The two-stage system can be considered to be a "host organism" with extended metabolic capabilities, being able to perform both carbon dioxide reduction and long-chain hydrocarbon synthesis. Thus, the system bypasses the need for transforming complex metabolic functions (CO_2 fixation or lipid synthesis) from one organism to another. With regard to the fan in - fan out model of metabolism, the first module represents the fan in, converting CO_2 and energy to acetyl-CoA, while the second module represents the fan out, utilizing acetyl-CoA for the synthesis of the macromolecular end products.

Recently, similar two-stage systems have been reported using different host organisms and experimental settings. Liu *et al*. (2015) combined light harvesting by nanowire semiconductor to acetate production by *S. ovata*. The acetate was further utilized for the growth and chemicals production by *E. coli* (C. Liu et al. 2015). In that study, genetically engineered *E. coli* strains were utilized for the production of n-butanol, polyhydroxybutyrate, or isoprenoids. In another study, *M. thermoacetica* was used for acetate production from H_2 and CO_2 (Hu et al. 2016). The acetate was used for the growth and triacylglyceride production by an engineered *Y. lipolytica* strain. These examples, together with the present study, emphasize the versatile nature of this kind of production platforms. In my study, wax esters and long-chain alkanes were produced from CO_2. As an easy-to-engineer chassis, *A. baylyi* can potentially be harnessed for the production of a wide range of compounds.

Two alternative microbial production systems for alkane production from CO_2 have been reported. First, the photoautotrophic production occurs naturally in cyanobacteria, and has also been enhanced by metabolic engineering (Savakis & Hellingwerf 2015; Wang et al. 2013). Compared with terrestrial plants, cyanobacteria are more efficient in utilizing the solar energy for biomass production (Ducat et al. 2011). However, the requirement for direct exposure to light complicates the cultivation of cyanobacterial cultivation when compared to acetogenic CO_2 reduction. A second system utilizes the bacterium *C. necator*, which is capable of CO_2 reduction using H_2 as the energy source. In a recent study, *C. necator* was engineered to produce alkanes, and the production of alkanes from CO_2 was demonstrated (Crépin et al. 2016). *C. necator* requires both O_2 and H_2 for efficient growth, which poses a safety risk especially at large-scale cultivations (Hawkins et al. 2013). In contrast, in the two-stage system utilizing two different bacteria, the H_2 and O_2-consuming phases are separated and can be carried out in different reactors. Another major difference of *C. necator* and the acetogens is that the former uses the Calvin-Benson-Bassham cycle, while the latter utilizes the Wood-Ljungdahl pathway for the CO_2 fixation.

Currently, all the microbial alkane production systems suffer from the same issue of low conversion efficiency. The two-stage production system may enable faster development towards higher efficiencies, as it allows the optimization of both of the parts independently of each other. The metabolic engineering efforts in this study were focused on the *A. baylyi* module, improving the WE and alkane production from acetate. While this hydrocarbon production part requires additional research, the acetate production module should also be improved. Currently, the acetate utilization by *A. baylyi* is faster than acetate production by the acetogens. Thus, by enhancing the acetate production, the overall process time could be shortened. For example, improved reactor design along with modifications to the medium composition and cultivation parameters have led to increased acetate productivities with *A. woodii* (Demler & Weuster-Botz 2011; Kantzow et al. 2015). Furthermore, the potential of these systems for scale-up and long-term operation should also be studied in order to potentially enable industrial-level applications in the future.

6 Conclusions

Reflecting the objectives and hypotheses (Chapter 3), the results of my study can be summarized as follows:

1) **A two-stage bacterial platform for the production of long-chain hydrocarbons from carbon dioxide and electricity was established.** In the first stage, CO_2 and H_2/electricity are utilized for acetate production by acetogenic bacteria. In the second stage, the acetate is utilized for growth and hydrocarbon production by *A. baylyi* ADP1. Of the studied acetogens, *S. ovata* and *A. woodii* were found to be the most efficient in supporting the growth of *A. baylyi*. The cysteine used as a reducing agent in the anaerobic acetogenic cultivation was found to inhibit the growth of *A. baylyi*, and the inhibition could be relieved by supplementing the medium with casein amino acids before the second phase. The modular nature of the system enabled the modification of both the acetate-producing and -consuming processes. Three different acetate-producing methods (microbial electrosynthesis, as well as gas fermentation in bottles and in bioreactor) were applied. On the other hand, the final product was modified by utilizing either wild type or genetically modified *A. baylyi*, resulting in the production of wax esters or long-chain alkanes, respectively. Proof-of-concept production of wax esters from CO_2 and electricity, as well as long-chain alkanes from CO_2 and H_2 were demonstrated, but additional work is required in order to improve the efficiency of the process.

2) **The fatty aldehyde production in *A. baylyi* was improved.** The overexpression of any of the three fatty acyl-CoA/ACP reductases tested was found to improve the fatty aldehyde production in *A. baylyi*, as measured by the previously developed *in vivo* biosensor. The studied reductases were the native *A. baylyi* FAR Acr1, a previously uncharacterized, putative reductase Ramo from *N. ramosa*, and the cyanobacterial fatty acyl-ACP reductase AAR from *S. elongatus*. The overproduced fatty aldehydes were found to be directed to different downstream pathways depending on the reductase utilized. With Acr1 or Ramo, the increased flux towards fatty aldehydes was reflected in the wax ester (WE) production by the native *A. baylyi* pathway. The expression of these reductases relieved the temperature dependency of WE accumulation in *A. baylyi*, leading to increased WE titer, productivity, and yield, indicating that the fatty aldehyde production is a central regulatory point in WE production. In contrast, the expression of AAR led to very small WE production, despite the high fatty aldehyde production. On the other

hand, AAR supported more efficient alkane production by the heterologous aldehyde-deformylating oxygenase (ADO), than the other two reductases. Thus, even though the fatty aldehydes produced by AAR are not efficiently directed for WE production, they can be utilized for long-chain alkane production in engineered ADP1. The mechanisms behind the differing behavior of the aldehydes produced by different enzymes remains unclear and requires further research.

3) **An alkane biosensor was developed and applied for studying heterologous alkane biosynthesis in *A. baylyi*.** The sensor consists of two parts that monitor different metabolites of the alkane biosynthesis pathway. In the first part, the bacterial luciferase LuxAB is used for the monitoring of the fatty aldehyde intermediate, whereas in the second part, a *gfp* gene placed under a native alkane-inducible promoter is used for the monitoring of alkanes. The LuxAB is constantly expressed, and the luminescence signal is dependent on the concentration of fatty aldehyde, whereas the expression of GFP is induced by the aliphatic alkanes. Combining both sensor elements and a heterologous alkane biosynthesis pathway in *A. baylyi* enabled the simultaneous monitoring of the fatty aldehyde and alkane production *in vivo*, and in real time. In the sensor strain, a gene essential for growth on alkanes was deleted, thus leading to a strain that is incapable of alkane degradation. The sensor was applied in the screening of different synthetic alkane-producing pathways, and the pathway consisting of AAR and ADO from *S. elongatus* was found to be the most efficient in alkane production. The two-layer configuration of the sensor enabled the evaluation of the two aldehyde- and alkane-producing components of the pathway. It was found that limiting the accumulation of the aldehyde intermediate by lowering the relative expression of AAR was beneficial for the alkane production. This was the first report of alkane production in the native alkane degrader *A. baylyi*. However, the alkane production was still very low, and improving the titer and yield represents an important target for future studies.

4) **Production of long-chain hydrocarbons by *A. baylyi* was demonstrated using acetate as the carbon source.** It was shown that acetate can be used as the carbon source for the production of wax esters by the wild type *A. baylyi*, or alkanes by the genetically engineered *A. baylyi*. Furthermore, as proof-of-concept for long-chain hydrocarbon production from carbon dioxide, the acetate was produced from CO_2 and H_2/electricity by the acetogens, and utilized for wax ester or alkane production by *A. baylyi*. The yield of hydrocarbon production was higher from acetate than from glucose, highlighting the suitability of acetate as the carbon source for *A. baylyi*. However, the toxicity of acetate in high concentrations limits the amount of biomass, and thus the product titer, that can be achieved in batch cultivations. The development of cultivation methods that would

allow efficient, high cell-density cultivations of *A. baylyi* on acetate was identified as an important target that would improve the efficiency of the platform.

To sum up, this study described the development of a novel bacterial platform for the production of long-chain hydrocarbons from carbon dioxide and electricity. While the platform holds potential for the sustainable production of liquid fuels and chemicals, it is clear that significant improvement in the efficiency is required in order to make the process feasible.

7 References

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ORIGINAL PUBLICATIONS

I

Twin-layer biosensor for real-time monitoring of alkane metabolism

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RESEARCH LETTER – Biotechnology & Synthetic Biology

Twin-layer biosensor for real-time monitoring of alkane metabolism

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One sentence summary: A twin-layer biosensor for studying alkane degradation or biosynthesis in *Acinetobacter baylyi* was developed and characterized.

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ABSTRACT

Intracellular metabolic sensors can be used for efficient screening and optimization of microbial cell factories. In particular, the sensors are useful in acquiring information about pathway dynamics and bottlenecks in a straightforward manner. Here, we developed a twin-layer biosensor that functions simultaneously at two levels: through transcription factor mediated sensing and enzyme-metabolite interaction, providing insights into the dynamics of alkane metabolism. In addition, the sensor can be used for monitoring either alkane degradation or biosynthesis, depending on the used cellular context. Alkanes are monitored using a fluorescent reporter green fluorescent protein placed under a native alkane-inducible promoter, whereas a bacterial luciferase producing bioluminescence signal enzymatically detects a specific metabolic intermediate in the alkane production/degradation pathway. First, we employed the sensor to investigate the native alkane degradation route in *Acinetobacter baylyi* ADP1. The highest fluorescence and luminescence signals were obtained for dodecane. Second, we constructed a non-native alkane synthesis pathway in *A. baylyi* ADP1, of which the functionality was confirmed with the sensor. The twin-layer approach provides convenient means to study and optimize the kinetics and performance of the heterologous pathway and will facilitate the development of an efficient cell factory.

Keywords: alkane; biosensor; LuxAB; GFP; *Acinetobacter baylyi* ADP1; real-time monitoring

INTRODUCTION

In synthetic biology, biological systems are harnessed for the production of valuable molecules by means of rational design and construction. The typical workflow consists of design, construction, characterization and revision of the synthetic systems. With the advances in computational modeling as well as synthetic DNA and cloning technologies, the phenotypical characterization of the constructed organisms remains the bottleneck for the efficient development of high-performing microbial hosts (Zhang, Jensen and Keasling 2015). This is mainly because the analysis of the metabolic end products and intermediates is laborious and time consuming. In rare exceptions, the positive changes are readily detectable as changes in the phenotype (e.g. colorful product such as lycopene), but usually the metabolites cannot be quantified in a high-throughput manner. Furthermore, for the identification of bottlenecks or imbalances in the metabolic network, the analysis of the end products alone is insufficient and should be complemented with the analysis of key metabolic intermediates.

One solution is to use a suitable biological sensor that is able to respond to changes in the concentration of a specific metabolite and report it with an easily quantifiable signal (Eggeling, Bott and Marienhagen 2015; Zhang, Jensen and Keasling 2015).

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Application of biosensors for the detection and quantitation of selected metabolites allows for high-throughput screening of producer strains (Schallmey *et al.* 2014). In an optimal case, the sensor allows for real-time, non-destructive analysis of key metabolites.

Metabolic sensors can work on different levels; they can report for example changes in promoter activity, protein or RNA expression, or concentration of a specific metabolite. By combining information from different levels, changes in the dynamics of the metabolism can be observed. Information about the pathway dynamics can reveal important aspects that would not be detectable or accessible with end metabolite analysis alone. Moreover, the sensors are often more sensitive and specific compared to conventional analyses methods. In this study, we introduce a biosensing system working on two levels: the system directly detects the presence of molecules of interest, alkanes, by a transcription factor mediated sensing, as well as provides information about the dynamic intermediate compounds, aldehydes, by an enzyme-metabolite interaction. The system can be applied for studying both the alkane degradation and synthesis, depending on the cell context. To highlight the modularity, the system was constructed using a bacterial strain *Acinetobacter baylyi* ADP1, which has not been employed for alkane synthesis before.

Microbial alkane production is a highly interesting option for the replacement of fossil fuels and chemicals (Schirmer *et al.* 2010; Choi and Lee 2013). However, current titers are too low for practical applications. One of the reasons for the low titers is the inefficient flux from acyl-CoA to alkanes through a toxic aldehyde intermediate. Several strategies have been tested in order to improve the production (Andre *et al.* 2013; Wang and Lu 2013), but the work is hindered by the lack of time- and cost-effective methods for the efficient screening of potential genetic modifications and producer strains. Thus, intracellular alkane biosensors could greatly facilitate the development of an efficient alkane-producing microbial factory (Kang and Nielsen 2016).

Various whole-cell sensors for alkane detection have been developed (Sticher *et al.* 1997; Minak-Bernero *et al.* 2004; Reed, Blazeck and Alper 2012; Zhang *et al.* 2012). These sensors are based on the induction of either a luciferase (LuxAB) or a green fluorescent protein (GFP) by an artificially constructed or naturally alkane-responsive promoter. However, those sensors have only been applied in the context of extracellular alkane detection. Recently, Wu *et al.* (2015) developed an alkane sensor for the detection of intracellular alkanes produced by the sensor strain. In the sensor, the alkanes produced by the cells activate the expression of GFP under an artificially constructed alkane-inducible promoter. This was the first demonstration of *in situ* alkane detection within the producer strain using a biosensor. The aforementioned reporters are based on a transcriptional activation of a reporter gene, and consequently show relatively slow responses.

Previously, we developed a simple and robust sensor for extracellular alkanes based on the novel synthetic biology chassis *A. baylyi* ADP1 (Santala, Karp and Santala 2012). This sensor relied on two important aspects of *A. baylyi* metabolism; first, its natural ability for efficient alkane uptake and degradation, and second, the generation of a specific intermediate of the alkane degradation pathway. The intermediate, long-chain aldehyde is a specific substrate for a light-producing bacterial luciferase LuxAB, thus eliminating the need for the addition of external substrate or development of synthetic induction systems.

In the current study, we wanted to improve our LuxAB-based alkane sensor by adding a second sensory level, allowing the detection of alkanes through a reporter transcript output, mediated by a ligand responsive transcription factor. This was achieved by placing a *gfp* gene under a native alkane-inducible promoter (Ratajczak, Geissdorfer and Hillen 1998b). As a result, the twin sensor simultaneously detects alkanes and alkane degradation/biosynthesis intermediates (long-chain aldehydes), offering insights into the dynamics of the metabolism. The twin sensor was employed for monitoring the natural alkane degradation route as well as a heterologous alkane biosynthesis pathway.

MATERIALS AND METHODS

Bacterial strains

The wild-type *Acinetobacter baylyi* ADP1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSM 24193) was used in the study. For plasmid construction and maintenance, *Escherichia coli* XL1-Blue (Stratagene, California, USA) was used.

Genetic modifications

The molecular work was carried out using established methods. The reagents and primers were purchased from ThermoFisher Scientific (Massachusetts, USA) and used according to the manufacturer's instructions. Antibiotics were used in concentrations of 25 μg ml^{-1} for chloramphenicol and 50 μg ml^{-1} for kanamycin and spectinomycin, when appropriate. Transformation of *A. baylyi* was carried out as described previously (Metzgar *et al.* 2004). All genome modifications were confirmed with PCR and sequencing.

The genetic cassette for the introduction of *gfp* to the genome of *A. baylyi* under the alkane-inducible promoter was assembled sequentially by restriction cloning using a previously described integration cassette *iluxAB_Cm*^{r}/pAK400c as the backbone (Santala *et al.* 2011a). The construct contains a *gfp* gene and a kanamycin resistance marker flanked by the upstream and downstream regions of *AlkM*. The upstream flanking region contained the alkane-inducible promoter. The 3′ and 5′ flanking regions were PCR-amplified from *A. baylyi* genome with primers tl01 & tl02 and tl09 & tl10, respectively (Table 1). A gene encoding superfolder GFP (sfGFP) was amplified from the BioBrick collection (BBa_I746915) with primers tl03 and tl04 and the kanamycin resistance marker from plasmid pBAV1K-T5-gfp (Bryksin and Matsumura 2010) with primers tl07 and tl08. *Acinetobacter baylyi* was transformed with the cassette and strains characterized based on their ability for n-alkane utilization. For the external alkane detection, a strain capable of alkane degradation was used (indicating that the integration of the cassette did not knock out the *alkM* gene). For internal alkane production, a strain with a functional *alkM* knockout was used in order to prevent the degradation of the synthesized alkanes.

For the introduction of the *luxAB* genes into the genome, a previously described cassette was employed (Santala *et al.* 2011b). This cassette is designed to knock out the ACIAD3383-3381 genes, thereby removing the endogenous fatty acyl-CoA reductase and pyruvate dehydrogenase activities. The *luxAB* genes were cut from the *iluxAB_Cm*^{r}/pAK400c plasmid using *NdeI* and *XhoI* restriction enzymes and cloned to the cassette.

For the alkane-producing strain, a construct was prepared where *aar (Synechococcus elongatus PCC7942_orf 1594)* and *ado (S. elongatus PCC7942_orf 1593)* genes are under T5/lac promoter in

Table 1. Primers used in the study.

Name	Sequence 5′ → 3′	Description
tl01	taagcgggtaccgtcgtaccatcctgtaatgtgaaatgag	AlkM 5′ flanking forward, KpnI
tl02	tacaggcaattgcatagtgaatcctttcttgttatcctc	AlkM 5′ flanking reverse, MfeI
tl03	gactaacaattgcgtaaaggcgaagagc	sfGFP forward, MfeI
tl04	aatgcctctagatcatcatttgtacagttcatccatacc	sfGFP reverse, XbaI
tl07	gcgagcctcgagaaggaagctaaaatggctaaaatgagaatatcaccgg	kanR forward, XhoI
tl08	aggaaactgcagctaaaacaattcatccagtaaaatataatattttattttctcc	kanR reverse, PstI
tl09	aacggccctaggtcagaccttattttaaaagagag	AlkM 3′ flanking forward, AvrII
tl10	attgccggcccccgaggccaaagcaggccgtatctgtaatg	AlkM 3′ flanking reverse, SfiI
tl24	cgtggaattggtaccctagatgttatttcttcaaaattatgg	pIM1463 1/2 forward, KpnI
tl25	gatgatctttctctagaagcggccgcgaattcgcatgctctagtaagcttctcc	pIM1463 1/2 reverse, XbaI
tl26	agggagtctagaactagtagcggccgctgcagaaaggagaagcttactagc	pIM1463 2/2 forward, XbaI
tl27	tgcagcggcccccgaggccaagcttacctgaaagccaat	pIM1463 2/2 reverse, SfiI

an integrative cassette. The integrative region of the plasmid pIM1463 (Murin *et al.* 2012) was PCR amplified in two parts with primers (tl24, tl25, tl26 and tl27) designed in such a way that the *gusA* gene becomes replaced by a BioBrick accepting cloning site (EcoRI-XbaI-SpeI-PstI). *Aar* and *ado* genes were codon-optimized and purchased from Genscript (New Jersey, USA) with appropriate restriction sites and ribosomal binding sites and placed under the promoter using standard methods. The construct contained flanking sites to facilitate the integration into a prophage region in the genome of *A. baylyi* ADP1.

Luminescent and fluorescent measurements

For the induction with external alkanes, the cells were grown overnight in the LB medium (5 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone) supplemented with 0.4% glucose. The measurement was done in 96-well plate, with 200 μl of the overnight culture and ∼40 mM of alkane added. Between the measurements, the plate was incubated in 30°C and shaking. Optical density (Multiscan Ascent, Thermo Labsystems, Finland; wavelength 540 nm), fluorescence (Fluoroskan Ascent FL, Thermo Labsystems, Finland) using the wavelengths 485 nm (half band width (HBW) 14±2 nm) for excitation and 538 nm (HBW 25±3 nm) for emission, and luminescence (Wallac 1420 Victor2, Perkin Elmer, Finland; 1 s measurement time) measurements were performed every 15 min.

For the measurement of internally produced alkanes, the cells were grown on Luria-Agar (LA) (5 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 15 g l^{-1} Agar-agar) on 48-well plate inside Xenogen *In Vitro* Imaging System (IVIS® Lumina, Caliper Life Sciences, USA) at 30°C. Luminescence and fluorescence (excitation: 445–490 nm, emission: 515–575 nm) signals were measured every hour. To investigate the possible overlap of the emission wavelength of the luciferase with the excitation profile of the GFP, the fluorescence emission was also measured without the excitation (the excitation filter was closed). Any 'autofluorescence' obtained in this setting would result from the luciferase exciting the GFP, but the signal was found to be negligible compared to the actual fluorescent signal. The relative fluorescence signal was calculated by dividing the fluorescence of the sensor strain with the fluorescence of wild-type *A. baylyi* ADP1 grown in the same conditions. For confirming the alkane production, GC-MS analysis was carried out (Supplementary File 1, Supporting Information).

For the visualization of the fluorescence from single colonies, the sensor cells were grown overnight in LB, diluted and plated on LA plates with 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG, ThermoFisher Scientific). From the control plates, the IPTG was omitted. The plates were incubated in 30°C for 24 h and visualized with a Safe Imager Blue Light Transilluminator (ThermoFisher Scientific).

RESULTS AND DISCUSSION

To detect the presence of alkanes by the reporter system, a native alkane-responsive promoter (P_{alkM}) of *Acinetobacter baylyi* was exploited in the construction of the sensor. The promoter is activated in the presence of alkanes by a transcriptional activator AlkR (Ratajczak, Geissdorfer and Hillen 1998b). For monitoring the levels of specific intermediates, long-chain aldehydes, a constitutively expressed bacterial luciferase LuxAB was employed. The bacterial luciferase uses aldehydes as a substrate in the reaction producing a fatty acid molecule and measurable light signal (bioluminescence).

The twin sensor for monitoring of the alkane degradation in *A. baylyi* (designated as EAS for external alkane sensor) is presented in Fig. 1 (left). The sensor was created as follows: a synthetic gene cassette was constructed consisting of the P_{alkM} promoter (sequence immediately upstream of *alkM*), a *gfp* gene and a kanamycin resistance marker. The cassette was flanked by homologous regions facilitating its integration to the genome by homologous recombination (Elliott and Neidle 2011). LuxAB was introduced using another genetic cassette, containing the *lux A* and *B* genes under constitutive promoter and flanking regions, facilitating the integration to the genetic locus, replacing the genes ACIAD3381–ACIAD3383 and thus eliminating the native aldehyde synthesis (Santala *et al.* 2011a).

Because *luxAB* is constitutively expressed, the detection of aldehydes by luminescence is not dependent on transcription or the presence of an inducer. Thus, the luminescence response is instant and dynamic. In contrast, the production of fluorescence signal is a slow process and the detected signal is cumulative in nature, due to the working mechanism of the reporter including induction event and protein expression as well as the relatively high stability of GFP.

The response of the twin sensor to external n-alkanes of various chain lengths is presented in Fig. 2. The alkanes induce transcription from the alkane-responsive promoter, which can be observed as the increase in the fluorescence signal. As the alkane degradation pathway activates, the formation of long-chain aldehydes is seen as the increase in the luminescence signal. Induction of the luminescence and fluorescence signals

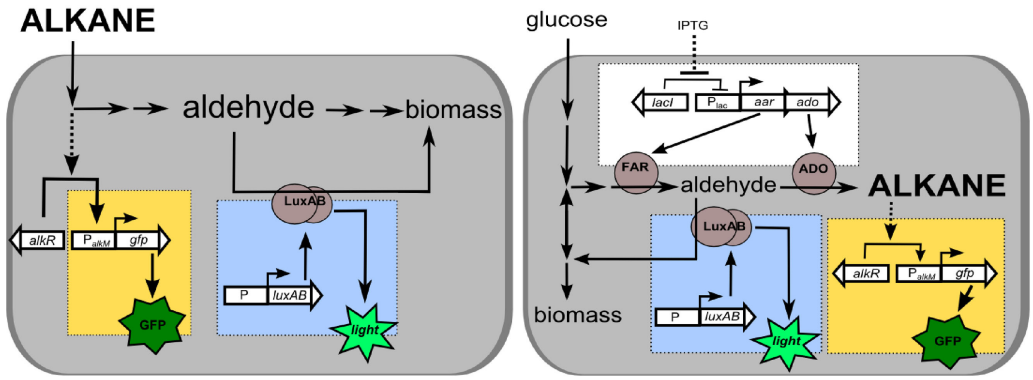

Figure 1. Twin-layer sensors for monitoring alkane degradation and synthesis. The sensors can be used for the monitoring of alkane degradation (left) or biosynthesis (right). The twin sensor components encompass a constitutively expressed *luxAB* encoding for bacterial luciferase, which produces bioluminescence upon interaction with the aldehyde intermediate (blue module), and an alkane inducible reporter consisting of the transcription factor AlkR and the cognate promoter P_{alkM} regulating the expression of GFP (yellow module). For alkane biosynthesis (right), a module containing the activities of a fatty acyl-CoA reductase (FAR) and an aldehyde-deformylating oxygenase (ADO) under inducible promoter system (*lacI*-P*lac*) was added.

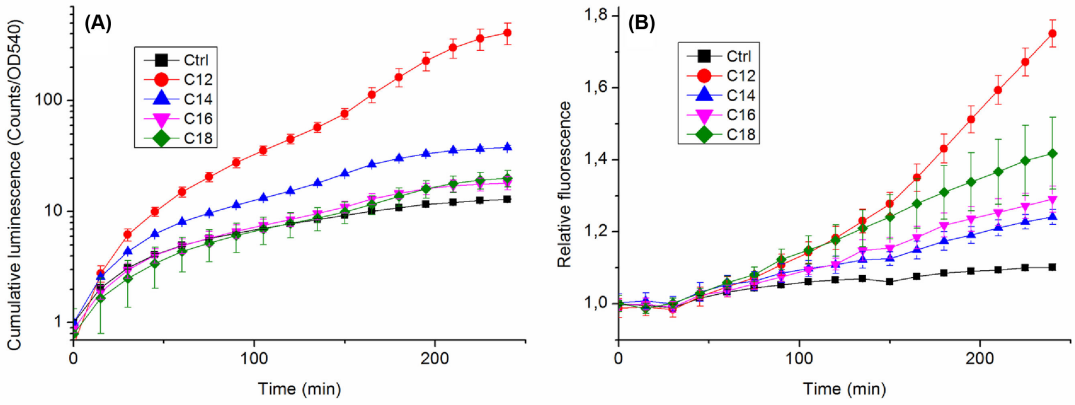

Figure 2. Luminescent and fluorescent responses of sensor cells to external alkanes. The EAS sensor cells (expressing LuxAB constitutively and GFP under an alkane-inducible promoter) were cultivated in LB overnight, divided into 96 well plate and aliphatic alkanes with carbon chain lengths of 12–18 added (final concentration 40 mM). Glucose (40 mM) was added to the control samples. The luminescent (**A**) and fluorescent (**B**) responses were monitored every 15 min. Relative fluorescence is defined as the fold change of the fluorescence signal divided by the OD_{540} of the sample. The luminescence signal is presented relative to the optical density. The optical densities are provided in Table S1 (Supporting Information). The signals are presented as average of three replicates. Error bars represent standard deviation.

behaves differently with varying alkane chain lengths. This can be attributed to the different chain-length preferences of LuxAB and the AlkR-P_{alkM} induction system. Among the tested alkanes, dodecane (C12) yields the highest signals in both luminescence and fluorescence, being consistent with our earlier results (Santala, Karp and Santala 2012). In the previous studies (Ratajczak, Geissdorfer and Hillen 1998b; Zhang *et al.* 2012), octadecane has been observed as the strongest inducer of AlkM expression. The differences can be due to different experimental settings or reporter systems in the genetic level. For example, the differences in solubility between the alkanes with different chain lengths might cause variation between the experimental setups. With octadecane (C18), the fluorescence induction is stronger than the luminescence, whereas with tetradecane the luminescence induction is stronger. For the luminescence signal, the differences in the induction can be partially explained with the substrate specificity of the luciferase; for example, tetradecanal (C14) is preferred over longer carbon chains by LuxAB (Colepicolo *et al.* 1989), explaining its relatively higher activity. The variations in the luminescence and fluorescence signal ratios in response to alkanes of different chain lengths further emphasize the importance of monitoring metabolic pathways at multiple levels.

Apart from the detection of the external alkanes, we wanted to use the sensor for studying the alkane biosynthesis. *Acinetobacter baylyi* exhibits relatively high tolerance towards hydrocarbon metabolites and naturally accumulates large amounts of acyl-CoA and aldehyde-derived hydrocarbons as storage compounds, thus making it an appealing host for microbial alkane production (Santala *et al.* 2011b; Santala, Karp and Santala 2012). Although *A. baylyi* has been previously employed as a robust host for genetic investigations, metabolic engineering and

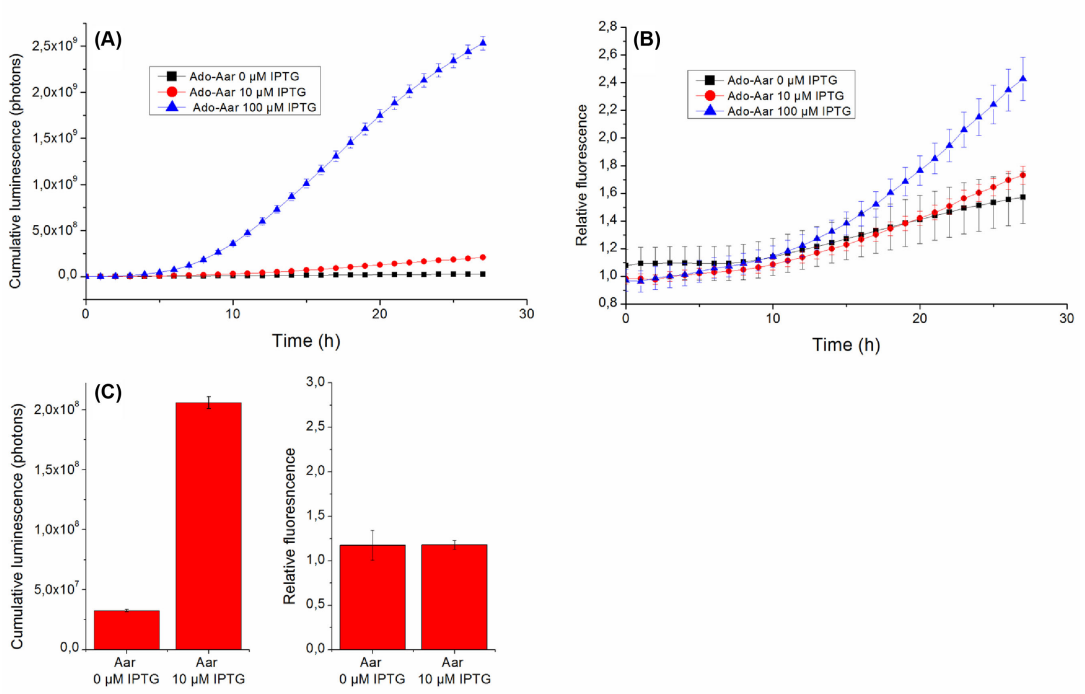

Figure 3. Monitoring the biosynthesis of alkanes. The IAS sensor cells (ADP1ΔACIAD3381-3383::*luxAB*, Δ*alkM*::*gfp*, harboring the alkane synthesis element with *ado* and *aar* under the IPTG-inducible lac/T5 promoter) were grown on LA plates in the presence of different IPTG concentrations. The constitutively expressed LuxAB measures the production of the aldehyde intermediate (**A**), while the expression of GFP is dependent on the activation of the alkane-inducible promoter P_{alkM} by alkanes (**B**). For control, responses of sensor cells expressing only Aar and not Ado were measured (**C**). The signals are presented as average of three replicates. Error bars represent standard deviation. Relative fluorescence was calculated by dividing the fluorescence signal of the sensor cells with the fluorescence signal of wild-type *A. baylyi* ADP1 cells grown in the same conditions.

synthetic biology (de Berardinis *et al.* 2009; Murin *et al.* 2012; Santala, Karp and Santala 2014; Santala *et al.* 2014; Kannisto *et al.* 2015), further research is required to investigate its potential for industrial use.

For the detection of internally produced alkanes, a different version of the twin sensor was used and a genetic module harboring the elements for alkane biosynthesis was added to the sensor strain (Fig. 1, right). In this sensor strain, alkane degradation is prevented by a knockout of *alkM*, which encodes for alkane hydroxylase and is essential for alkane degradation in *A. baylyi* (Ratajczak, Geissdorfer and Hillen 1998a). Alkane biosynthesis has been achieved in *Escherichia coli* by the expression of two cyanobacterial genes, *aar* and *ado* (Schirmer *et al.* 2010), which encode enzymes with the activities of fatty acyl-ACP reductase and aldehyde-deformylating oxygenase (ADO), respectively. We applied the two widely used components to investigate alkane biosynthesis in *A. baylyi*. The sensor strain was transformed with a construct containing *ado* and *aar* under a lac/T5 promoter and homologous regions to facilitate the integration of the cassette to the genome. To our knowledge, this is the first time when a naturally alkane degrading strain is engineered to produce alkanes. In this twin sensor/producer strain, designated as IAS (for internal alkane sensor), the same reporter elements LuxAB and GFP allow the monitoring of long-chain aldehydes produced by Aar and alkanes produced by Ado, respectively (Fig. 1, right). Similarly to EAS strain, the gene cassette eliminating ACIAD3381-3383 was used for the insertion of *luxAB*. The ACIAD3383 gene encodes for a native fatty acyl-CoA reductase of *A. baylyi*. It was desirable to remove this activity in order to eliminate the endogenous fatty aldehyde production. The ACIAD3381 encodes for a pyruvate dehydrogenase, a deletion of which has been associated with potentially higher wax ester production (Santala *et al.* 2011b). Thus, it seemed possible that its deletion could also be beneficial for alkane production. ACIAD3382 encodes for a homocysteine synthase, and its deletion is neutral in terms of growth and lipid production (Santala *et al.* 2011b); the ACIAD3382 gene was deleted for practical reasons to allow the deletion of ACIAD3383 and ACIAD3381 with a single cassette. It is also notable that LuxAB consumes aldehyde molecules in the light-producing reaction. However, the fatty acid molecules produced in the reaction are recycled by the cells, which somewhat alleviates the possible effect on alkane production. Furthermore, we have previously used a similar detection system for the analysis of wax ester production pathway, and have found that the luciferase does not have a significant influence on the production (Santala *et al.* 2011a). Thus, the overall effect of the LuxAB activity on the production of alkanes can be considered negligible.

In order to study the alkane biosynthesis, Aar and Ado were expressed under an IPTG-inducible promoter with different concentrations of the inducer. The luminescence and fluorescence signals were measured in real time during the cell growth.

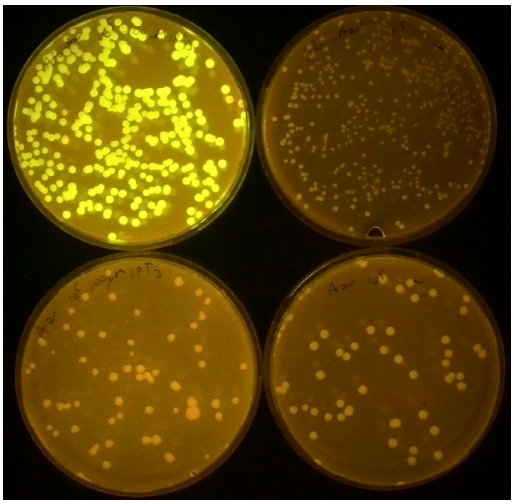

Figure 4. Detection of fluorescence from single colonies on agar plate. The alkane-producing sensor cells were plated from liquid culture on agar plate with 100 μM IPTG (top left) or without IPTG (top right) and incubated for 24 h. The bottom row is the sensor cells with only Aar and no Ado, with and without IPTG. The plates are illuminated with a blue light transilluminator.

In the presence of the inducer, a response was seen in the luminescence and fluorescence signals, indicating the production of alkanes via the aldehyde intermediate (Fig. 3). In both signals, a positive correlation was seen with the IPTG concentration and the signal strength, indicating a dynamic behavior of the sensor. The alkane production of the Ado-Aar expressing strain was also confirmed with GC-MS analysis (Supplementary File 1). A moderate induction (10 μM IPTG) of Aar alone (for control) resulted in luminescence but not fluorescence production, as expected. However, with higher inducer concentrations the expression of Aar resulted in inconsistent cell growth, potentially caused by the toxic effect of long-chain aldehydes.

Biosensors can speed up the development of efficient producer strains by allowing for high-throughput screening of different genetic modifications. Currently, practical technologies allowing the selection of single cells in a high-throughput manner are limited to fluorescence-activated cell sorting, which requires special instrumentation. Thus, it would be highly convenient to be able to screen the best phenotypes directly from colonies grown on plates. To assess the applicability of our twin sensor for high-throughput screening, individual cells were plated on agar plates and the signal strength was evaluated from single colonies. The fluorescence was readily detected from single colonies grown in the presence of the inducer (Fig. 4), suggesting that the sensor would be amenable to high-throughput screening directly on plates.

A major challenge in metabolic engineering is to balance the metabolic pathway in a way that the flux is directed to the desired product and accumulation of potentially toxic intermediates is minimized (Jones, Toparlak and Koffas 2015). One option is to screen for various natural or modified enzymes and their expression levels to find a combination that results in maximal product formation. In the alkane biosynthesis pathway, the fatty aldehyde production must be carefully balanced in order to provide ADO with sufficient substrate load while avoiding the accumulation of the fatty aldehydes, which can cause toxic or inhibitory effects (Cao *et al.* 2016). The twin configuration of the sensor enables the detection of both the intermediate and the end product, which allows more targeted optimization of the production pathway than a sensor working on a single level. The optimal production strain should show high fluorescence-to-luminescence ratio, indicating that aldehydes are effectively converted to alkanes and do not accumulate in the cells. It is well known that ADOs are relatively slow enzymes, with k_{cat} values ~1 min^{-1} (Marsh and Waugh 2013). Various strategies have been attempted to increase the turnover rate, including the removal of inhibition (Andre *et al.* 2013) and improving the substrate availability by scaffolding (Sachdeva *et al.* 2014). Another approach to improving the alkane synthesis could involve protein engineering of ADOs. The sensor presented here could serve as an efficient tool for screening the variants.

In this study, we developed and characterized a novel twin biosensor for the analysis of alkane metabolism. The sensor can be applied for the detection of both external and internally produced alkanes in real time. The twin configuration of the sensor makes it possible to simultaneously detect signals from two different levels, offering insights into the dynamics of the metabolism. Furthermore, this is the first report describing alkane production in the promising synthetic biology chassis *A. baylyi*. The presented sensor system holds potential for speeding up the metabolic engineering work aiming for efficient alkane production.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

AUTHOR CONTRIBUTIONS

TL, SS and VS designed the study. TL performed the molecular and microbiological work, analyzed the data and wrote the manuscript. SS and VS supervised and coordinated the study. All authors approved the final version of the manuscript.

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Conflict of interest. None declared.

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Supplementary File 1.

Optical density data from the measurement of external alkane induction

The cells were grown overnight in the LB medium (5 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone) supplemented with 0.4% glucose. The cultures were divided onto 96-well plate and 40 mM of alkanes added. The optical densities measured with Multiscan Ascent (Thermo Labsystems, Finland) are presented in Supplementary Table 1. The measurements were done with cultures near the stationary phase to avoid possible effects of alkanes on growth.

Supplementary Table 1. Optical density raw data for Figure 2.

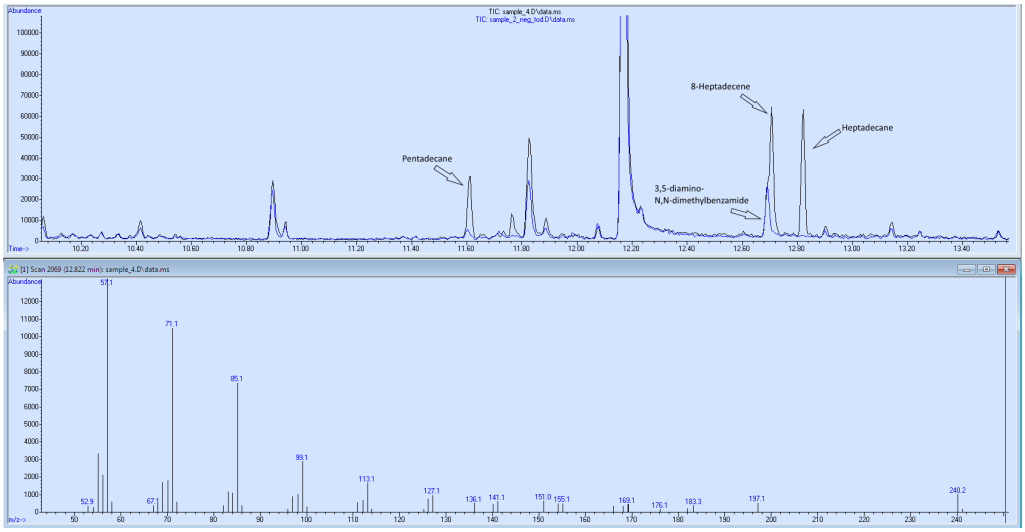
wild type																		
OD540	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**	**195**	**210**	**225**	**240**	**min**
C12	1.12	1.13	1.15	1.17	1.19	1.21	1.23	1.24	1.25	1.26	1.27	1.26	1.27	1.28	1.28	1.28	1.28	
C14	1.12	1.13	1.15	1.16	1.18	1.19	1.21	1.22	1.22	1.24	1.25	1.24	1.26	1.27	1.27	1.28	1.27	
C16	1.12	1.13	1.14	1.15	1.17	1.18	1.20	1.21	1.21	1.23	1.24	1.23	1.25	1.26	1.26	1.26	1.27	
C18	1.14	1.12	1.14	1.14	1.16	1.17	1.19	1.20	1.21	1.22	1.22	1.22	1.24	1.24	1.25	1.26	1.26	
glucose	1.09	1.09	1.10	1.11	1.13	1.14	1.15	1.16	1.16	1.17	1.17	1.18	1.19	1.20	1.20	1.21	1.20	
sensor cells																		
OD540	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**	**195**	**210**	**225**	**240**	**min**
C12	1.17	1.18	1.20	1.22	1.24	1.27	1.28	1.29	1.30	1.31	1.32	1.31	1.33	1.33	1.33	1.33	1.33	
C14	1.17	1.18	1.20	1.21	1.23	1.25	1.26	1.27	1.28	1.29	1.30	1.30	1.31	1.31	1.32	1.32	1.32	
C16	1.15	1.16	1.18	1.19	1.22	1.23	1.24	1.25	1.26	1.27	1.27	1.28	1.29	1.29	1.30	1.30	1.30	
C18	1.15	1.17	1.17	1.19	1.21	1.23	1.24	1.25	1.26	1.27	1.27	1.27	1.29	1.29	1.30	1.30	1.30	
glucose	1.11	1.13	1.14	1.15	1.17	1.17	1.19	1.20	1.20	1.22	1.21	1.22	1.24	1.23	1.25	1.24	1.24	

GC-MS analysis of alkanes produced by the sensor cells

The sensor cells harboring *ado* and *aar* genes were cultivated in 50 ml volume of MA/9 minimal salts medium (Kannisto *et al.*, 2014) supplemented with 0.2% casein amino acids, 5% glucose and 100 µM IPTG. The cells without the *ado* gene were used as a negative control (induction with 10 µM IPTG because of the effect on growth with higher concentrations). The cells were grown for 68 hours at 20°C, after which cells from 7 ml of the culture were collected by centrifugation. The total lipids from the cell pellet were extracted with Bligh and Dyer method (methanol-chloroform extraction) as described earlier (Santala et al. 2011). The chloroform phase was used for the GC-MS analysis.

GC-MS analysis was done with Agilent Technologies 6890N/5975B system, column HP-5MS 30 m x 0.25 mm with 0.25 µm film thickness, He flow 4.7 ml/min, 1µl splitless injection, oven program: 55°C hold 5 min, 55°C - 280°C 20°/min ramp, 280°C hold 3 min. Scan 50-500 m/z, 1.68 scan/sec.

GC-MS analysis showed the presence of heptadecane, 8-heptadecene and pentadecane in the samples extracted from the alkane producing cells (expressing Ado and Aar). These products are consistent with the data obtained from the *Escherichia coli* strain expressing the same genes (Schirmer *et al.*, 2010). No other alkanes or alkenes were detected. Furthermore, no alkanes or alkenes were detected in the samples of negative controls (only Aar expression). Peaks were identified based on the NIST library (Version 2.2 /Jun 2014). The identity of heptadecane peak was further confirmed and its quantitation performed with an external standard (Sigma-Aldrich). Based on the external calibration curve, the heptadecane titer was approximately 400 µg/L. Supplementary Figure 1 shows the overlaid total ion chromatograms from a sample and negative control. The analysis was confirmed with replicate samples.



Supplementary Figure 1: Top: An overlay of total ion chromatograms of the sample from alkane producing cells (black) and negative control (blue). Below: Mass spectrum of the heptadecane peak with retention time of 12.82.

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Production of long chain alkyl esters from carbon dioxide and electricity by a two-stage bacterial process

by

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Production of long chain alkyl esters from carbon dioxide and electricity by a two-stage bacterial process

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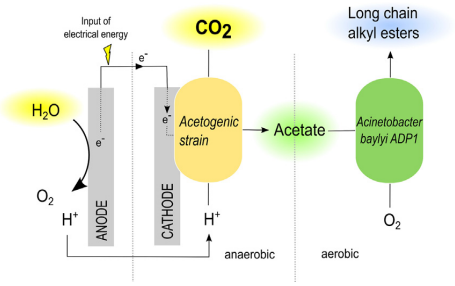
HIGHLIGHTS

- Microbial production of wax esters from CO_2 and electricity was demonstrated.
- CO_2 can be reduced to acetate in microbial electrosynthesis by *Sporomusa ovata.*
- Acetate can be converted to long chain alkyl esters by *Acinetobacter baylyi.*
- These two processes were coupled to produce long chain alkyl esters from CO_2.
- Potential for production of broad range of carbon-neutral biofuels or chemicals.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial electrosynthesis (MES) is a promising technology for the reduction of carbon dioxide into value-added multicarbon molecules. In order to broaden the product profile of MES processes, we developed a two-stage process for microbial conversion of carbon dioxide and electricity into long chain alkyl esters. In the first stage, the carbon dioxide is reduced to organic compounds, mainly acetate, in a MES process by *Sporomusa ovata.* In the second stage, the liquid end-products of the MES process are converted to the final product by a second microorganism, *Acinetobacter baylyi* in an aerobic bioprocess. In this proof-of-principle study, we demonstrate for the first time the bacterial production of long alkyl esters (wax esters) from carbon dioxide and electricity as the sole sources of carbon and energy. The process holds potential for the efficient production of carbon-neutral chemicals or biofuels.

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1. Introduction

In a carbon neutral future, the energy and carbon used in the fuels and chemicals must come from the sun and CO_2, respectively. Advances in photovoltaic technologies enable efficient harvesting of solar energy (Polman et al., 2016). However, the usability of electricity as an energy source is limited in many applications, including transportation. Production of renewable electricity (wind or solar) is also intermittent by nature, which leads to large fluctuations in production. Consequently, an efficient storage method for the electrical energy is required in order to utilize the full potential of these green technologies.

Acetogenic bacteria (acetogens) are capable of reducing CO_2 into multicarbon molecules, such as acetate and ethanol, using hydrogen as the electron donor (Drake et al., 2008). Besides the

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hydrogen gas, some acetogens are able to accept electrons from a cathode of a bioelectrochemical system in a process called microbial electrosynthesis (MES) (Lovley and Nevin, 2013; May et al., 2016; Nevin et al., 2011; Tremblay and Zhang, 2015). Thus, the reduction of CO_2 to value-added multicarbon molecules by microbial catalysts is seen as an attractive possibility. Compared with photosynthesis-derived biomass production strategies, the advantages of MES are high energy efficiency and versatility (Tremblay and Zhang, 2015). The reducing power (electrons) for MES can be obtained from various renewable and sustainable sources, such as solar or wind electricity, sulfide oxidation, or wastewater treatment (Tremblay and Zhang, 2015; Tremblay et al., 2017).

In MES systems, ca. 80–95% of consumed electrons are diverted to the end products (Nevin et al., 2010; Tremblay and Zhang, 2015). Thus, MES coupled with electricity from solar cells could be an efficient way of storing the solar energy in chemical bonds. The process can be considered as a kind of artificial photosynthesis, involving the conversion of carbon dioxide, water and sunlight into oxygen and multicarbon molecules, but with significantly greater efficiency compared with natural photosynthesis (Lovley and Nevin, 2011).

The main product of MES is typically acetate (Aryal et al., 2017, 2016; Bajracharya et al., 2017; Chen et al., 2016; Jourdin et al., 2015; LaBelle et al., 2014), which itself has value as an industrial chemical. However, in order to exploit the MES system to produce a greater variety of chemicals and biofuels, and especially products that are formed in pathways thermodynamically 'uphill', a more biochemically versatile production platform needs to be developed. Efforts have been made to alter the product profile of the acetogens by metabolic engineering or process design (Phillips et al., 2015; Ueki et al., 2014). However, introducing complex non-native pathways to acetogens may be difficult because of limited knowledge about their genetics and lack of established gene editing tools.

One solution is to employ a second microorganism to convert the end products of MES into more complex molecules. The advantage of this approach is the possibility to use a well-known organism with suitable metabolism and existing tools and know-how for sophisticated genetic engineering. This kind of two-stage approach has been just recently demonstrated by Liu et al. (2015) and Hu et al. (2016). Liu et al. (2015) used a hybrid semiconductor nanowire-bacteria system with an acetogen *Sporomusa ovata* to convert the solar energy and carbon dioxide to acetate and further produced several commodity chemicals from the acetate with genetically engineered *Escherichia coli* cells. Hu et al. (2016) produced acetate from CO_2 and H_2 with *Moorella thermoaceticum* and used a yeast *Yarrowia lipolytica* to convert the acetate to lipids. However, products with long hydrocarbon chains have not been previously produced with microbial electrosynthesis from CO_2 and electricity.

Wax esters (WEs) are high-value oleochemicals with a chemical structure of a fatty acid esterified with a long chain 1-alcohol. They have various industrial uses, including cosmetics and the production of lubricants or surfactants (Carlsson et al., 2011; Miwa, 1984). In addition, WEs can be hydrolyzed to yield fatty acid and fatty alcohol, both of which have industrial value (Carlsson et al., 2011). Because of the long hydrocarbon tails, WEs have high energy content and can be used for biofuel production (Canoira et al., 2006).

Long carbon chains are energetically expensive to produce, making their overproduction using acetogens challenging (Rabaey et al., 2011). To highlight the power of the two-stage approach, we built a microbial system which produces long alkyl chains (WEs with an average of 32 carbons/molecule) from carbon dioxide and electricity (Fig. 1). For the conversion of acetate to WEs, we utilize *Acinetobacter baylyi* ADP1, which has proven to be a suitable chassis for synthetic biology and especially lipid production (de Berardinis et al., 2009; Elliott and Neidle, 2011; Kannisto et al., 2015; Santala et al., 2011b, 2014). *A. baylyi* has high specific growth rate on acetate (1.22 h^{-1} (Du Preez et al., 1981), compared with for example that of *E. coli* (0.3 h^{-1} (Andersen and von Meyenburg, 1980)). Furthermore, its natural ability to produce storage lipids, namely triacylglycerols and WEs, makes it an appealing organism for the upgrading of the end-products of MES.

Towards the two-stage process, we first characterized the wax ester production of *A. baylyi* ADP1 from acetate. Second, we screened four different acetogenic bacteria grown on CO_2/H_2 for their acetate production and compatibility with *A. baylyi* ADP1. Finally, we demonstrate WE production from carbon dioxide and electricity by a two-stage process with *S. ovata* and *A. baylyi* ADP1.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. baylyi ADP1 (DSM 24193) was used in the study. The acetogenic bacteria (*S. ovata,* DSM 2662*; Clostridium ljungdahlii,* DSM 13528*; Clostridium aceticum,* DSM 1496*; Acetobacterium woodii,* DSM 1030) were ordered from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and revived in their DSMZ recommended media. For routine maintenance, the acetogens were cultivated in DSM 311 medium without organic carbon substrate and with a headspace of 80% H_2 and 20% CO_2. All cultivations were performed at 30 °C, except the microbial electrosynthesis, which was performed at ambient temperature (25 °C).

2.2. Bacterial cultivations

2.2.1. Wax ester production from acetate by A. baylyi

For the initial acetate utilization experiment, *A. baylyi* was cultivated in 50 ml of MA/9 minimal salts medium (Kannisto et al., 2014) supplemented with 0.2% casein amino acids and 0–200 mM acetate as carbon and energy sources. The cells were grown for 24 or 48 h in 250 ml flasks and the samples were collected for HPLC and NMR analyses.

2.2.2. Comparison of the different acetogens

For the comparison of the acetogens, the four different acetogenic strains were cultivated in DSM 311 medium without organic carbon substrate. Three replicates of 50 ml cultures were initiated with a 5 ml inoculum, the headspace pressurized with H_2/CO_2 at 1.5 bar and incubated at 30 °C and 150 rpm shaking. The bottles were repressurized daily and acetate formation followed by HPLC. After 8 days, the cells were centrifuged (7000*g*, 15 min) and the supernatants (50 ml) transferred to 250 ml flasks and used for *A. baylyi* cultivation. The pH of the medium was adjusted by adding 5 ml of 400 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer (pH 7.2). Casein amino acids (0.2%) were also added to counter the growth inhibition caused by the cysteine present in the anaerobic medium. *A. baylyi* was grown in the flasks and acetate consumption was followed with HPLC. At the end of the cultivation, samples were collected for the NMR analysis. For control, *A. baylyi* was grown in unused DSM 311 medium (*i.e.* the same medium where the acetogens were grown in the first phase). *A. baylyi* was pregrown overnight at 30 °C in MA/9 supplemented with 20 mM acetate and 5 ml was used for inoculation.

2.2.3. Acetate production by microbial electrosynthesis

Microbial electrosynthesis was performed as described earlier (Nevin et al., 2010; Tremblay et al., 2015). A graphite stick cathode (36 cm^2) and anode (36 cm^2) were suspended in 250 ml of DSM

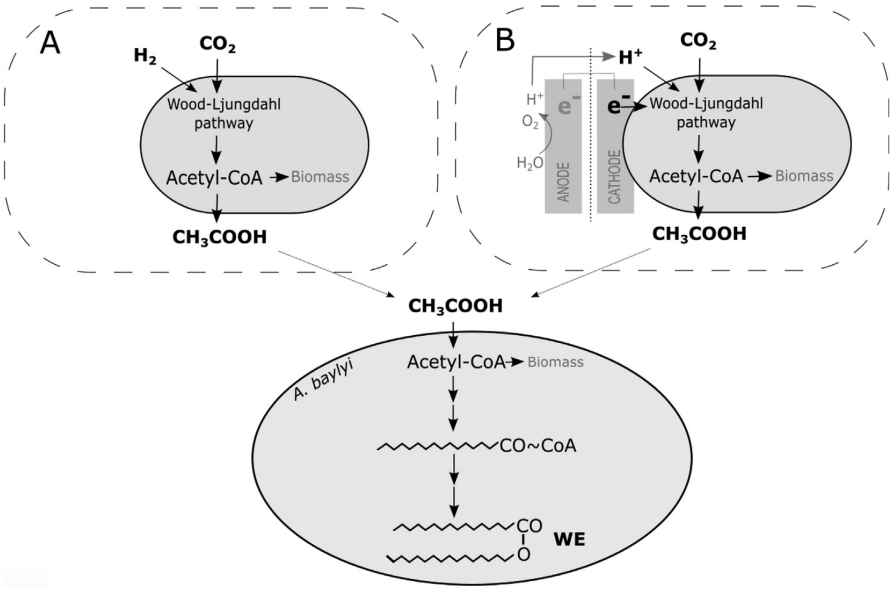

Fig. 1. The principle of the production system. The conversion of acetate to value-added long chain oleochemicals (wax esters, WE) was established using *Acinetobacter baylyi* ADP1 (bottom). The WE production module was connected with an upstream acetate production module, where acetogenic bacteria were used to produce acetate form CO_2 and H_2 gas (A) or CO_2 and electricity (B). In the case (A), the substrates are provided in gaseous form. In the case (B), the acetogenic cells are grown in the cathode chamber of a bioelectrochemical system, and electrical energy is used to provide electrons through the cathode. When either A or B is connected to the WE production module, a net production of wax esters from CO_2 is established, the sole source of energy being either H_2 gas or electricity. CoA; Coenzyme A, WE; Wax ester.

311 medium in two chambers separated by a Nafion 115 ion-exchange membrane (Ion Power, Inc., New Castle, DE, USA). A *S. ovata* strain (met-T18-2) previously developed by adaptive laboratory evolution (Tremblay et al., 2015) was used in this experiment. The cells were pregrown with H_2/CO_2 and transferred to the cathode chamber of the reactor. The cathode was poised at −690 mV (versus standard hydrogen electrode) with a potentiostat (ECM8, Gamry Instruments, USA). At this cathode potential, it is likely that a large part of the electrons transferred from the cathode to *S. ovata* are shuttled via H_2 (May et al., 2016). During the experiment both cathode and anode chambers were continuously flushed with N_2/CO_2 (80:20). The potential, acetate formation, and optical density of the reactor were monitored daily.

2.2.4. Conversion of acetate from MES to wax esters

After 10 days, the MES reactor was stopped and the medium from the cathode chamber was used for *A. baylyi* cultivation. The medium was centrifuged (7000g, 10 min) and sterile filtered, and the pH adjusted by adding 10% (vol/vol) 400 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 7.2). Three 50 ml aliquots of the medium were transferred to flasks and used for *A. baylyi* cultivation at 30 °C and 300 rpm shaking. *A. baylyi* was grown in the flasks and acetate consumption was followed with HPLC. At the end of the cultivation, samples were collected for the NMR analysis. For control, *A. baylyi* was grown in unused DSM 311 medium (the same as used in the MES reactor). *A. baylyi* was pregrown overnight at 30 °C in MA/9 supplemented with 20 mM acetate and 5 ml was used for inoculation.

2.3. Metabolite analysis

Acetate formation and consumption were monitored with high performance liquid chromatography (HPLC) using a LC-20AC prominence liquid chromatograph (Shimadzu, Japan) and Rezex RHM-Monosaccharide H + (8%) column (Phenomenex, USA) with 5 mM H_2SO_4 as the mobile phase.

2.4. Wax ester analysis with NMR spectroscopy

Wax esters were quantitatively analyzed by nuclear magnetic resonance (NMR) spectroscopy as described previously (Santala et al., 2011a). Briefly, the cells from 40 ml of the culture were collected by centrifugation, freeze-dried and lipids extracted with methanol-chloroform extraction as described previously (Santala et al., 2011b). The lipid fraction was analyzed with ^{1}H NMR, which allows the quantitative detection of the protons of α-alkoxy-methylene groups specific for WEs.

2.5. Calculations

Palmityl palmitate $C_{32}H_{64}O_2$ (a typical size of WEs in *A. baylyi* (Fixter et al., 1986 and our unpublished data)) was used as an average WE molecule in the calculations. The production of acetate from two CO_2 molecules requires 8 electrons, and the conversion of 16 acetate molecules to palmityl palmitate requires 60 electrons. Thus, in total the production of a WE molecule from CO_2 requires 188 electrons. The coulombic efficiency of MES was calculated by dividing the electrons required for acetate (8 per acetate molecule) with the electrons consumed in the cathode. In the case of WE production from acetate, the efficiency was calculated by dividing the electrons required for the production of WE (188 per WE molecule) with the electrons in the consumed acetate (8 per acetate molecule). Yields (carbon/carbon) were calculated as the ratio of carbon atoms in the consumed acetate and in the produced WEs. For calculations of WE content in milligrams, the molar mass of palmityl palmitate (480 g/mol) was used.

3. Results and discussion

3.1. Wax ester production from acetate

Several *Acinetobacter* species have been reported to efficiently utilize acetate as carbon source. Additionally, WE production from acetate has been reported for some strains (Fixter et al., 1986). As the first step towards the two-phase production system, we characterized the growth and WE production of *A. baylyi* ADP1 in different acetate concentrations. The cells were cultivated in 100 or 200 mM acetate for 48 h, and growth, acetate consumption, and WE production were analyzed. The results are presented in Table 1. *A. baylyi* tolerated both acetate concentrations without any adaptation phase, albeit less biomass was obtained with 200 mM acetate. The introduced acetate concentrations are in the same range as the amounts of the acetate produced with various MES approaches (May et al., 2016), underlining the suitability of *A. baylyi* ADP1 for the acetate upgrading. The WE production from acetate was analyzed after 24 and 48 h. The highest WE titer, 190 μmol/L (corresponding to approximately 91 mg/L) was observed with 100 mM initial acetate concentration after 24 h. At that point, 69 mM of acetate had been consumed, resulting in a 4.4% yield (carbon/carbon). After the acetate had been completely utilized, the WEs were degraded, as has been observed previously with other carbon sources (Fixter et al., 1986; Santala et al., 2011a).

3.2. Wax ester production from carbon dioxide and hydrogen

Next, we studied the WE production from carbon dioxide and hydrogen in a two-stage process, where acetate is produced by the acetogens in the first phase and consumed by *A. baylyi* in the second phase. In order to demonstrate the simplicity and applicability of the set-up, we studied the possibility to inoculate *A. baylyi* directly to the same medium that has been used for the acetogenic cultivation. Specifically, we wanted to find an acetogenic strain that is compatible with *A. baylyi*, in terms of culture medium and possible side products potentially produced by the acetogens. We evaluated the applicability and performance of four different acetogenic species for the two-stage process. The acetogens were cultivated on H_2/CO_2, and the medium and growth temperature were chosen to be close to the optimum for *A. baylyi*. The acetate production was measured (Fig. 2A) and the suitability of the supernatant for *A. baylyi* cultivation and WE production was tested (Fig. 2B–D). Of the tested strains, *S. ovata* and *A. woodii* performed better in terms of acetate production and *A. baylyi* growth and WE production than *C. aceticum*. *C. ljungdahlii* exhibited poor growth and negligible acetate production in the studied conditions (data not shown).

Interestingly, we found that the cysteine used in the anaerobic medium induces a long lag-phase in the *A. baylyi* cultivation, even if the medium was aerated before the aerobic phase. The effect could be reduced with the addition of 0.2% casein amino acids to the *A. baylyi* cultivation (data not shown). A similar effect has been reported with *E. coli* (Kari et al., 1971).

When provided with the casein amino acids, *A. baylyi* grew well and produced WEs from the acetate-containing supernatant of *S. ovata* or *A. woodii* (Fig. 2B–D). Because of the tendency of *A. baylyi* to degrade the WEs in carbon-limited conditions, the consumption of acetate was monitored during the cultivation and the experiment stopped before the acetate was completely utilized from the medium. The highest WE production, 38 μmol/L (corresponding to approximately 18 mg/L) was observed with the supernatant of *A. woodii* cultivation. At that point, 17 mM of acetate had been consumed, resulting in a 3.6% yield (carbon/carbon). A small amount of WEs (12 μmol/L) was also detected from the control samples (*A. baylyi* cultivation in unused medium) due to the use of the casein amino acids in the medium. The supernatant of *C. aceticum* did not support *A. baylyi* growth or WE production efficiently, potentially because *C. aceticum* produced little acetate, but likely consumed the casein amino acids from the medium.

3.3. Wax ester production from carbon dioxide and electricity

To demonstrate the production of long chain alkyl esters from electricity and CO_2, we set up a two-stage process in which *A. baylyi* ADP1 is used to convert the end products of MES into long chain WE molecules. *S. ovata* was chosen for the electrosynthesis experiment based on its previous high performance in MES (Chen et al., 2016; Nevin et al., 2010, 2011) and promising results in our experiments with H_2/CO_2. A *S. ovata* strain (met-T18-2) previously developed by adaptive laboratory evolution (Tremblay et al., 2015) was grown in the MES bioreactor with carbon dioxide and electricity as sources of carbon and energy, respectively, and the current and acetate formation were monitored (Fig. 3A). After the electrosynthesis phase, the medium was collected and used for *A. baylyi* cultivation as such. As expected, the acetate produced by *S. ovata* was used by *A. baylyi* for growth and WE production (Fig. 3B–C). *A. baylyi* utilized approximately 13 mM of acetate and produced 38 μmol/L WEs (carbon/carbon yield 4.6%). Again, the cells were harvested before complete consumption of the acetate. No WEs were detected from *A. baylyi* cells grown in the control medium (unused DSM 311 medium).

In this study, the production of WEs was studied with three different settings; directly from acetate, from acetate produced from H_2 and CO_2 and from acetate produced with MES. The molar yields (carbon/carbon) for WE production from acetate were similar (from 3.6% to 4.6%) in each of the three approaches, suggesting that the supernatant of acetogenic cultivations is an equally good a substrate for *A. baylyi* as acetate provided in the defined media. The variation in the WE titers is potentially caused by the different

Table 1
Wax ester production from acetate. *A. baylyi* ADP1 was cultivated in minimal salts medium with two different acetate concentrations (initial concentrations approximately 100 mM (low) and 200 mM (high)) and without acetate. The cell dry weight (CDW), acetate concentration and wax ester (WE) production were measured. The average and standard deviation from two replicates are shown. n.a; not analyzed.

		0 h	24 h	48 h
CDW (g/L)	No Acetate	n.a.	0.43 ± 0.01	0.46 ± 0.08
	Low Acetate	n.a.	1.48 ± 0.02	1.72 ± 0.08
	High Acetate	n.a.	0.37 ± 0.03	0.89 ± 0.17
Acetate (g/L)	No Acetate	0	0	0
	Low Acetate	6.50 ± 0.06	2.41 ± 0.32	0
	High Acetate	13.06 ± 0.20	13.64 ± 0.04	12.73 ± 1.3
WE (μmol/L)	No Acetate	n.a.	0	0
	Low Acetate	n.a.	185 ± 10	0
	High Acetate	n.a.	17.9 ± 1.2	136 ± 1.8

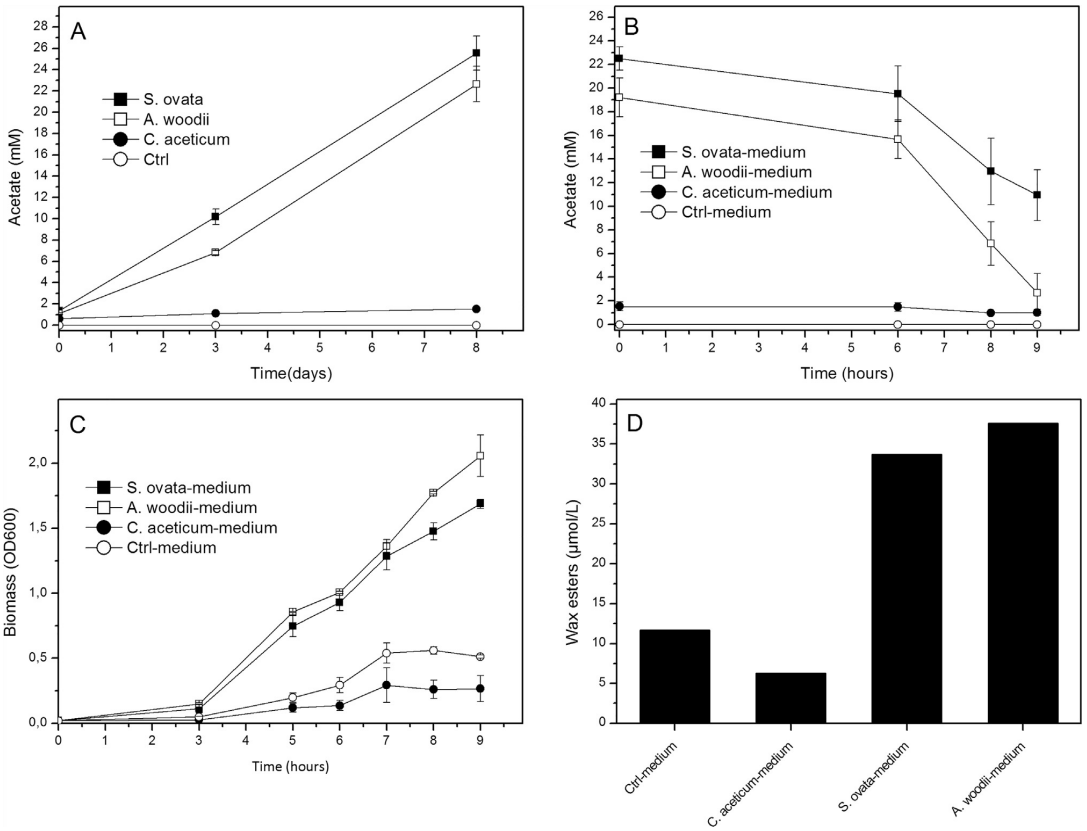

Fig. 2. Production of wax esters from carbon dioxide and hydrogen. A two-phase system was established, where in the first phase, three acetogenic strains were used to produce acetate from carbon dioxide and hydrogen (A). In the second phase, *A. baylyi* was grown in the medium from each acetogenic cultivations. The acetate consumption (B) and optical density (C) were monitored during the *A. baylyi* cultivation. The cultivations were stopped after nine hours, lipids extracted and wax ester content analyzed by NMR (D). The experiment was run in triplicates, and the average and standard deviation are shown (A–C). For the wax ester analysis, the three samples were pooled together (D). The control in (A) is the unused medium. The control in (B–D) is the culture of *A. baylyi* grown in the control medium from (A).

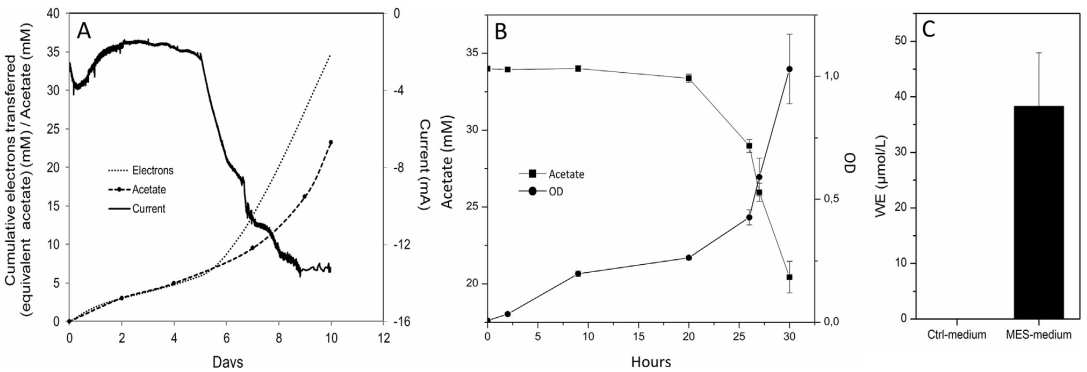

Fig. 3. Production of wax esters from carbon dioxide and electricity. A two-phase cultivation was established for the production of wax esters from carbon dioxide and electricity. First, *S. ovata* was grown in a bioelectrochemical system with carbon dioxide as the sole carbon source and electrons provided by a potentiostat as the sole source of energy. The current consumption and acetate formation were monitored (A). The Electrons curve corresponds to acetate concentration if all the consumed electrons were transferred to acetate, whereas the Acetate curve represents the real acetate concentrations measured with HPLC. After ten days, the medium from the cathode chamber was divided for three *A. baylyi* cultivations. The optical densities and acetate consumption were monitored and averages and standard deviations are shown in (B). At the end of the cultivation, the cells were collected, lipids extracted and wax esters (WE) analyzed with NMR (C). The average and standard deviation of the WE production for the three replicates is shown. The control cultivation for *A. baylyi* was performed with unused medium (identical to the medium used in the MES reactor). The Acetate curve in (A) was set to start from zero for illustrative purposes, even though there was approximately 14 mM of residual acetate in the medium in the beginning of the electrosynthesis phase.

acetate concentration at the end of the cultivation, as *A. baylyi* tends to degrade the WEs in low-carbon conditions.

The MES approach also enabled the calculation of the coulombic efficiencies (the fraction of the supplied electrons that end up in the end product). The average coulombic efficiency of the acetate production (the MES phase) was approximately 70%. The coulombic efficiency of the WE production from acetate was 6.6%, resulting in the total coulombic efficiency of 4.6% from CO_2 and electricity to WE. These values serve as a benchmark for future development of the system.

The critical aspects to be improved include the acetate productivity and titer of the MES system, and the efficiency of WE production from the acetate by *A. baylyi*. In particular, it would be beneficial to improve the WE production from relatively low acetate concentrations. The wild type *A. baylyi* ADP1 was used in this study, but potentially higher productivities could be achieved with genetically engineered strains (Kannisto et al., 2017; Santala et al., 2011b). Furthermore, *A. baylyi* has been recently engineered to produce long chain n-alkanes, which could serve as a drop-in replacement for current traffic fuels (Lehtinen et al., 2017). Thus, this system holds potential for the production of carbon neutral traffic fuel directly from carbon dioxide and electricity. Additionally, the productivity of the system could potentially be improved by optimizing the medium and culture parameters, such as the carbon/nitrogen ratio and temperature (Fixter et al., 1986). The two parts of the system work in a modular fashion, which enables them to be further developed independently of each other. Microbial electrosynthesis is a novel, rapidly developing field and advances made in the efficiency would likely benefit the overall process.

This study demonstrates for the first time the bacterial production of long chain neutral lipids from electricity and carbon dioxide as the sole sources of carbon and energy. The dual-culture approach enables the production of versatile end products, depending on the characteristics of the production strain. Production of commodity chemicals such as n-butanol and polyhydroxybutyrate has been demonstrated with a similar system with *E. coli* (Liu et al., 2015). In this study, the production of lipid compounds with >30 carbon chains was demonstrated, further emphasizing the system potential and the possibility to produce a broader range of products.

4. Conclusions

In this study, a proof of principle for long chain alkyl ester production from electricity and carbon dioxide was demonstrated. The process holds potential for producing carbon-neutral biofuels or chemicals. The two-stage cultivation process allows for the optimization of both parts individually and further development is required for both parts in order to enhance the overall productivity of the system.

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III

Improved fatty aldehyde and wax ester production by overexpression of fatty acyl-CoA reductases

by

Tapio Lehtinen, Elena Efimova, Suvi Santala, and Ville Santala, 2018

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RESEARCH

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Improved fatty aldehyde and wax ester production by overexpression of fatty acyl-CoA reductases

Tapio Lehtinen*, Elena Efimova, Suvi Santala and Ville Santala

Abstract

Background: Fatty aldehydes are industrially relevant compounds, which also represent a common metabolic intermediate in the microbial synthesis of various oleochemicals, including alkanes, fatty alcohols and wax esters. The key enzymes in biological fatty aldehyde production are the fatty acyl-CoA/ACP reductases (FARs) which reduce the activated acyl molecules to fatty aldehydes. Due to the disparity of FARs, identification and in vivo characterization of reductases with different properties are needed for the construction of tailored synthetic pathways for the production of various compounds.

Results: Fatty aldehyde production in *Acinetobacter baylyi* ADP1 was increased by the overexpression of three different FARs: a native *A. baylyi* FAR Acr1, a cyanobacterial Aar, and a putative, previously uncharacterized dehydrogenase (Ramo) from *Nevskia ramosa*. The fatty aldehyde production was followed in real-time inside the cells with a luminescence-based tool, and the highest aldehyde production was achieved with Aar. The fate of the overproduced fatty aldehydes was studied by measuring the production of wax esters by a native downstream pathway of *A. baylyi*, for which fatty aldehyde is a specific intermediate. The wax ester production was improved with the overexpression of Acr1 or Ramo compared to the wild type *A. baylyi* by more than two-fold, whereas the expression of Aar led to only subtle wax ester production. The overexpression of FARs did not affect the length of the acyl chains of the wax esters.

Conclusions: The fatty aldehyde production, as well as the wax ester production of *A. baylyi,* was improved with the overexpression of a key enzyme in the pathway. The wax ester titer (0.45 g/l) achieved with the overexpression of Acr1 is the highest reported without hydrocarbon supplementation to the culture. The contrasting behavior of the different reductases highlight the significance of in vivo characterization of enzymes and emphasizes the possibilities provided by the diversity of FARs for pathway and product modulation.

Keywords: Fatty acyl-CoA reductase, FAR, Fatty aldehyde, Wax ester, *Acinetobacter baylyi* ADP1

Background

Microbial synthesis of oleochemicals is an attractive option for the production of substitutes for the petrochemicals and fossil fuels [1, 2]. For the production of the long carbon chains required in oleochemicals, the fatty acid biosynthetic pathway is one of the few existing metabolic pathways [2]. Fatty acids and their activated forms (fatty acyl-CoAs and -ACPs) are precursors for a range of industrially relevant products, including alkanes, fatty aldehydes, fatty alcohols, triacylglycerols and wax esters [2]. Microbial production of these molecules has been achieved by the expression of native and/or heterologous enzymes in various host organisms [2, 3]. Although significant advancements in the understanding and manipulation of microbial lipid metabolism have been achieved, further consideration of the behavior and interaction of enzymes in different cell contexts is required in order to optimize the production and to diversify the range of possible products.

Of the bioproducts derived from acyl-CoA, aldehydes are of particular interest, as they represent industrially

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relevant molecules with a range of applications, from flavors and fragrances to precursors for pharmaceuticals [4]. The microbial production of volatile short-chain aldehydes has been improved by metabolic engineering [5]. In addition, aliphatic long-chain aldehydes are central intermediates in the biosynthesis of various industrially relevant lipid molecules, such as alkanes, fatty alcohols, and wax esters. Thus, the biosynthesis of these molecules would potentially benefit from increased long-chain aldehyde production. The key enzymes in aldehyde synthesis are fatty acyl-CoA (or -ACP) reductases (FAR). Various such reductases have been studied, including Aar from *Synechococcus elongatus* PCC 7942 [6], Aar-homologs from other cyanobacteria [7], and Acr1 from *Acinetobacter baylyi* ADP1 [8]. Notably, reductases found in marine bacterium *Marinobacter aquaeolei* VT8 [9, 10] or plants [11] further reduce the fatty aldehyde intermediate to fatty alcohol. Acr1 and Aar have been characterized not to reduce aldehydes [8, 12], and are thus more suitable for aldehyde production.

Depending on the cellular context and prevailing native or non-native enzyme activities, fatty aldehydes may have various fates in a cell, such as reduction to fatty alcohols, oxidation to fatty acids, or conversion to alkanes. For example, alkanes can be produced in a non-native microbial host by the expression of Aar and aldehyde-deformylating oxygenase (Ado), another enzyme originating from cyanobacteria [6, 13]. Aar catalyzes the reduction of acyl-ACP (or -CoA) to fatty aldehyde and Ado the conversion of the aldehyde to alkane [6]. In addition, the properties of the alkanes can be controlled by the selection of key enzymes with desired substrate specificities. Examples of this strategy include the expression of a modified thioesterase in *Escherichia coli* to modify the chain lengths of the alkanes produced with a synthetic pathway [14]. Another example of a pathway using fatty aldehyde as an intermediate compound is the synthesis of wax esters (WE), which are naturally produced by some bacterial species. The WE synthesis pathway in *Acinetobacter baylyi* ADP1 has been partially characterized in previous studies [8, 15]: the proposed pathway consists of three steps: (1) reduction of fatty acyl-CoA to fatty aldehyde by the fatty acyl-CoA reductase Acr1, (2) reduction of fatty aldehyde to fatty alcohol by a yet uncharacterized aldehyde reductase(s), and (3) esterification of fatty aldehyde with fatty acyl-CoA by a bifunctional wax ester synthase/diacyl glycerol acyl transferase (WS/DGAT) (Fig. 1).

In addition, *A. baylyi* ADP1 has been established as a robust chassis for synthetic biology, metabolic engineering, and genetic studies [13, 16–21]. It is particularly well suited for studying the fatty aldehyde and lipid production pathways, because of the tendency to naturally accumulate storage lipids. The acyl-CoA producing pathway is constantly active in favorable conditions [22], which simplifies the metabolic engineering process compared with other model organisms that require substantial modifications to the central carbon metabolism in order to promote efficient lipid synthesis [23]. In particular, the modularity of the WE pathway has been previously demonstrated by substituting the natural fatty acyl-CoA reductase Acr1 of *Acinetobacter baylyi* with a heterologous reductase complex LuxCDE in order to produce modified wax esters [24]. The genome of *A. baylyi* ADP1 has been sequenced and a metabolic model, as well as expression vectors and induction systems, are available [25–28]. Genetic engineering of *A. baylyi* ADP1 is straightforward due to the tendency for natural transformation and homologous recombination [20], enabling genome engineering in a high-throughput manner using automated systems [29].

As fatty aldehydes are rapidly metabolized in the cells, their analysis is often limited to indirect detection methods, such as conversion to alkanes [7] or alcohols [30].

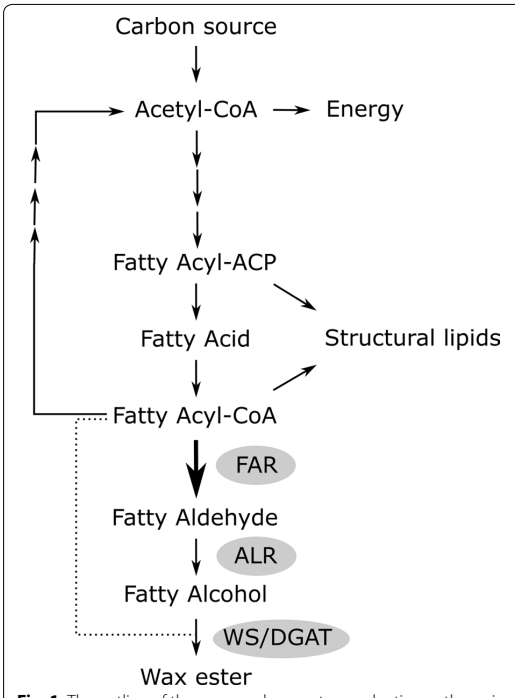

Fig. 1 The outline of the proposed wax ester production pathway in *A. baylyi*. Wax esters are produced from fatty acyl-CoA in three steps (see text for details). In this study, the effect of the overexpression of different FARs on the pathway was studied. *CoA* coenzyme A, *ACP* acyl carrier protein, *FAR* fatty acyl-CoA reductase, *ALR* aldehyde reductase, *WS/DGAT* wax ester synthase/diacylglycerol acyl transferase

We have previously developed a specific tool for monitoring the aldehyde production in the cells in real-time [13, 22, 31]. The tool is based on a bacterial luciferase LuxAB, which utilizes fatty aldehyde as a substrate in a reaction that produces visible light. Thus, aldehyde production can be directly detected in real-time during cell growth, offering a convenient tool for screening and optimization of the aldehyde-producing enzymes. In this study, we utilized the previously developed real-time aldehyde monitoring tool and the natural WE production machinery of *A. baylyi* ADP1 to study the fatty aldehyde overproduction and to elucidate the fate of the overproduced fatty aldehydes in the cells. In addition to the previously characterized FAR enzymes Acr1 and Aar, an uncharacterized putative dehydrogenase from a bacterium *Nevskia ramosa* was included in the study.

Methods

Bacterial strains and genetic modifications

The bacterial strains used in the study are listed in Table 1. The wild type *A. baylyi* ADP1 (DSM 24193, Deutsche Sammlung von Microorganismen und Zellkulturen, Germany) was used in the study. For cloning and plasmid amplification, *E. coli* XL1-Blue (Stratagene, USA) was used. The molecular work was carried out using established methods. The reagents and primers were purchased from ThermoFisher Scientific (USA) and used according to the manufacturer's instructions. Transformation of *A. baylyi* was carried out as described earlier [32]. Antibiotics were used for the selection with concentrations of 25 µg/ml for chloramphenicol and 50 µg/ml for kanamycin and spectinomycin. All genetic modifications were confirmed with PCR and sequencing.

For construction of the background strains (S1 and W1), a previously described gene cassette was used [32]. Briefly, the cassette contains chloramphenicol resistance marker and homologous regions to facilitate genomic integration. The cassette is designed to knock out the ACIAD3383–3381 genes, thereby removing the endogenous fatty acyl-CoA reductase Acr1 and pyruvate oxidase PoxB activities. For S1, the kanamycin resistance marker was used, and for W1 the marker was chloramphenicol resistance [32]. For the S1 strain, the *luxAB* genes were cut from the *iluxAB_Cm*^{r}/pAK400c plasmid [22] using *Nde*I and *Xho*I restriction enzymes and cloned to the cassette under T5/*lac* promoter. ADP1 was transformed with either of the constructs and selected with the respective antibiotic.

For FAR overexpression, a construct was prepared where the corresponding FAR gene is under T5/*lac* promoter in an integrative cassette. The integrative region of the plasmid pIM1463 [25] was PCR amplified in two parts with primers tl24, tl25, tl26 and tl27 (Additional file 1: Table S2) and designed in such a way that the *gusA* gene becomes replaced by a Biobrick RFC10 accepting cloning site (*Eco*RI-*Xba*I-*Spe*I-*Pst*I). The T5/*lac* promoter and the *lacI* gene were preserved as they are in the original plasmid. The construct contained flanking sites to facilitate the integration into a prophage region in the genome of *A. baylyi* ADP1. *Acr1* (ACIAD3383) was amplified from the genome of *A. baylyi* ADP1 and *ramo* (an open reading frame encoding for a putative short chain dehydrogenase WP_022976613.1) from the genome of *Nevskia ramosa* (DSM 11499) with primers tl17 and tl18 and tl33 and tl34, respectively (Additional file 1: Table S2). *Aar* (*S. elongatus PCC7942_orf 1594*) was codon-optimized and purchased from Genscript (USA) with appropriate restriction sites and ribosomal binding site. The three genes were separately cloned to the Biobrick cloning site of the constructed cassette. The nucleotide sequences of the three genes, as well as the sequence and plasmid map of the expression cassette, are provided in the Additional file 1.

A control strain for the S1 strain was a previously described strain *A. baylyi* ADP1Δ*poxB*::i*luxAB_Cm*^{r} [22], in which the gene ACIAD3381 is replaced with a cassette containing the *luxA* and *luxB* genes. A control strain for the W1 strain was *A. baylyi* ADP1Δ*poxB*::*Kan*^{r}/*tdk* [32], where the gene ACIAD3381 is replaced with a kanamycin resistance marker [29]. Both control strains were transformed with the empty expression cassette (expressing only *lacI* under the T5/*lac* promoter).

Table 1 Bacterial strains used in the study

Name	Genotype	Description
S1	ADP1Δ*ACIAD3381–3383::luxAB,kan*^{r}	Background strain for FAR overexpression with LuxAB for luminescence measurements (native FAR deleted)
iluxAB	ADP1Δ*ACIAD3381::luxAB,cm*^{r}	Control strain for S1 (with LuxAB), carrying native FAR (Acr1)
W1	ADP1Δ*ACIAD3381–3383::cm*^{r}	Background strain for FAR overexpression without LuxAB
ADP1Δ3381	ADP1Δ*ACIAD3381::kan*^{r}	Control strain for W1 (without LuxAB), carrying native FAR (Acr1)

ACIAD3381—*poxB*; ACIAD3383—*acr1*

Medium and culture conditions

A modified minimal salts medium MA/9 was used for the cultivations. The medium contained (per liter): 5.52 g $Na_2HPO_4{\cdot}2H_2O$, 3.4 g KH_2PO_4, 1 g NH_4Cl, 0.008 g nitrilotriacetic acid, 0.5 mg $FeCl_3$, 11.1 mg $CaCl_2$ and 240 mg $MgSO_4$. The medium was supplemented with 0.2% w/v casamino acids and 5% D-glucose. IPTG (isopropyl β-D-1-thiogalactopyranoside) was used for induction in concentrations indicated in the results section. For the luminescent measurements, the cells were incubated inside Xenogen In Vitro Imaging System (IVIS® Lumina, Caliper Life Sciences, USA) in liquid culture on 48-well plate (1 ml medium/well) at 30 °C or room temperature, and without shaking. The total flux of photons from each well was measured every hour with a 10-min exposure time. For WE production experiments, the cells were cultivated in tubes (5 ml volume) or flasks (50 ml volume) for 48 h with 300 rpm shaking and temperature as indicated in the result section. The optimal temperature for A. baylyi growth is 30 °C, and the growth is slower at lower temperatures. The cultivation time (48 h) is long enough to ensure that all the cultures have reached stationary phase at the time of WE analysis.

Lipid extraction and analysis

Small-scale lipid extraction for the thin layer chromatography (TLC) analysis was carried out as follows. Cells from equal volumes (1–3 ml) of the cultures were collected by centrifugation and suspended in 500 µl of methanol. Next, 250 µl of chloroform was added and samples were incubated with shaking for 1 h. Then 250 µl of chloroform and 250 µl of PBS added and incubated for 1 h with gentle mixing. The samples were centrifuged and the lowest phase used for TLC analysis. The TLC analysis was carried out using 10 × 10 cm Silica Gel 60 F254 HPTLC glass plates with 2.5 × 10 cm concentrating zone (Merck, USA). Mobile phase was *n*-hexane: diethyl ether: acetic acid, with proportions 90:15:1, respectively. Iodine was used for visualization. Jojoba oil was used as an external wax ester standard.

For the fatty aldehyde and fatty alcohol analysis by gas chromatography-mass spectrometry (GC–MS), cells from 10 ml of the cultures were collected and the lipids extracted as described above. Additionally, the lipids from the supernatant were extracted by mixing 1 ml of the supernatant and 1 ml of ethyl acetate vigorously for 10 min. The lower phase (chloroform) from the biomass extraction and the upper phase (ethyl acetate) from the supernatant extraction were applied to the GC–MS analysis. GC–MS analysis was performed with Agilent Technologies 6890N/5975B system, column HP-5MS 30 m × 0.25 mm with 0.25 µm film thickness, He flow 4.7 ml/min, 1 µl splitless injection, oven program: 55 °C hold 5 min, 55–280 °C 20°/min ramp, 280 °C hold 3 min. Scan 50–500 *m/z*, 1.68 scan/s. Identification of the peaks was based on the NIST library (Version 2.2/Jun 2014) and external standards (1-hexadecanol and 1-octadecanol, Sigma-Aldrich, USA).

For the wax ester analysis with NMR and GC, the cells from 40 ml of the cultures were collected by centrifugation (30,000*g*, 30 min) and the cell pellets were freeze-dried with ALPHA 1–4 LD plus freeze-dryer (Martin Christ, Germany). The extraction of lipids from freeze-dried biomass and the quantitative ^{1}H NMR analysis of wax esters was carried out as described earlier [22]. The areas of the peaks in the NMR spectrum are directly proportional to the molar concentration of each functional group. The content of wax esters in total lipid extracts was calculated from the integrated signal at 4.05 ppm which is characteristic for protons of α-alkoxy-methylene group of esters (–CH_2-COO-CH_2–). For the calculation of the WE titer in grams per liter, an average molar mass of 506 g/mol was used (corresponding to a WE with 34 carbons and one double bond).

For the analysis of the wax ester composition, the wax esters were purified from the lipid extract and analyzed by NMR and gas chromatography (GC). Purification and isolation of wax esters were performed by preparative TLC, employing aluminum sheets precoated with silica gel 60 F254 (Merck) and *n*-hexane: dichloromethane 3:2 as mobile phase. The products with R_f 0.42 were scraped from TLC plates and extracted from silica gel with chloroform with 5% of ethanol. The solutions were filtered from silica gel and solvents were removed in vacuum. The isolated wax ester fractions were analyzed by ^{1}H NMR spectroscopy. The average degree of unsaturation and the average length of fatty chains were calculated from NMR spectra as described earlier [33]. For GC, the isolated wax esters were hydrolyzed and transmethylated and peaks were qualitatively identified based on external standards. See Additional file 1 for details about NMR and GC analyses.

Results

We selected two previously characterized and one putative reductase from different organisms and studied the effect of their expression on aldehyde production in *A. baylyi* ADP1. The first one was the endogenous fatty acyl-CoA reductase Acr1 from *A. baylyi*. In addition, Aar from *S. elongatus* (PCC7942_orf1594) and an Acr1-homolog (WP_022976613.1, here designated Ramo) from *N. ramosa* were studied. Aar is characterized to accept both acyl-ACP (acyl carrier protein) and acyl-CoA (coenzyme A) as substrates [6], but Acr1 only accepts acyl-CoA [8]. Ramo has not been previously studied and thus the function as a reductase was not known. The gene was

identified with a BLAST search based on its homology to *acr1*. A preliminary in vivo characterization of Ramo in *E. coli* indicated fatty aldehyde production associated with its expression (Additional file 1).

To generate a background strain for the study, the wild type ADP1 was transformed with a genetic cassette containing the *luxA* and *luxB* genes and a kanamycin resistance marker. The construct also contained homologous flanking regions facilitating its integration to the genome and deletion of the genes ACIAD3381–3383 by homologous recombination. The gene ACIAD3383 encodes the natural fatty acyl-CoA reductase Acr1, and thus the natural aldehyde production related to the wax ester production pathway was removed [8, 22]. The gene ACIAD3381 encodes a pyruvate oxidase, and the deletion of this gene has been associated with higher WE production in an earlier study [32]. In addition to these two genes, the ACIAD3382 gene was deleted for practical reasons to allow the deletion of ACIAD3383 and ACIAD3381 with a single cassette. ACIAD3382 encodes for a homocysteine synthase, and its deletion has been shown to be neutral in terms of growth and lipid production [32]. The strain used in this study has a genotype ADP1Δ3381–3383::luxAB,kan^r and was designated S1. A control strain was the previously described *A. baylyi* ADP1Δ*poxB*::i*luxAB_Cm*^{r}, designated here as iluxAB strain [22], in which only ACIAD3381 is replaced by the luxAB-containing cassette. This strain has the wild type expression of Acr1, and therefore can be used as a control for studying the effect of the overexpression.

The three different FARs were separately cloned under T5/lac promoter in a cassette containing the *lacI* gene, a spectinomycin resistance marker, and homologous sequences facilitating its integration to a prophage region of ADP1 genome [25] (Fig. 2). The constructed cassettes were introduced to the S1 strain.

Our previously developed bioluminescence-based tool enables the monitoring of the aldehyde production in the cells in real-time [13, 22, 31]. The aldehyde production can be directly detected during cell growth. Thus, it was expected that increasing the expression of FARs would increase the long chain aldehyde production, which would be seen as an increase in the luminescent signal.

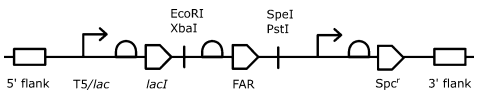

Fig. 2 The gene cassette used for the overexpression of the FARs. The cassette contains the *lacI* and FAR genes under T5/*lac* promoter, a spectinomycin resistance marker under its own promoter and homologous flanking sites facilitating the integration of the cassette to the prophage region of *A. baylyi* genome

The cells were grown at 30 °C, the expression of the FARs was induced with different concentrations of IPTG and the luminescence response monitored in real time as the cells grew (Fig. 3). For each of the three genes, the luminescence signal could be detected, indicating that the reductases were functionally expressed and provided aldehydes as suitable substrates for LuxAB. Moreover, the luminescence production associated with the expression of the putative dehydrogenase Ramo suggests that its expression leads to fatty aldehyde production in this cellular context. With all the genes, the luminescence signals increased with increasing IPTG concentrations. The signals were also markedly higher than the signal from the control strain iluxAB, indicating increased internal aldehyde production (Fig. 3). The IPTG concentration did not have a significant effect on the luminescence of the iluxAB strain (Additional file 1: Figure S2) confirming that changes in the expression levels of *luxA* and *luxB* are not limiting the luminescence production, as has been shown earlier [22]. The highest tested concentration of 1 mM IPTG yielded the highest luminescence response for Aar and Acr1, whereas for Ramo the highest luminescence signal was obtained with 100 µM IPTG (Additional file 1: Figure S3). With Ramo, the signal obtained with 1 mM IPTG was slightly lower than with 100 µM, possibly due to the increased metabolic burden associated

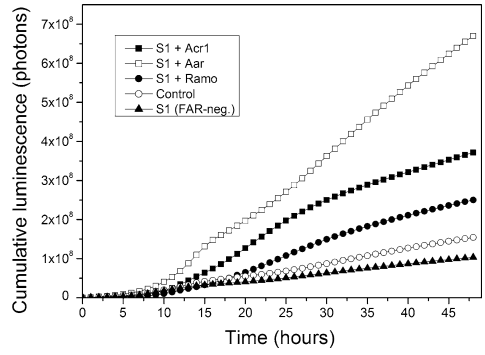

Fig. 3 The effect of FAR overexpression on aldehyde production. All the strains express the luciferase LuxAB, which reacts with fatty aldehydes to produce the measured luminescence signal. The S1 strain expressing Acr1, Aar or Ramo under an IPTG-inducible promoter were cultivated at 30 °C for 48 h and the luminescence signals measured. The control strains were iluxAB, which has a wild type expression of Acr1 and S1, which is FAR negative. Various IPTG concentrations were tested and the ones that yielded the highest signal are presented here. The concentrations were 1 mM for Acr1 and Aar, and 0.1 mM for Ramo. The full data with all the tested IPTG concentrations is presented in Additional file 1: Figures S2, S3. The signals are presented as averages of two independent replicates. Standard deviations were negligible and thus omitted for clarity

with the high expression level. These data demonstrate that the aldehyde production in *A. baylyi* can be increased in a controllable manner with the overexpression of any of the three enzymes tested.

In order to study the effect of the temperature on the luminescence production, the experiment was performed also at room temperature (ca. 24 °C). At this temperature, the luminescence signals from the overexpression strains were close to the wild type strain (Additional file 1: Figure S4). The effect of the increasing IPTG induction on the luminescence signal was similar as at 30 °C (i.e. the highest signals were obtained with the highest IPTG concentration (1 mM) with Acr1 and Aar, whereas with Ramo the highest signal was obtained with 100 µM IPTG).

Fatty aldehydes are usually rapidly metabolized by the cells [34, 35]. Thus, the fate of the overproduced fatty aldehydes in the cells was studied. For this purpose we utilized the natural wax ester production pathway present in *A. baylyi* [8, 15], for which fatty aldehyde is a specific intermediate. Based on the luminescence results, we hypothesized that at least part of the overproduced fatty aldehydes would be directed to wax ester synthesis pathway and thus lead to increased WE production.

The S1 strains overexpressing Aar, Ramo, or Acr1 were cultivated at 30 °C with the IPTG concentration that led to the highest luminescence production, and WEs were analyzed with thin layer chromatography. It was found that the overexpression of Acr1 or Ramo resulted in markedly higher WE production compared with the control cells with wild type Acr1 expression (Fig. 4). On the other hand, the Aar-expressing strain produced only a small amount of WEs, despite the higher luminescence signal. The amount of WEs produced by the Aar-expressing strain was under the detection limit of TLC (Fig. 4), but the production was confirmed with NMR analysis (17 mg/g CDW, 60 mg/l). Thus, intriguingly, even though the aldehydes produced by the different enzymes can be detected with the LuxAB-based sensor, they seem to be diverted to different downstream pathways. The FAR-negative background strain did not produce wax esters, as expected.

Wax ester production in *Acinetobacter* is strongly affected by temperature, with higher production in low temperatures [24]. Thus, we wanted to study the effect of the overexpression of FAR (endogenous or heterologous) on the WE production in different temperatures. We hypothesized that the overexpression of a key enzyme in the pathway could at least partly unravel the temperature dependency of the WE production. Because the aldehydes produced by Aar were found not to be directed to WEs in the cells, it was excluded from this experiment. In this point, the background strain was changed from the *luxAB*-containing S1 to W1. The strain W1 is otherwise identical to S1 but lacks the *luxAB* genes and has a

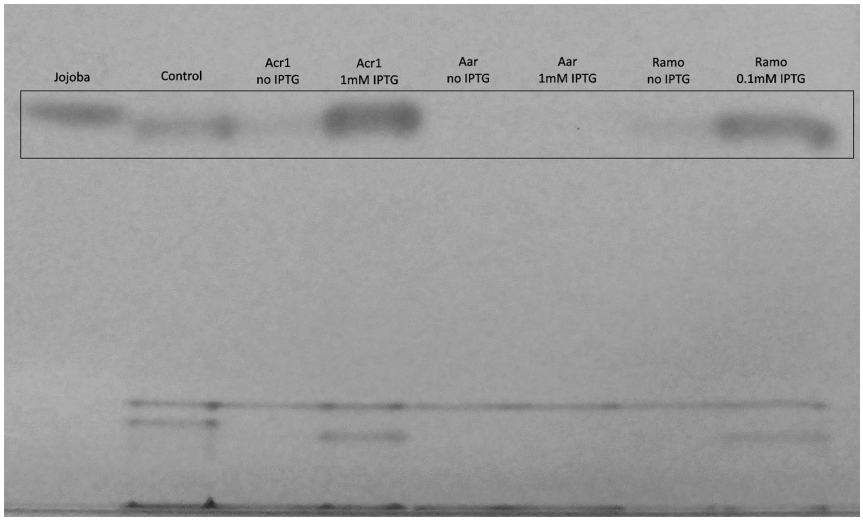

Fig. 4 Thin layer chromatography analysis demonstrating the effect of the FAR overexpression on the WE production. The S1 strain harboring different FARs under an IPTG-inducible promoter were cultivated at 30 °C for 48 h with or without IPTG, the lipids extracted and analyzed with thin layer chromatography. The control strain iluxAB has the wild type expression of Acr1. Jojoba oil (1 mg/ml) was used as a wax ester standard. Equal volume of each culture were used for the extraction. Two replicates were analyzed and a representative picture is shown. The clarity of the picture was enhanced by adjusting the contrast

chloramphenicol resistance marker instead of the kanamycin marker (ADP1ΔACIAD3381–3383::cm^r).

W1 strains harboring either *acr1* or *ramo* were cultivated at 20, 30 and 37 °C for 48 h. The inducer concentrations were chosen according to the highest luminescence signals obtained in the previous experiment; 1 mM for Acr1 and 0.1 mM for Ramo. According to quantitative NMR analyses, the overexpression of the reductases at 30 °C resulted in more than two-fold increase in the WE content of the cells for both Acr1 and Ramo compared with the control strain (Table 2). On the contrary, the overexpression at 20 °C did not significantly improve the production in comparison with the control strain (Table 2). At 37 °C, the overexpression did not improve the WE production (based on TLC analyses). The increase in the WE production was accompanied by only a small decrease in cell growth. Thus, the titer, productivity, and yield were also enhanced by approximately twofold at 30 °C (Table 2). The WE titer (0.45 g/l), productivity (9.4 mg/(l h)) and yield (0.040 g/g glucose) were highest for the Acr1 overexpressing strain grown at 20 °C, but the overexpression of the FARs enhances the WE production at 30 °C to a level comparable to that of at 20 °C (Table 2). Thus, the aldehyde overproduction effectively decouples the temperature dependency of WE production, enabling high production at the optimal growth temperature (30 °C) of *A. baylyi*.

The physical and chemical properties of WEs are largely determined by the length of the carbon chain and the level of unsaturation. In *Acinetobacter*, the wax esters typically consist of carboxyl and alcohol moieties with 16 or 18 carbons, the total length of the carbon chain being 32–36 [36]. The fatty acyl chain length of WEs is probably largely determined in the point of termination of the fatty acyl chain elongation. However, it is possible that FARs affect the composition of WEs for example through a biased substrate specificity [24]. In order to determine whether the overexpression of Acr1 or Ramo has an effect on the carbon chain length, we studied the composition of the WEs with NMR and gas chromatography. The cells were cultivated at 30 °C for 48 h and the WEs extracted, purified and analyzed. The composition of WEs was found to be similar to those of the wild type, with approximately 34 carbons and one double bond in a wax ester molecule (Additional file 1).

Because the fatty aldehydes produced by Aar were not efficiently directed to WE synthesis, we investigated alternative fates for the aldehydes. Even though aldehydes inside the cells are usually rapidly metabolized, fatty aldehydes could potentially be secreted out from the cells and thus accumulate in the culture medium. Another possibility is the conversion of fatty aldehydes to fatty alcohols by endogenous enzymes [7, 34]. The W1 cells overexpressing Aar or Acr1 were cultivated at 30 °C with 1 mM IPTG induction, and fatty aldehydes and fatty alcohols were analyzed from both the biomass and the supernatant by GC–MS. The fatty aldehyde concentration was below the detection limit in all samples both in the supernatant and in the biomass. Fatty alcohols were detected from the biomass samples but not from the supernatant. The fatty alcohol concentration was decreased in the Aar-overexpressing strain compared to the Acr1-strain and the wild type (Additional file 1: Table S1).

Discussion

Fatty acyl-CoA/ACP reductases are key enzymes in the production of fatty acyl-derived molecules, and earlier studies have indicated that this step is limiting the production of downstream products, such as fatty alcohols, in synthetic pathways [37, 38]. Thus, the identification and in vivo characterization of modular and orthogonal reductases that can be utilized in diverse metabolic engineering strategies is of high importance. In this study, we studied the effect of the overexpression of three different

Table 2 The effect of the FAR overexpression on the WE production in different temperatures

T (°C)	Strain	CDW (g/l)	WE content[a] (mg/g CDW)	WE titer[a] (g/l)	Productivity[a] (mg/(l h))	Yield[a] (g/g glucose)
30	Control	5.6 ± 0.2	35 ± 2	0.20 ± 0.01	4.1 ± 0.3	0.014 ± 0.001
	W1 + Acr1	4.3 ± 0.5	93 ± 3	0.40 ± 0.06	8.4 ± 1.3	0.032 ± 0.003
	W1 + Ramo	5.3 ± 0.3	78 ± 8	0.41 ± 0.07	8.6 ± 1.4	0.025 ± 0.005
20	Control	4.0 ± 0.06	110 ± 0	0.43 ± 0.01	9.0 ± 0.2	0.035 ± 0.002
	W1 + Acr1	3.7 ± 0.02	125 ± 11	0.45 ± 0.04	9.4 ± 0.8	0.040 ± 0.002
	W1 + Ramo	4.1 ± 0.08	109 ± 5	0.45 ± 0.03	9.4 ± 0.6	0.040 ± 0.007

The W1 (ADP1Δ3381–3383::cm^r) cells expressing either Acr1 or Ramo under an IPTG-inducible promoter were cultivated at 30 and 20 °C for 48 h, the lipids extracted and wax esters measured with NMR. The average and standard deviation of two independent cultivations are shown. The control strain was ADP1Δ3381, which has the wild type Acr1 expression

CDW cell dry weight, *WE* wax ester

[a] An average molar mass of 506 g/mol for wax esters used in the calculations

FARs in *A. baylyi* ADP1. Two well-studied aldehyde-producing (as opposed to alcohol-producing) FARs were selected for the study (Aar and Acr1). In addition, in an attempt to broaden the array of available reductases, an uncharacterized putative dehydrogenase from *Nevskia ramosa* was included in the study.

First, the effect of FAR overexpression on aldehyde production was analyzed with a bioluminescence-based sensor. It was shown that the aldehyde production in *A. baylyi* can be increased and controlled by regulating the expression any of the three studied enzymes. The highest luminescence signal was obtained with Aar. In an earlier study [30], Acr1 was more efficient in aldehyde production than Aar in *E. coli*. The difference can be due to the different detection method; Liu et al. converted the aldehydes to alcohols before analysis whereas we employed a direct luciferase-based method for the detection of aldehydes, further emphasizing the importance of monitoring the target metabolite of interest in determining the enzyme activity.

The metabolic fate of the overproduced fatty aldehydes was studied by utilizing the natural wax ester production pathway. It was shown that the increased aldehyde production correlated with increased wax ester production with Acr1 and Ramo. Thus, the aldehyde overproduction is reflected also in the downstream production efficiency. The overexpression did not impose a large burden to the cells, as demonstrated by only a small degrease in cell growth. Consequently, the increase in the WE content of the cells was reflected as an increase in also titer, productivity, and yield.

In contrast to Acr1 and Ramo, the fatty aldehydes produced by Aar were not efficiently directed towards the WE production. The reasons for this are not clear, but it can be speculated that the subcellular localization of the enzymes affect the flux of the metabolites through the WE synthesis pathway. Acr1 is speculated to be a membrane-associated protein [8], whereas Aar is soluble [12]. WS/DGAT has also been observed to be associated with the membranes [39], and it is therefore plausible to assume the whole wax ester synthesis pathway to be spatially closely arranged in the cells, ensuring efficient delivery of the intermediates between the enzymes. Since Aar is a soluble enzyme, the aldehydes produced by Aar might not be efficiently directed to these downstream enzymes. Another possible reason could be the fact that Aar also accepts acyl-ACPs as substrates, which could affect the balance of the activated acyl molecules in the cell, negatively affecting the wax ester production.

Fatty aldehydes are usually efficiently converted to the corresponding alcohols by endonous aldehyde reductases in the cells [6, 34]. However, the Aar-overexpressing strain accumulated less fatty alcohols than the wild type or the Acr1-overexpressing strain, indicating that the flux from Aar to the yet uncharacterized endogenous fatty aldehyde reductase(s) of *A. baylyi* is inefficient. As the aldehyde reduction to alcohol is the next step in the WE production pathway (Fig. 1), this indicates that the aldehyde reductase is responsible for the inefficient WE production of the Aar-overexpressing strain.

It is also possible that the subcellular distribution of fatty aldehydes affects the signal obtained with the LuxAB biosensor. The luminescent signal is expected to reflect the concentration of fatty aldehydes available for the luciferase enzyme. The aldehydes produced by Aar might be more readily available for the luciferase, which could partially explain the high luminescent signals obtained. If this is the case, Aar could potentially be an optimal reductase for the production of other aldehyde-derived products, such as alkanes. We have previously produced alkanes in the *A. baylyi* host by the expression of the two-step cyanobacterial pathway involving Aar and Ado (aldehyde deformylating oxygenase) [13]. In this pathway, Aar produces fatty aldehydes, which are converted to alkanes by Ado. Thus, the successful implementation of this pathway in *A. baylyi* indicates that the aldehydes produced by Aar are readily available for other enzymes in the cell, even though they do not support efficient wax ester synthesis.

Interestingly, while aldehydes produced by Aar were not directed to WE synthesis, the expression of another heterologous enzyme (Ramo) promoted efficient WE synthesis. The performance of Ramo was comparable to that of the endogenous Acr1, demonstrating that it is possible to improve the WE production by the expression of a heterologous enzyme. To our knowledge, this is the first report describing the heterologous expression of Ramo (WP_022976613.1) from *Nevskia ramosa*. The protein is annotated as a short chain dehydrogenase based on sequence, but little is known about the enzyme's substrate, end product, or other characteristics. In this study, we observed a correlation with fatty aldehydes and the expression of the *ramo*, indicating that fatty aldehyde is at least one of the end products in the cellular context of ADP1. Further studies would need to be carried in order to determine the details of the substrate specificity and other characteristics of the enzyme. Various reductases are widely used in metabolic engineering, and the results presented here suggest Ramo to be a useful part in the toolbox.

The contrasting behavior of Aar and Ramo underlines that even enzymes of seemingly similar function need to be studied in the context of the production chassis. In addition, an enzyme's activity is not necessarily reflected in the quantity of the final product of the pathway. Thus, to avoid futile use of cellular resources and substrates,

biological sensors that allow the detection of intermediate metabolites in real time are valuable tools in determining the in vivo activity and efficiency of heterologous enzymes. On the other hand, the differential behavior of the FARs provide possibilities to construct pathways with diverse products.

WE synthesis in *A. baylyi* has been previously observed to be more efficient at lower temperatures [24]. At the optimal growth temperature (30 °C), carbon is efficiently directed to biomass formation and not WE synthesis. With lower temperatures, the growth is slower but a larger proportion of carbon is directed to wax esters [24]. This may be due to the general tendency of *A. baylyi* to produce more WEs at lower growth rates [36], or for example adaptation to the colder environment. Based on the data presented here, FAR overexpression effectively decouples this temperature dependency and enables simultaneous high WE production and high growth rate at 30 °C. This demonstrates the natural regulatory mechanisms for WE accumulation can be overridden by the expression of a key enzyme in the pathway.

The storage lipid accumulation in various microorganisms is promoted by an excess of available carbon source [36, 40, 41]. Thus, a common strategy to improve the lipid production is to perform a two-phase cultivation, where biomass is first accumulated in an optimal growth medium and in the second phase, a medium with high carbon-to-nitrogen ratio is used to promote efficient lipid synthesis. In this work, we studied the effect of FAR overexpression in the aldehyde formation as well as the quality and quantity of WEs in a simple one-phase cultivation without optimization of the carbon to nitrogen ratio. Such optimization combined to the FAR overexpression could potentially further increase the WE titer. Even without this optimization, the WE titer (0.45 g/l) and productivity (9.4 mg/(l h)) achieved in this study are higher than previously reported with microbial production systems involving de novo synthesis of the fatty acyl chains (i.e. without hydrocarbon supplementation to the cultivation).

Conclusions

In this work, the fatty aldehyde production in *A. baylyi* was increased by the overexpression of any of the three fatty acyl-CoA reductases. With two of the reductases (Acr1 and Ramo), the increased aldehyde production was associated with an increase of the downstream product, wax esters. The wax ester titer and productivity reported here are the highest among the literature without hydrocarbon supplementation to the cultivation. With the third reductase (Aar), the overproduced fatty aldehydes were not directed to the WE synthesis, highlighting the importance of in vivo characterization of heterologous enzymes.

Additional file

Additional file 1. Supplementary data and methods.

Authors' contributions

TL, SS, and VS designed the study. TL performed the molecular and microbiological work, analyzed the data and wrote the manuscript. TL and EE carried out the lipid analytics. EE, SS, and VS participated in manuscript drafting. SS and VS supervised and coordinated the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Additional File 1

Improved Fatty Aldehyde and Wax Ester Production by Overexpression of Fatty Acyl-CoA Reductases

Tapio Lehtinen, Elena Efimova, Suvi Santala, Ville Santala

Initial characterization of Ramo

In order to identify novel candidate genes for the fatty aldehyde production, homologs for *A. baylyi* Acr1 were searched with BLAST from the non-redundant protein sequence database. Among the top results, a putative short chain hydrogenase (WP_022976613, designated here as Ramo) from *Nevskia ramosa* was selected for further studies. *Nevskia Ramosa* is an aerobic bacterium specialized in living in the air-water interface. The selection of this homolog was supported by 1) The fact that the whole genome sequence for *N. ramosa* was available also in the beginning of this study and 2) the genomic sequence revealed the presence of a WS/DGAT homolog, indicating a possible wax ester production pathway, and thus supporting the possibility that WP_022976613 could be involved in fatty aldehyde metabolism.

The *ramo* gene was PCR amplified from *N. ramosa* genome and cloned into expression vector pMIK30 under T5/*lac* promoter. The aldehyde-producing activity of Ramo was characterized *in vivo* in *Escherichia coli*. *E. coli* KRX cells harboring a luxAB/pIX plasmid (ColE1 origin of replication) were transformed with the *ramo*/pMIK30 plasmid (p15A origin of replication). The bacterial luciferase luxAB catalyzes a reaction converting fatty aldehyde to fatty acid and visible light. Thus, the aldehyde production in the cells can be monitored by following the luminescence. The cells were cultivated overnight and diluted to optical density of 0.5. The expression of Ramo was induced with IPTG, cells grown for 4 hours, and the aldehyde production measured using the LuxAB system. An increasing luminescent signal was observed with increasing IPTG concentration (Figure S1), indicating that the expression of *ramo* leads to fatty aldehyde production *in vivo*.

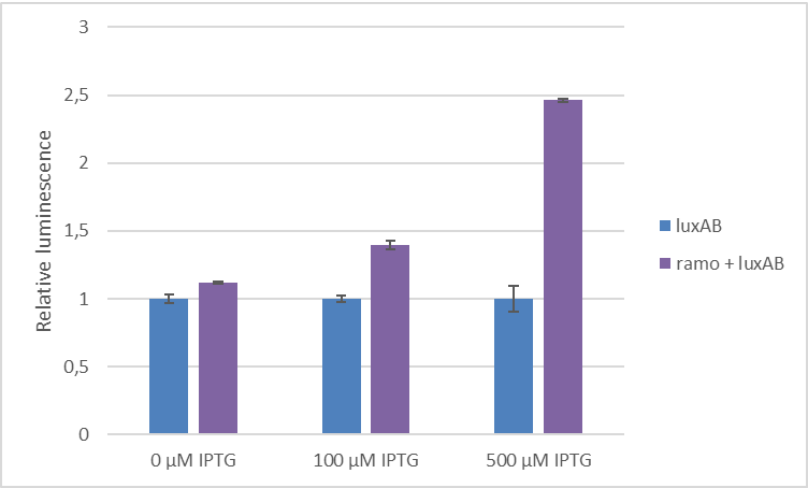

Figure S1. *In vivo* measurement of aldehyde production by Ramo. *E. coli* KRX cells harboring the *ramo* gene under an IPTG-inducible promoter and the *lux A* and *lux B* genes were cultivated with different IPTG concentrations and the luminescence signal measured. The fold change of the luminescence signal relative to the control strain harboring only the *lux A* and *B* genes is presented.

Luminescent measurements

The luminescent data complementing the data in Figure 3 are presented in Figures S2 and S3. The luminescent data from a similar experiment performed at lower temperature (room temperature, ca. 24°C) is presented in Figure S4.

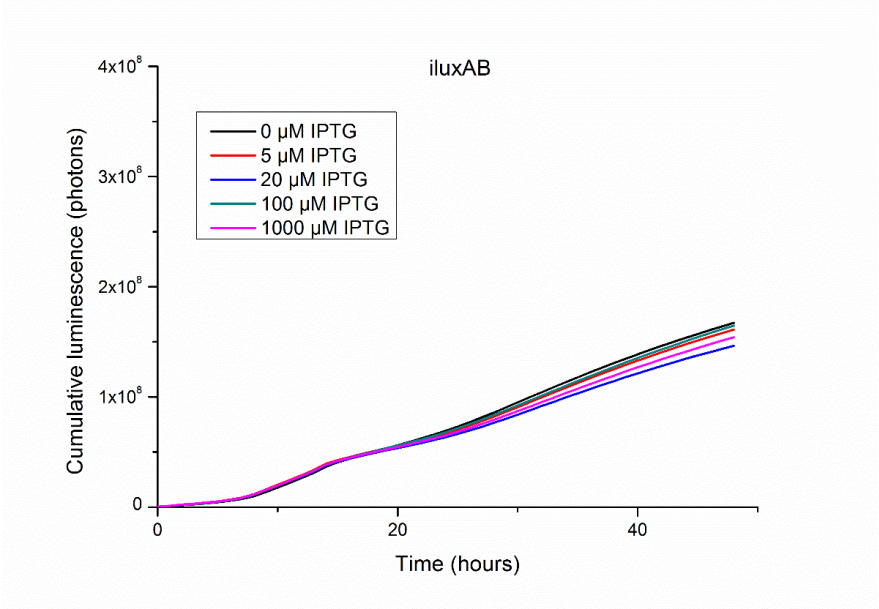

Figure S2. The luminescence production with the wild type Acr1 expression. The iluxAB cells harboring the empty expression cassette (with *lacI*, but not the FAR genes) were cultivated for 48 hours at 30°C and the luminescence signals measured. This strain has the wild type Acr1 expression, and thus the aldehyde production is expected to be independent of IPTG concentration. The data is from the same experiment as Figure 3 and Figure S3. The averages of two replicates are shown.

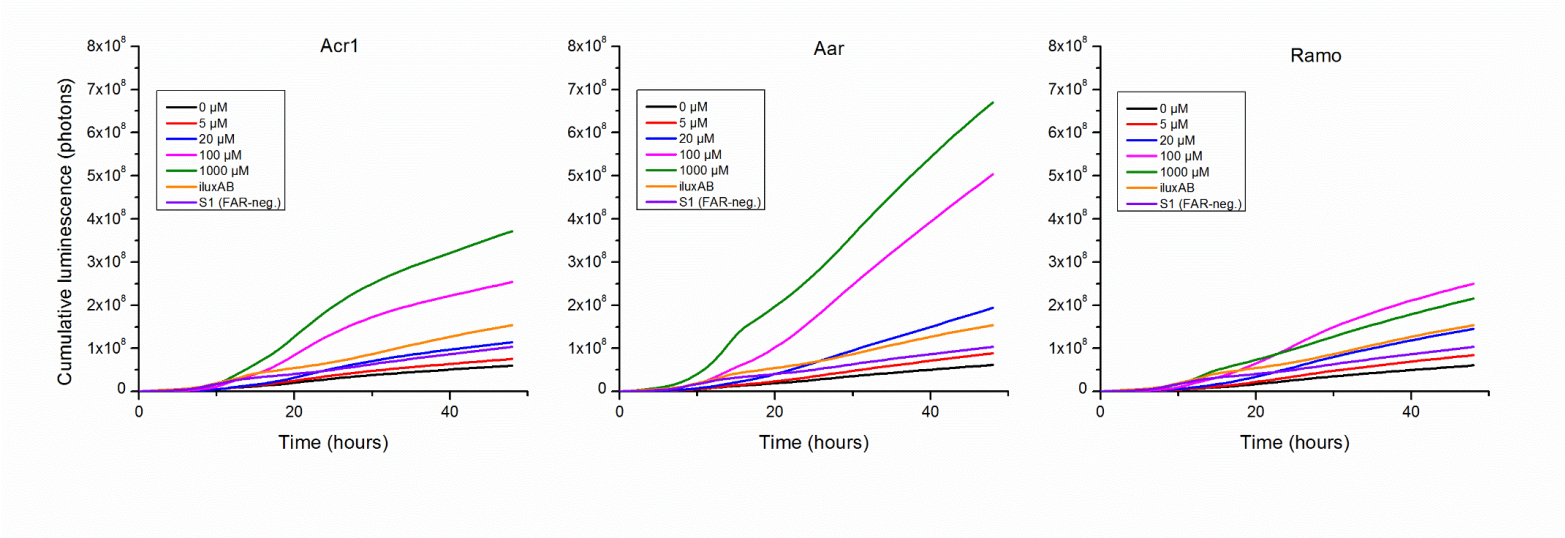

Figure S3. Luminescence production with different expression levels of the three genes. The S1 strain expressing Acr1, Aar or Ramo under an IPTG-inducible promoter were cultivated with varying concentrations of IPTG at 30°C for 48 hours and the luminescence signals measured. The data is from the same experiment, but is presented in separate graphs for clarity. The control strains iluxAB (with wild type Acr1 expression) and S1 (FAR-negative strain) are shown in each graph as a reference. The averages of two replicates are shown.

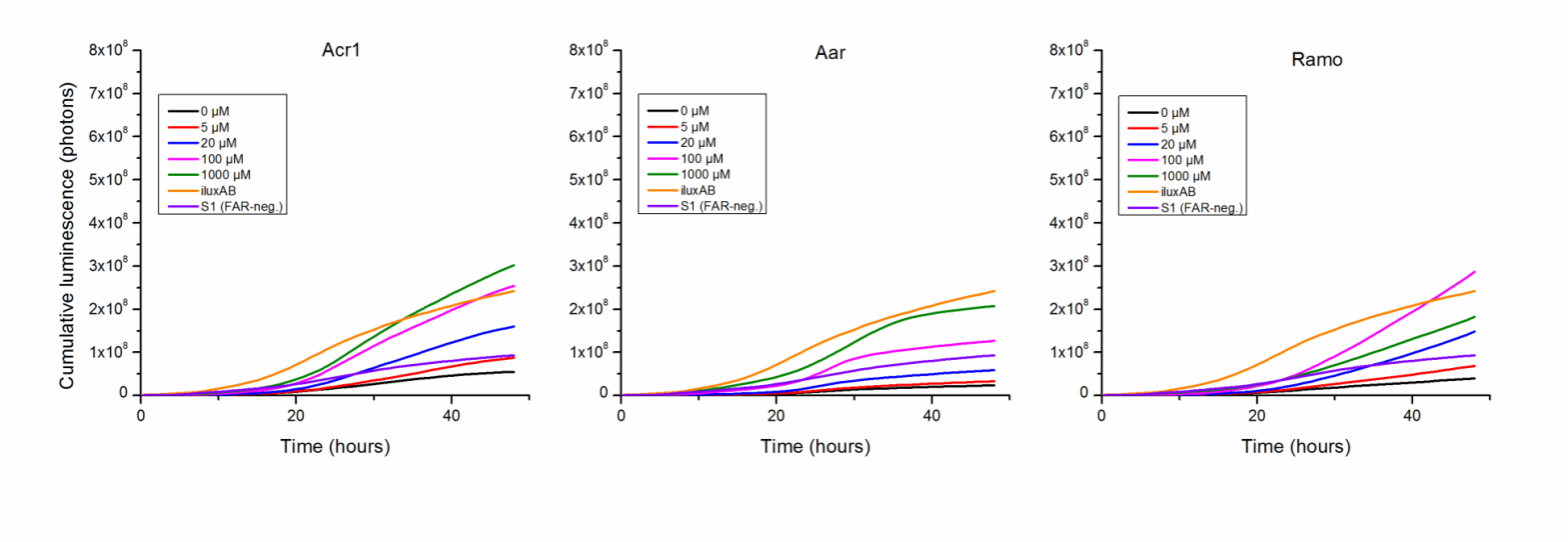

Figure S4. Luminescence production at room temperature with different expression levels of the three FARs . The S1 strain expressing Acr1, Aar or Ramo under an IPTG-inducible promoter were cultivated with varying concentrations of IPTG at room temperature (ca. 24°C) for 48 hours and the luminescence signals measured. The data is from the same experiment, but is presented in separate graphs for clarity. The control strains iluxAB (with wild type Acr1 expression) and S1 (FAR-negative strain) are shown in each graph as a reference. The averages of two replicates are shown.

Analysis of WE composition by NMR

Wax esters isolated by preparative TLC were analyzed by ^{1}H NMR. Examples of ^{1}H NMR spectra of a total lipid extract and a wax fraction isolated from it are given in Figures S5 and S6. For all the studied samples (W1 strain overexpressing either Acr1 or Ramo, and the control strain ADP1Δ3381) the ^{1}H NMR spectra looked similar.

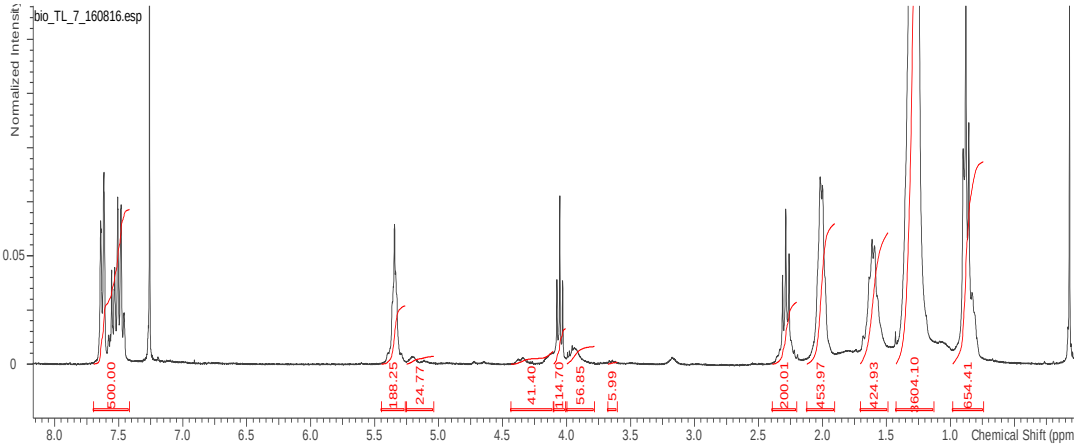

Figure S5. The ^{1}H NMR spectrum of the total lipid extract from the biomass sample W1 + Acr1.

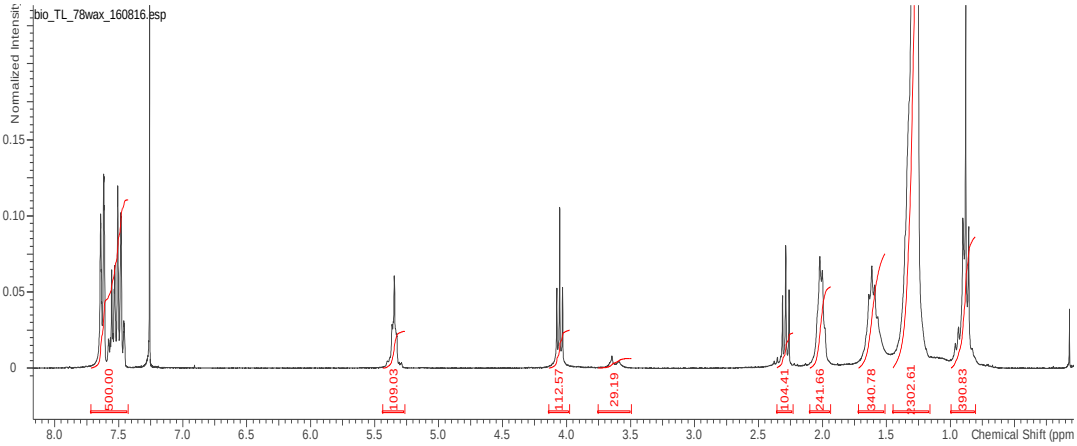

Figure S6. The ^{1}H NMR spectrum of wax ester fraction isolated by preparative TLC from the total lipid extract of the biomass sample W1 + Acr1.

The average degree of unsaturation and the average length of fatty chains were calculated from NMR spectra as described earlier [1]. The analysis revealed no significant differences between the samples, and suggested an average structure of a WE molecule with 33-34 carbons and approximately one double bond.

Analysis of WE composition by GC

Wax esters isolated by preparative TLC were hydrolyzed and transmethylated using a modified procedure of Morrison and Smith [2]. The solution (14%) of BF_3/methanol (Sigma-Aldrich Co.) was added to a dry sample of wax esters (1 mL/4-16 mg of a sample), containing methyl ester of fatty acid $C_{27:0}$ as internal standard (1 mg/sample), in a sealed glass vial and the reaction mixture was heated at 100°C for 1 h. After cooling to room temperature an equal volume of water was added and the mixture was extracted with n-hexane (2 x 2 mL). Hexane phases were combined, the solvent was removed under a stream of nitrogen and a residue was dissolved in dichloromethane (1 mL) to obtain a sample for gas-chromatography (GC).

GC-analysis of fatty acid methyl esters (FAME) and fatty alcohols obtained after hydrolysis and transmethylation of wax fractions was carried out using Thermo Finnigan gas chromatograph equipped with a flame ionization detector. A 100% dimethylpolysiloxane column (Agilent Technologies) DB-1 (30m x 0.32 mm i.d., film thickness 0.25 µm) was used. Helium was the carrier gas at a flow rate of 1 mL/min; split flow was 50 mL/min; injection and detector temperature were 250°C. The oven temperature was programmed from 140°C for 5 min and then at the rate of 4°/min to 250°C and at 250°C with final hold for 10 min. The identification of major peaks was based on qualitative comparison with the C_8-C_{24} FAME mix (Supelco®, Sigma-Aldrich, carbon chains $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:1n-7}$, $C_{16:0}$, $C_{18:3n-3}$, $C_{18:2n-6}$, $C_{18:1n-9}$, $C_{18:0}$, $C_{20:0}$, $C_{22:1n-9}$, $C_{22:0}$, $C_{24:0}$) and the fatty alcohols (FAL) mixture ($C_{12}H_{25}OH$, $C_{14}H_{29}OH$, $C_{16}H_{33}OH$, $C_{18}H_{37}OH$) prepared in the lab.

The gas chromatogram of wax ester fractions is presented in Figure S7. Based on a comparison with the reference samples, the WEs are composed of fatty chains with 16 or 18 carbons and one or zero double bond. Taken together, both the GC and NMR methods suggest that the overexpression of Ramo or Acr1 does not significantly affect the chain length distribution or the degree of unsaturation of WEs and that WEs are predominantly composed of acyl chains with 16 or 18 carbons, with an average of one double bond per WE molecule.

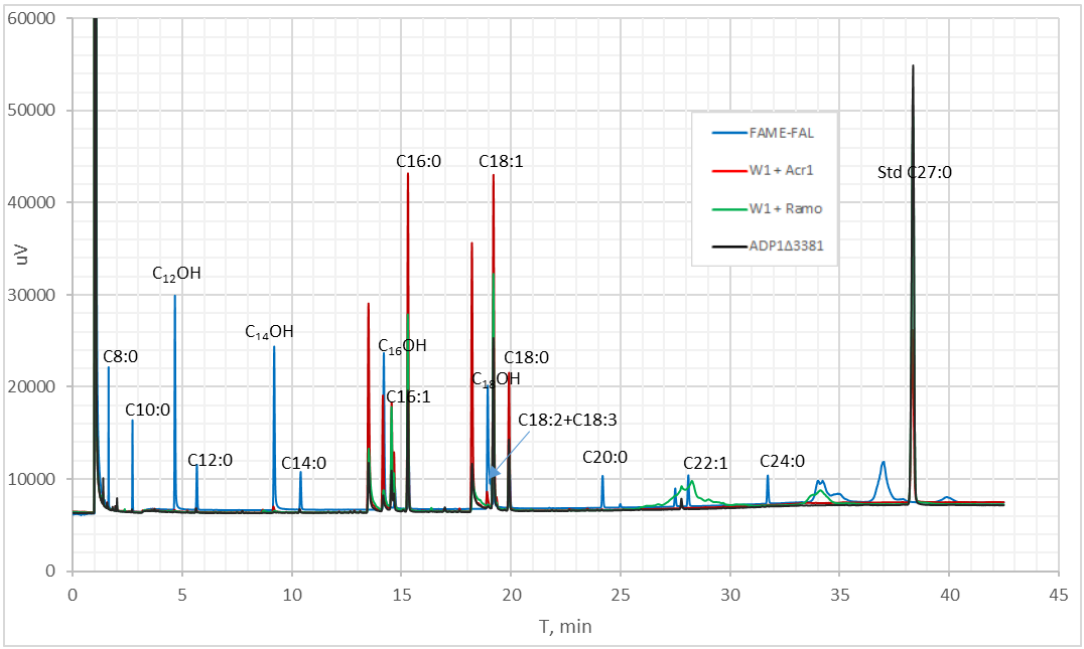

Figure S7. The gas chromatogram of purified, hydrolyzed and transmethylated samples. The control strain ADP1Δ3381 has the wild type Acr1 expression.

Analysis of fatty alcohols by GC-MS

Table S1. Fatty alcohol production of the FAR-overexpressing strains.

Strain	CDW (g/L)	1-Hexadecanol (mg/L)	1-Octadecanol (mg/L)
Control	8.7 ± 0.05	1.7 ± 0.4	0.59 ± 0.1
W1 + Acr1	8.5 ± 0.1	8.1 ± 0.4	2.6 ± 0.2
W1 + Aar	8.1 ± 0.15	0.15 ± 0.02	0.15 ± 0.02

The W1 (ADP1Δ3381-3383::cm^r) cells expressing either Acr1 or Aar under an IPTG-inducible promoter were cultivated at 30 °C for 48 hours, the lipids extracted and fatty alcohols measured with GC-MS. The average and standard deviation of two independent cultivations are shown. The control strain was ADP1Δ3381, which has the wild type Acr1 expression. CDW, cell dry weight.

Table S2. Primers used in the study.

Name	Sequence 5' --> 3'	Description
tl24	CGTGGAATTGGTACCCTAGATGTTATTTCTTCAAAATTATGG	pIM1463 1/2 forward, KpnI
tl25	GATGATCTTTCTCTAGAAGCGGCCGCGAATTCGCATGCTCTAGTAAGCTTCTCC	pIM1463 1/2 reverse, XbaI
tl26	AGGGAGTCTAGAACTAGTAGCGGCCGCTGCAGAAAGGAGAAGCTTACTAGC	pIM1463 2/2 forward, XbaI
tl27	TGCAGCGGCCCCCGAGGCCAAGCTTACCTGAAAGCCAAT	pIM1463 2/2 reverse, SfiI
tl17	TGGAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGATATCAATCAGGG AAAAACGCG	Acr1 forward, Biobrick
tl18	GTTTCTTCCTGCAGCGGCCGCTACTAGTATTATTACCAGTGTTCGCCTGGG	Acr1 reverse, Biobrick
tl33	CGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGAATTACTTCGTCACCGG	RAMO, XbaI
tl34	GGCCGCTACTAGTATTATTACCAATGCTCGCCCTTG	RAMO, SpeI

The nucleotide sequence of *acr* (ACIAD 3383):

TTGATATCAATCAGGGAAAAACGCGTGAACAAAAAACTTGAAGCTCTCTTCCGAGAGAAT
GTAAAAGGTAAAGTGGCTTTGATCACTGGTGCATCTAGTGGAATCGGTTTGACGATTGCA
AAAAGAATTGCTGCGGCAGGTGCTCATGTATTATTGGTTGCCCGAACCCAAGAAACACTG
GAAGAAGTGAAAGCTGCAATTGAACAGCAAGGGGGACAGGCCTCTATTTTTCCTTGTGAC
CTGACTGACATGAATGCGATTGACCAGTTATCACAACAAATTATGGCCAGTGTCGATCAT
GTCGATTTCCTGATCAATAATGCAGGGCGTTCGATTCGCCGTGCCGTACACGAGTCGTTT
GATCGCTTCCATGATTTTGAACGCACCATGCAGCTGAATTACTTTGGTGCGGTACGTTTA
GTGTTAAATTTACTGCCACATATGATTAAGCGTAAAAATGGCCAGATCATCAATATCAGC
TCTATTGGTGTATTGGCCAATGCGACCCGTTTTTCTGCTTATGTCGCGTCTAAAGCTGCG
CTGGATGCCTTCAGTCGCTGTCTTTCAGCCGAGGTACTCAAGCATAAAATCTCAATTACC
TCGATTTATATGCCATTGGTGCGTACCCCAATGATCGCACCCACCAAAATTTATAAATAC
GTGCCCACGCTTTCCCCAGAAGAAGCCGCAGATCTCATTGTCTACGCCATTGTGAAACGT
CCAAAACGTATTGCGACGCACTTGGGTCGTCTGGCGTCAATTACCTATGCCATCGCACCA
GACATCAATAATATTCTGATGTCGATTGGATTTAACCTATTCCCAAGCTCAACGGCTGCA

CTGGGTGAACAGGAAAAATTGAATCTGCTACAACGTGCCTATGCCCGCTTGTTCCCAGGC
GAACACTGGTAA

The nucleotide sequence of *ramo* (encoding for a putative short chain dehydrogenase WP_022976613.1):

ATGAATTACTTCGTCACCGGCGCCACCGGTTTCATCGGCAAGCATCTGATCGAGCGCCTGCTGGCGCGCCCGGATGCC
ACCATCCACGTGCTGGTCCGCGCATCTTCGGAAGACAAGTTCGCCGCCTTGCAGGAGCGCTACGGTGATGCCGGCGA
CCGGCTGCAGATGGTCGCTGGCGACATCACCACGCCGGGCCTGGTGTCCGCCGCGGAGCTCAAGAAGCTGAAGGGC
AAGGTCGGACACGTGTTTCACCTGGCCGCCGTCTACGACATGAACATGGACGATGCGACCGGCGATCGCATCAACAA
CGAAGGCACGCGCAACACCGTGGCCTTCGCCAACAGTCTGGGCGGCGACGTGGTGCTGCATCACGTGTCGAGCGTG
GCGGTGGCCGGCGGCGATTTCGTCGGCACTTTCACCGAAGCGATGTTCGACGAAGGCCAGCCGGTCAAGCATCCGTA
CTTCCGGACCAAGTTCCAGTCCGAGAAGATCGTCCGTGACGAAGCCAAGGTGCCGTTCCGGGTCTATCGTCCCGGTGC
TGTTGTCGGCCATTCGAAGACCGGCGAGATGGACAAGATCGACGGCCCTTACTACTTCTTCAAGACCATCCAGAAGCT
CAGCCACCGGATTCCGAAGTGGCTGCCACTGCTCGGCATCGAAGGCGGCAAGGTGCCGATCGCGCCGGTCGACTACA
TCGCCGATGCGCTCGATGCGATCGCCCACAAGGACGGCCTGAACGGCCAGACCTTCCATCTGGTGCAGTCGAACAGC
CCCAGCGTCGGCGACCTGATCCAGTCGATCCTGAAGGCCGCCCACGGGCCGCGCTTCAAGAAAAAGTTCGAGCTGCC
GACGATGCCGGCCTCGATGCGCAAGTTCGGCGGCCAGATGGGCGGCGCCTTGCCGGCCAGCGTCAAGAAGCAGATC
GCCAAGGCGATCGGCGCACCGCTGTCGGTGCTCGGCTACATCACCAACCGTGCCGTGTTCGACGACAAGAACGCCCG
CGCCGCGCTCAAGGGCACCGGCATCAAGTGCCCGGAATTCCGCGAGTACGCGAAGCACCTGTGGTCGTACTGGGAGC
AGTTCCTCGACATCCACTACGAGCCGAGTGCCGAGCTGATCGCCAAGGTGAAGGGCAAGGTGATCCTGGTCACCGGC
GCCTCATCGGGCATCGGCTTCACCACCGCGAAGAACCTGGCGATTGCCGGCGCCCGGGTCATCCTGGTGGCGCGTAC
CGAATCGAACCTGATCGAAACTCAGGAGATCATTTCCCGGGCCGGCGGCGAGAGCTATGTCTACCCCTGCGATCTGAT
CGACATGAAGGCGATCGACGCCATGGCCGCGAAAGTGCTGCGCGACTTCGGCCACGTCGACATCCTGATCAACAACG
CCGGCCGCTCGATCCGCCGCGCAGTGATGGAAAGCTTCGACCGCTTCCATGACTTCGAGCGGACCATGGAGCTGAAC
TACTACGGCGCCGTGCGCCTGATCATGGCGCTGCTGCCGACGATGACCGCGCGCAAGAACGGCCACATCATCAACAT
CAGCTCGATCGGCGTGCTGGCCAACGCGGCACGCTTCTCGGCCTATGTCGCCTCCAAGGCCGCGCTCGATGCCTTCAC
CCGCTGCCTGTCGGCCGAAGTGAAAGGCAGCAACATCCGCACCACGGTGATCTACATGCCGCTGGTGCGCACGCCGA
TGATCGCTCCGACCAAGATCTACAGCTACGTGCCGACCTGGTCCCCGGACGACGCCGCCGACACCGTGATCAAGGCG
ATCCTCGACGAACCGAAATCGATCGCGACAACTCTGGGTACTGCCGCTGCGGTCAGCTATGCGATCTGGCCGAAGGT
CAACGACTACATCCTGTCGAAAGGCTTCCAGCTGTTCCCGTCATCAACCGCCGCACGTGGCTCGAAGGACAAGGACTC
GAAGGCCGACAAGCCGACCCTCGAACAGGTGGTGTTCGCGAATGTGTTCAAGGGCGAGCATTGGTAA

The nucleotide sequence of *aar* (*S. elongatus PCC7942_orf 1594),* codon-optimized and including the restriction sites and the RBS*:

GAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGTTTGGTTTAATTGGCCATTTGACCTCGCTGGAACA
GGCACGTGATGTTAGTCGTCGCATGGGCTATGATGAATATGCGGATCAGGGTCTGGAATTTTGGAGTTCTGCCCCGCC
ACAAATTGTGGATGAAATCACCGTTACATCTGCTACAGGCAAAGTGATTCATGGTCGTTATATCGAAAGCTGTTTTCTG
CCGGAAATGCTGGCAGCGCGTCGCTTTAAAACTGCCACGCGCAAAGTTTTAAATGCTATGAGTCATGCACAGAAACAT
GGTATTGATATCAGCGCGCTGGGTGGCTTTACCTCAATCATCTTTGAAAACTTTGATTTAGCCTCTTTGCGTCAAGTAC
GCGATACCACACTGGAATTTGAACGTTTTACTACGGGCAACACCCATACAGCGTATGTAATTTGTCGCCAGGTCGAAG
CCGCTGCAAAAACTCTGGGTATTGATATCACTCAAGCGACGGTTGCCGTGGTTGGTGCTACGGGCGATATTGGTTCGG
CAGTATGCCGTTGGTTGGATCTGAAATTAGGTGTCGGCGATTTGATTCTGACTGCTCGTAATCAGGAACGCCTGGATA
ACTTACAAGCAGAACTGGGTCGTGGCAAAATTTTACCTTTGGAAGCGGCCTTACCGGAAGCGGATTTTATTGTATGGG
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TTGATTGGCAGATCATGAGTGCAGCAGAAATGGCACGTCCTGAACGCCAAATGTTTGCATGCTTTGCGGAAGCCATGC
TGTTAGAATTTGAAGGCTGGCATACCAATTTTTCATGGGGTCGTAACCAGATTACAATCGAAAAAATGGAAGCGATCG
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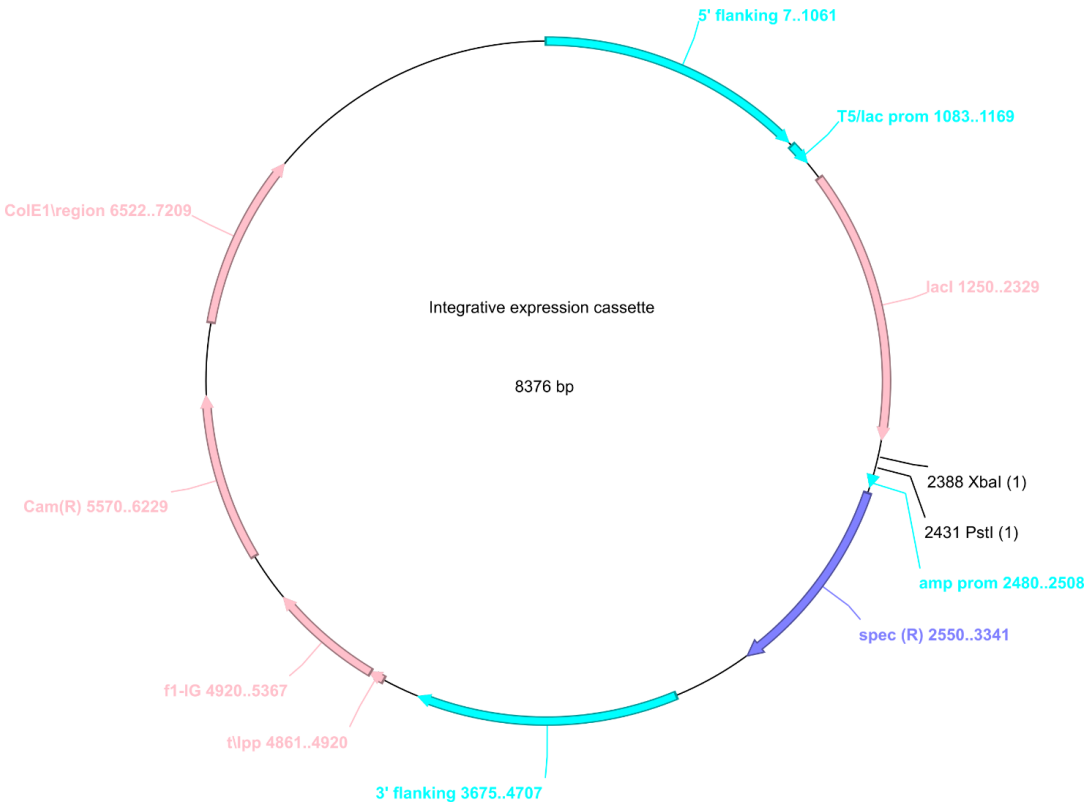

Figure S8. The integrative expression cassette. The integrative cassette used for the FAR overexpression includes the *lacI* gene and the cloning site with XbaI and PstI restriction sites under the T5/lac promoter, as well as the spectinomycin resistance marker under its own promoter. The FAR genes were cloned to this plasmid using XbaI and PstI. The 5' and 3' flanking sites facilitate the homologous recombination of the integrative part of the plasmid to the *A. baylyi* genome. Other relevant components of the plasmid backbone are the ColE1 origin of replication as well as the chloramphenicol resistance marker in the non-integrative part of the plasmid.

The nucleotide sequence of the integrative expression cassette:

GGTACCCTAGATGTTATTTCTTCAAAATTATGGATTAATTTAAATTGTTGAGCCGACATTTTATTACCCTCTTATCAAACC
GTACCTTTCACATAACGAATGAATGAATACCGTACATGGAGTGCGGCCAACCCACAGCGAACATCATATTTCGCATCC
ATCACCGTACGGTTTTCCGTTTTAAGCTCTGCCCATGATCTATCATGGAAATAACGGCTAATGATCACCTGCATCCACTC
AAGTGTCGTTTCACTGTCTGTACCATTAATAATATCCAGTACTAAACGTTGTACGGCACGAGCTTCATTATCGTTAATCT
GACACGACACTTTGTGACGTATAGCTTGTTGTACTCCTTGAGCATCACAAAGGTAATAAGCAATAAGTTTAGCTCGATC
TTTCTTCTTTACACGTACCTTGGCTTTCTTCATTGCAATAGCAATCGGGCTATCGGAATACTGTCCACCACGACAAGAAC
GTTGCCACGCTCCACATTGGCGTAACCAATCTGGCAAATCATACTTGCTCCAATCCACCGTCTGCATAATGTGCACTGC
TGTATTCATCTCATCACCTAATTTGTTTCAAGTTAAATTTTATAAGCGTTATTGTTTTATGGTTCTGCCTGCTCCTCTACC
GATCTAAAACGACAAGTTTCGAGATAATCCAGTACTCGAACTGCACCGCGTTTACCGTGTCGGTTTTTCACTACAATCA
GCTCTGTGATTCCCATCGGTTTGGTTGAGTCTTCTGGATCGGTTAGGGGGTTAACAAGGATGATCTGGTCTGCATCTT
GCTCGATTTGTCCAGATTCTTTGATATCTGATGCTTTAGGACGTTTGCCCTTCTCTGCCTCACGGTTGAGCTGTACCAGT
GCAATGACAGGACATTCAAACTCTTTCGCCATGGATTTTAATTCACGGCTGATGGAACTGACTTCCTGAAAGCGATCTT
TCTTGCTCGGGTCTCTGAGCAGTTGTAAATAATCCACGATGATGCAGCCCAATTCCTTGTAACGGCGTTTGGCTCGACG
TGCATAGGAACGGACCTCACTCAAGCTTACTAGAACTATAGCTAGTCGAGAAATCATAAAAAATTTATTTGCTTTGTGA
GCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAGAATTAATTCATTAAAGAGGAGAAAT
TAACCATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGGCCCTGAGGGCCGTGAAACCAGTAACGTTAT
ACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGA
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CAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAA
ATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAAGCG
GCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCT
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CCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCC
CATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGC
GGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCA
CTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGT
GCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAG
GATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCA
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Production of alkanes from CO_2 by engineered bacteria

by

Tapio Lehtinen, Henri Virtanen, Suvi Santala, and Ville Santala, 2018

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Biotechnology for Biofuels

RESEARCH

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Production of alkanes from CO_2 by engineered bacteria

Tapio Lehtinen*, Henri Virtanen, Suvi Santala and Ville Santala

Abstract

Background: Microbial biosynthesis of alkanes is considered a promising method for the sustainable production of drop-in fuels and chemicals. Carbon dioxide would be an ideal carbon source for these production systems, but efficient production of long carbon chains from CO_2 is difficult to achieve in a single organism. A potential solution is to employ acetogenic bacteria for the reduction of CO_2 to acetate, and engineer a second organism to convert the acetate into long-chain hydrocarbons.

Results: In this study, we demonstrate alkane production from CO_2 by a system combining the acetogen *Acetobacterium woodii* and a non-native alkane producer *Acinetobacter baylyi* ADP1 engineered for alkane production. Nine synthetic two-step alkane biosynthesis pathways consisting of different aldehyde- and alkane-producing enzymes were combinatorically constructed and expressed in *A. baylyi*. The aldehyde-producing enzymes studied were AAR from *Synechococcus elongatus*, Acr1 from *A. baylyi*, and a putative dehydrogenase from *Nevskia ramosa*. The alkane-producing enzymes were ADOs from *S. elongatus* and *Nostoc punctiforme*, and CER1 from *Arabidopsis thaliana*. The performance of the pathways was evaluated with a twin-layer biosensor, which allowed the monitoring of both the intermediate (fatty aldehyde), and end product (alkane) formation. The highest alkane production, as indicated by the biosensor, was achieved with a pathway consisting of AAR and ADO from *S. elongatus*. The performance of this pathway was further improved by balancing the relative expression levels of the enzymes to limit the accumulation of the intermediate fatty aldehyde. Finally, the acetogen *A. woodii* was used to produce acetate from CO_2 and H_2, and the acetate was used for alkane production by the engineered *A. baylyi*, thereby leading to the net production of long-chain alkanes from CO_2.

Conclusions: A modular system for the production of drop-in liquid fuels from CO_2 was demonstrated. Among the studied synthetic pathways, the combination of ADO and AAR from *S. elongatus* was found to be the most efficient in heterologous alkane production in *A. baylyi*. Furthermore, limiting the accumulation of the fatty aldehyde intermediate was found to be beneficial for the alkane production. Nevertheless, the alkane productivity of the system remained low, representing a major challenge for future research.

Keywords: Alkane, Aldehyde, Drop in, Biofuel, CO_2, Carbon dioxide, Acetate, Biosensor, *Acinetobacter baylyi* ADP1, Acetogen

Background

Microbially produced alkanes have been proposed as potential replacements for the current fossil-derived fuels and chemicals. Alkanes would be ideal substitutes for the currently used fossil fuels, because they can drop in directly in the current infrastructure and engines and have high-energy density and low hygroscopicity, preferred especially in aviation and diesel fuels [1, 2]. However, realization of alkane production in an environmentally viable way requires that alkanes are produced from sustainable substrates with high efficiency. Currently, most of the microbial alkane production studies have focused on alkane production from biomass-derived sugars. In contrast, carbon dioxide could allow bypassing the biomass step and serve as sustainable

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carbon source for large-scale production, provided that efficient methods and microbial hosts can be developed for its utilization.

The challenge of alkane production from carbon dioxide can be divided in two parts: (1) the reduction of carbon dioxide to organic compounds, and (2) the conversion of these compounds to long-chain alkanes. These processes impose different, and in part contrasting, requirements for the host metabolism, and are thus regarded difficult to achieve efficiently in any single organism. For example, the most efficient CO_2 fixation pathways require anaerobic conditions and elevated CO_2 concentrations [3, 4]. Due to the anaerobic metabolism, the organisms employing these pathways have limited capacity for the production of highly energy-intensive molecules, such as long-chain alkanes [5].

Homoacetogenic bacteria (homoacetogens) are known for their ability to efficiently reduce carbon dioxide to acetate by the Wood–Ljungdahl pathway [6]. The energy for the acetogenesis can be obtained from hydrogen gas, carbon monoxide, synthesis gas, organic compounds, or in the form of electrons directly from the cathode surface of a bioelectrochemical system in a process called microbial electrosynthesis [7–9]. Homoacetogens have been studied for decades and are already being used in industrial scale [9]. Thus, they present an attractive option for the fixation of carbon dioxide into organic compounds. However, due to the limitations of their anaerobic energy metabolism, they are unlikely to be able to efficiently produce molecules with higher energy content [10, 11]. Furthermore, metabolic engineering of homoacetogens is hampered by the lack of established gene editing tools. One solution to circumvent these issues and to broaden the metabolic capability is to employ more than one organism in the production system. For example, the acetate produced from CO_2 by the acetogens can be upgraded to value-added, high-energy molecules by heterotrophic organisms such as *Acinetobacter baylyi* [12] or *Yarrowia lipolytica* [13]. Hu et al. produced acetate with *Moorella thermoacetica* from CO_2 and H_2, and utilized *Y. lipolytica* for the conversion of the acetate into triacylglycerol. We have previously studied the conversion of acetate produced by acetogens either from CO_2 and H_2, or by CO_2 and electricity (microbial electrosynthesis) into long alkyl chains. In addition, mixed cultures have been utilized for the chain elongation of acetate to higher carboxylic acids, such as caproate and caprylate [14]. However, alkane production from acetate has not been reported to date.

Alkanes have been produced in various microbial hosts by the heterologous expression of a cyanobacterial two-step pathway. The pathway involves the reduction of fatty acyl-ACP to fatty aldehyde, which is subsequently converted to *n*-alkane. In cyanobacteria, the first step is catalyzed by acyl-ACP reductase (AAR) and the second step by aldehyde deformylating oxygenase (ADO) [15]. Besides the cyanobacterial AAR homologs, fatty aldehyde-producing reductases have been characterized from various organisms [16–18]. Even though these reductases are not known to participate in alkane production in their native context, they can be utilized as part of synthetic alkane-producing pathways in heterologous hosts. Similarly, an alkane-producing enzyme CER1 from *Arabidopsis thaliana* has been used in heterologous alkane production instead of the cyanobacterial ADOs [19, 20].

The last enzyme of the pathway, ADO, is regarded as the bottleneck for the alkane biosynthesis, due to its low activity [21]. Cyanobacteria naturally produce only small amounts of alkanes, and thus the low activity is not an issue in the native context. However, in the (heterologous) overproduction, the low activity of ADO can cause accumulation of the pathway intermediates. Especially the fatty aldehyde, which is the substrate for ADO, is problematic due to its toxic nature [22]. In addition, endogenous enzymes, such as aldehyde reductases [23] and dehydrogenases [24], compete for the fatty aldehyde substrate with ADO, leading to the loss of the valuable long acyl chains from the alkane-producing pathway. Thus, efficient alkane production requires careful balancing of the enzyme activities to provide ADO with sufficient amount of substrate, while avoiding these negative effects of fatty aldehyde accumulation. Furthermore, while the individual components (especially the cyanobacterial AARs and ADOs) of the pathway have been thoroughly studied [25–27], less attention has been paid to the performance of synthetic pathways comprised of enzymes from different origins.

We have previously developed a twin-layer biosensor for the monitoring of the alkane biosynthesis pathway [28]. The sensor consists of two parts, and is able to monitor both the intermediate fatty aldehyde and the end product alkane. The first part of the sensor consists of constantly expressed bacterial luciferase LuxAB, which utilizes fatty aldehyde as a substrate in a reaction that produces visible light. Thus, the concentration of fatty aldehydes is reflected in the luminescence signal. The second part of the sensor consists of a *gfp* gene under a native alkane-inducible promoter. Both luminescence and fluorescence can be measured in real time during cell growth, making the sensor a powerful tool for the screening and optimization of the alkane biosynthesis pathways in a high-throughput manner.

Acinetobacter baylyi ADP1 is a promising chassis for genetic studies, metabolic engineering, and lipid production [29–33]. *A. baylyi* does not naturally produce alkanes, but the fatty aldehyde intermediate is part of its

natural storage lipid metabolism [34], suggesting potential for long-chain alkane production. We have previously shown that alkanes can be produced by the expression of the cyanobacterial pathway (AAR and ADO) [28]. In addition, *A. baylyi* grows well on acetate, and we have previously shown it to be a suitable chassis for the upgrading of the acetate produced by acetogens into long-chain lipid compounds [12]. Furthermore, *A. baylyi* ADP1 has tendency for natural transformation and homologous recombination, supporting straightforward genome editing [29].

In this study, we establish alkane production from acetate in metabolically engineered *A. baylyi* ADP1. By utilizing the alkane biosensor, we compare the performance of different synthetic alkane biosynthesis pathways, and demonstrate the importance of pathway balancing for improved alkane production. Further, we demonstrate the net production of alkanes from CO_2 and H_2 by a two-stage bacterial process combining an acetogen *Acetobacterium woodii* and the *A. baylyi* engineered for alkane production (Fig. 1).

Results

As the first step towards the alkane production from CO_2, we started by constructing nine synthetic alkane biosynthesis pathways and compared their performance in alkane production from acetate. We have previously demonstrated increased fatty aldehyde production in *A. baylyi* with the expression of any of the three different reductases: Acr1, AAR and a putative dehydrogenase from *Nevskia ramosa*, designated here as Ramo [35]. Acr1 is an endogenous fatty acyl-CoA reductase from *A. baylyi* that is involved in the natural storage lipid (wax ester) production pathway. AAR from *Synechococcus elongatus* is a fatty acyl-ACP/CoA reductase employed in the alkane production pathway in cyanobacteria, and earlier studies indicate that it is the most efficient among the cyanobacterial homologs [25]. Ramo, on the other hand, is a putative short-chain dehydrogenase from the bacterium *Nevskia ramosa*. Ramo was initially identified based on its sequence homology with Acr1, and its expression was shown to increase the fatty aldehyde and wax ester production in *A. baylyi* [35]. These three fatty aldehyde-producing enzymes were included in this study. The alkane-producing enzymes selected for the study

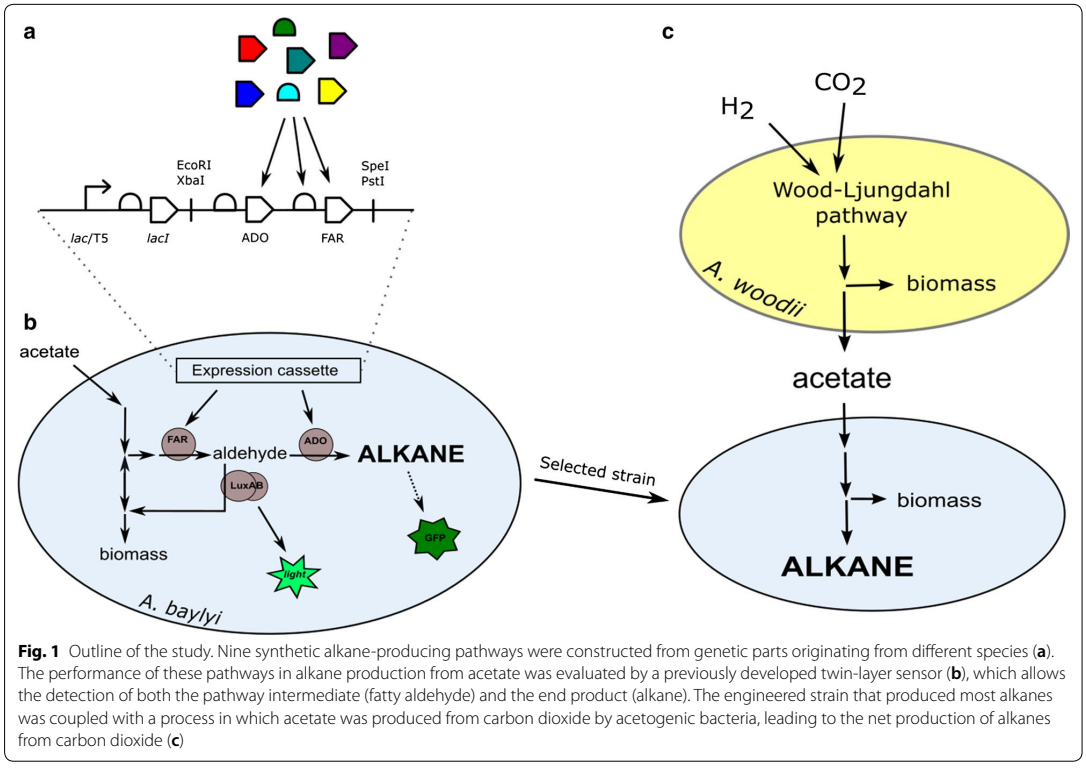

Fig. 1 Outline of the study. Nine synthetic alkane-producing pathways were constructed from genetic parts originating from different species (**a**). The performance of these pathways in alkane production from acetate was evaluated by a previously developed twin-layer sensor (**b**), which allows the detection of both the pathway intermediate (fatty aldehyde) and the end product (alkane). The engineered strain that produced most alkanes was coupled with a process in which acetate was produced from carbon dioxide by acetogenic bacteria, leading to the net production of alkanes from carbon dioxide (**c**)

were ADOs from *Synechococcus elongatus* (SeADO) and *Prochlorococcus marinus* (PmADO), and CER1 from *Arabidopsis thaliana*, all of which have been previously utilized in heterologous alkane production [15, 20].

The genes encoding the pathway enzymes were cloned under T5/*lac* promoter as synthetic operons containing first the alkane-producing and then the aldehyde-producing gene, both preceded by ribosomal binding site (RBS) sequences (Fig. 1b). The resulting nine constructs were transformed to the previously developed biosensor strain [28]. *A. baylyi* naturally degrades alkanes, but the *alkM* gene essential for alkane degradation [36] has been deleted from the sensor strain [28].

For the comparison of alkane production, the sensor cells harboring the different pathways were cultivated in 96-well plate in minimal medium supplemented with 50 mM acetate. The expression of the alkane biosynthesis genes was induced with either 10 or 100 µM IPTG and the luminescence and fluorescence signals measured in real time during cell growth (Fig. 2). The highest luminescence signal, indicating highest fatty aldehyde production, was obtained with the CER1-AAR pair. On the contrary, the highest fluorescence signal, indicating the highest alkane production, was obtained with SeADO-AAR pair with the 100 µM IPTG induction. This pair was selected for further studies.

Optimal production requires that the enzyme activities in the pathway are balanced, thus avoiding the accumulation of intermediates while maintaining high flux through the pathway. Thus, we next set out to optimize the relative expression levels of ADO and AAR. Changes in the relative expression were achieved by modulating the RBS of AAR in the synthetic operon. Three RBS:s with predicted strengths "weak", "medium" and "strong" based on *E. coli* characterization were chosen (Biobrick parts BBa_0031, BBa_0032, and BBa_0030, respectively). The original strong RBS (BBa_0034) of ADO was not changed. The three synthetic operons with varying AAR RBSs were again expressed in the sensor strain. The cells were cultivated in the 96-well plate, and luminescence and fluorescence measured in real time. As expected, the highest luminescence signal was obtained with the strongest RBS, indicating that the highest expression level of AAR led to the highest fatty aldehyde production (Fig. 3, upper panel). In contrast, the highest fluorescence signal was obtained with the intermediate strength RBS,

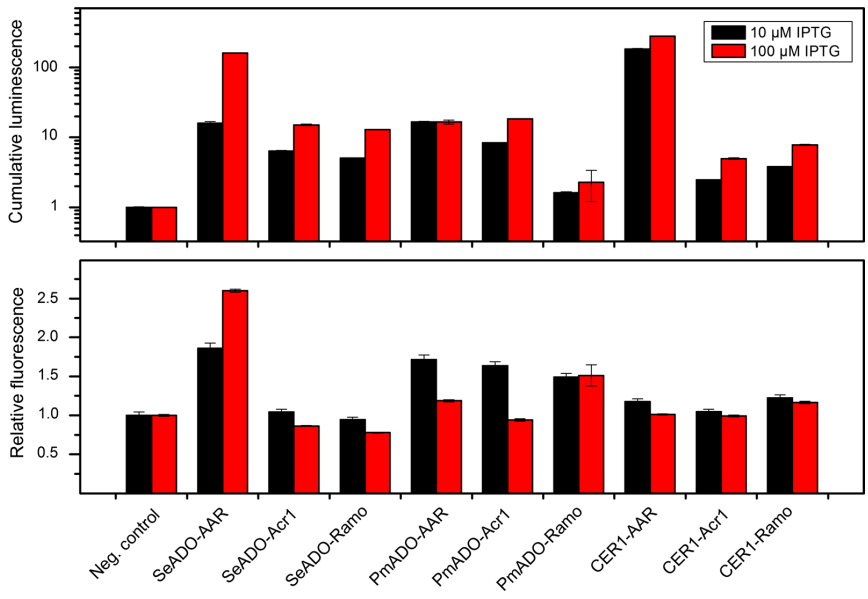

Fig. 2 Comparison of the synthetic pathways. The sensor cells containing different alkane biosynthesis pathways were cultivated in minimal medium with 50 mM acetate. The expression of the alkane biosynthesis genes was induced with 10 or 100 µM IPTG, and the luminescence (upper panel) and fluorescence (lower panel) signals produced by the sensor cells were measured. The cumulative luminescence signal reflects the amount of fatty aldehyde produced by the cells throughout the cultivation (relative to the control), and the relative fluorescence signal reflects the activation of the *gfp* expression in response to the alkanes produced by the cells. The negative control strain is the sensor strain without the alkane biosynthesis pathway. Average and standard deviation of two replicates are shown

suggesting that relatively lower expression of AAR leads to more balanced metabolism and higher alkane production (Fig. 3, lower panel). Thus, this strain was utilized in the subsequent alkane production experiments.

The application of acetate as the substrate in high concentrations is restrained by its growth-inhibiting nature [37]. Although *A. baylyi* grows well on reasonably high acetate concentrations [12, 38], carbon limitation is still inevitable in batch cultivations. To circumvent this issue and allow higher growth and alkane production, we next established a fed-batch cultivation strategy in a 1-L benchtop bioreactor. The engineered alkane production strain was cultivated in the reactor in minimal medium. The cultivation was initiated with a batch phase in 500 mL of the medium with 0.2% casein amino acids as the sole carbon source. After that, the expression of the alkane biosynthesis operon was induced with 100 µM IPTG and the feed initiated. The feed solution contained 80 g/L acetic acid, and the rate was manually controlled to keep the acetate concentration in the reactor between 30 and 60 mM to avoid carbon limitation. The cultivation was continued for 25 h after the beginning of the feed, and cell growth, acetate consumption, and alkane production were monitored (Fig. 4). During the cultivation, the cells consumed approximately 290 mmol acetate (Fig. 4). The cells produced approximately 1.6 µmol heptadecane, the final titer being 540 µg/L [productivity 21 µg/(L*h) and yield (carbon/carbon) 0.005%].

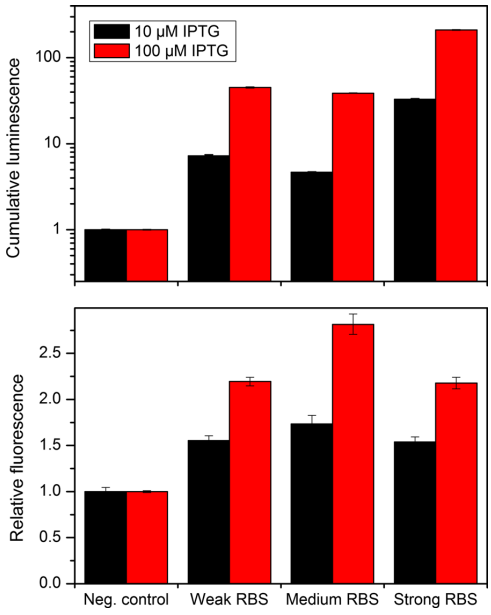

Fig. 3 Balancing the relative expression levels of ADO and AAR. The alkane biosynthesis pathway consisting of SeADO and AAR was balanced by altering the ribosomal binding site (RBS) of AAR, while the RBS of SeADO was remained unchanged (strong). The sensor cells harboring the pathways with different RBS strengths were cultivated in minimal medium with 50 mM acetate. The expression of the alkane biosynthesis genes was induced with 10 or 100 µM IPTG, and the luminescence (upper panel) and fluorescence (lower panel) signals were measured. The cumulative luminescence signal reflects the amount of fatty aldehyde produced by the cells throughout the cultivation (relative to the control), and the relative fluorescence signal reflects the activation of the *gfp* expression in response to the alkanes produced by the cells. The negative control strain is the sensor strain without the alkane biosynthesis pathway. Average and standard deviation of two replicates are shown

Acetogens are known for their ability to efficiently reduce carbon dioxide to acetate. To achieve alkane production from carbon dioxide, we employed the acetogen *A. woodii* for the production of acetate from CO_2 and H_2. *A. woodii* was grown in a bioreactor in 600 mL of minimal medium, and the medium was continuously flushed with H_2/CO_2 gas. In addition, the medium was supplemented with 4 g/L yeast extract to support the growth of *A. woodii*. The yeast extract is expected to contribute only to biomass formation and not to acetate production, as demonstrated earlier [39]. During a 6-day cultivation, *A. woodii* grew to an optical density of 2.3 and produced approximately 190 mM acetate (Fig. 5a). The medium from this cultivation, including the *A. woodii* biomass, was used as the feed for the alkane-producing *A. baylyi* as such. *A. baylyi* was grown in a bioreactor similarly as earlier. After the initial batch phase with 0.2% casein amino acids as the carbon source, the feed was initiated, and the alkane production induced with 100 µM IPTG. The rate of the feed was manually controlled to keep the acetate concentration in the reactor between 30 and 70 mM. Due to the relatively dilute feed, the volume of the cultivation increased from an initial 300 mL to the final 800 mL. *A. baylyi* utilized the feed for growth and alkane production (Fig. 5b), resulting in the net production of alkanes from CO_2 and H_2. During the 9-h cultivation, *A. baylyi* produced 0.25 µmol heptadecane, the final titer being 74 µg/L [productivity 8.2 µg/(L*h)], and consumed approximately 74 mmol of acetate (carbon/carbon yield 0.003%).

Discussion

In the production of bulk chemicals, such as liquid fuels, required in massive quantities, the sustainability of the raw material is of special importance. Carbon dioxide would be an ideal carbon source due to its abundance, independency of land usage, and the urgent need to limit its concentration in the atmosphere [40]. On the other hand, alkanes could serve as drop-in replacements to

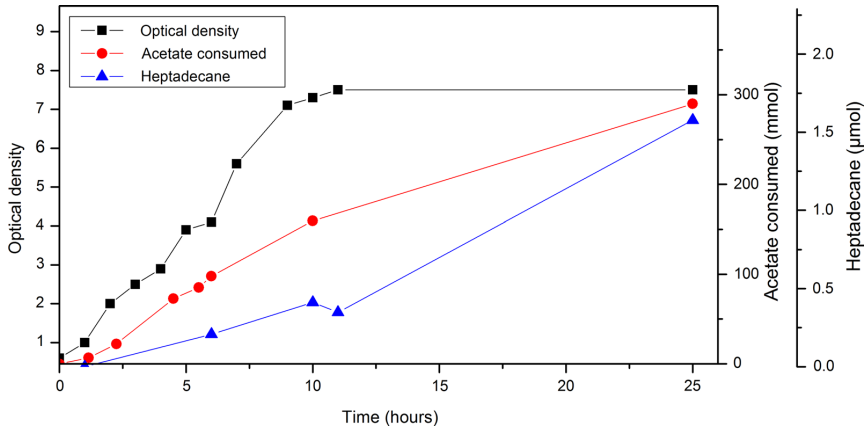

Fig. 4 Alkane production from acetate. The selected alkane-producing *A. baylyi* strain was cultivated in a bioreactor in minimal salts medium in fed-batch mode. The feed contained 80 g/L acetic acid, and the feed rate was manually controlled to keep the acetate concentration in the reactor between 30 and 60 mM. Acetate consumption was calculated based on the measured acetate concentrations in the reactor and the volume of the acetic acid solution fed to the reactor

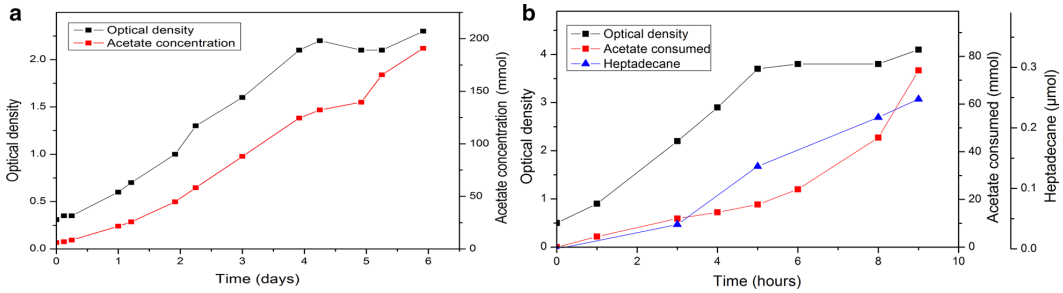

Fig. 5 Alkane production from CO_2 and H_2. *Acetobacterium woodii* was grown with CO_2 and H_2, and cell growth and acetate production were measured (**a**). After 6 days, the acetate-containing *A. woodii* medium was used as the feed for alkane-producing *A. baylyi* in fed-batch culture (**b**). The feed rate was manually controlled to keep the acetate concentration in the medium between 30 and 70 mM. *A. baylyi* utilized the feed for growth and alkane production (**b**). Due to the relatively dilute feed (containing approximately 190 mM acetate), the culture volume of the *A. baylyi* cultivation increased from 300 to 800 mL during the process

current fossil-derived liquid fuels. Microbes hold potential for both the production of alkanes, and the reduction of CO_2, but significant advancements are required to establish an efficient microbial process for the conversion of CO_2 to alkanes.

In this study, we demonstrated the microbial production of alkanes from CO_2 and H_2 by a system combining the strengths of two different bacterial species. For the CO_2 reduction, we employed the acetogen *A. woodii*, which is able to efficiently produce acetate from CO_2 and H_2 [39]. To convert the acetate to alkanes, and to achieve the net production of alkanes from CO_2, we studied heterologous alkane production in *A. baylyi* ADP1, which has been previously used to upgrade acetate to long-chain lipid molecules [12]. *A. baylyi* does not naturally produce alkanes, but we have previously demonstrated alkane production by the heterologous expression of the two-step cyanobacterial pathway [28]. In this study, we utilized the previously developed alkane biosensor [28] for the evaluation and optimization of different synthetic alkane-producing pathways. The native fatty acyl-CoA reductase Acr1 has been deleted from the sensor strain, thus disrupting the native wax ester synthesis pathway [28].

Microbial alkane production can be achieved in heterologous hosts by the expression of aldehyde-producing

fatty acyl-CoA/ACP reductase (AAR) and aldehyde deformylating oxygenase (ADO) [41]. We have previously found that the fatty aldehydes produced in *A. baylyi* may have different metabolic fates depending on the reductase that is producing them. Specifically, the aldehydes produced by Acr1 or Ramo were efficiently directed to fatty alcohols and further to wax esters by native *A. baylyi* enzymes, but the aldehydes produced by cyanobacterial AAR were not [35]. Furthermore, it has been speculated previously that the cyanobacterial AAR and ADO might interact with each other and thus ensure efficient delivery of the fatty aldehyde from AAR to ADO [27]. Thus, it is reasonable to assume that there might be differences in the efficiency of the aldehyde delivery from AAR to ADO in synthetic pathways utilizing enzymes originating from different species. To investigate the function of alkane biosynthesis pathways consisting of different enzymes, we started by constructing and comparing nine synthetic pathways that comprised of different aldehyde- and alkane-producing enzymes.

For the comparison of the synthetic pathways, we utilized the previously developed twin-layer alkane biosensor, which allows the monitoring of both the aldehyde intermediate and the end product alkane. The monitoring of the aldehyde intermediate, in addition to the end product alkane, yields valuable information about the functionality of the aldehyde-producing enzymes in this context, and enables rational evaluation of potential pathway bottlenecks. Three aldehyde-producing reductases (AAR, Acr1, Ramo) and three alkane-producing enzymes (SeADO, PmADO, CER1) were selected for the study. The expression of any of the aldehyde-producing reductases in combination with any of the alkane-producing enzymes was associated with aldehyde production, as measured by the biosensor. The results are in agreement with our earlier study, where the fatty aldehyde production was increased in *A. baylyi* by the overexpression of any of these reductases [35].

Based on the biosensor output, there are significant differences on the efficiency of alkane production from the aldehydes depending on the enzyme pair. The combination of CER1-AAR led to the highest aldehyde production, whereas the SeADO-AAR pair led to the highest alkane production. Interestingly, alkane production by SeADO was significantly higher when AAR was used to provide the aldehydes, than with the other two reductases (Acr1 or Ramo). With the other two alkane-producing enzymes (PmADO, and CER1), the difference was not so clear, and they seem to accept the aldehydes equally well from any of the reductases tested.

For synthetic pathways to work efficiently, the enzyme activities need to be balanced to avoid the accumulation of pathway intermediates and the metabolic burden caused by unnecessary high protein expression [42]. In the case of the alkane biosynthesis pathway, the relatively low activity of ADO compared to AAR may lead to the accumulation of the fatty aldehyde, potentially hampering the efficiency of alkane production. Thus, we next set out to balance the relative expression of ADO and AAR for improved alkane production. The relative expression of AAR was altered by modifying the strength of the RBS, and the effects on the aldehyde and alkane production were measured with the biosensor. The strongest RBS yielded the highest aldehyde production, as expected. In contrast, the alkane production was higher when an RBS with intermediate strength was used. This suggests that lowering the AAR expression leads to more balanced metabolism, thus avoiding the harmful effects of the fatty aldehyde accumulation, and resulting in higher alkane production. This result is in agreement with a recent study, where it was concluded that the accumulation of fatty aldehydes was not beneficial for the alkane production in *E. coli* [43].

The RBSs utilized in this study have not been previously characterized in *A. baylyi*. The luminescence signal of the biosensor is expected to correlate with the aldehyde production [28]. As the RBS modification was applied to the aldehyde-producing part (AAR) of the pathway, the luminescence signal could be used to estimate the strength of each RBS in *A. baylyi*. The "strong" RBS resulted in the production of approximately five-fold stronger luminescence signal, as compared to the "weak" RBS. On the other hand, the signal produced with the "medium" RBS was similar to that of the "weak". Further development and characterization of RBS toolbox with wider range would benefit the metabolic engineering of *A. baylyi*.

Alkane biosensors have been suggested as a potential tool for speeding up the strain development and the screening of novel and/or altered enzymes for alkane production [41]. Even though various alkane biosensors have been developed [28, 44, 45], this is the first time a sensor is used for the evaluation of the performance of different pathway configurations. The twin-layer sensor utilized here allows the monitoring of both the key intermediate and the end product of the alkane biosynthesis pathway, offering insights into the function of the pathway. The successful utilization of the sensor for the pathway balancing exemplifies the benefits of biosensors in the development of efficient production strains.

In many microbes, including *A. baylyi*, efficient lipid production is promoted by an excess of the carbon source. Typically, favorable conditions are achieved by maintaining a high carbon-to-nitrogen ratio in the culture medium. With acetate, however, high concentrations are not feasible due to its toxicity. Consequently,

batch cultivations are rapidly depleted of acetate, leading to carbon limitation and inefficient lipid production. In this study, we utilized a fed-batch cultivation strategy to support better growth and alkane production from acetate. In the cultivations, acetate concentration was maintained between 30 and 70 mM to provide the cells with sufficient carbon and energy source while avoiding the toxic effects of higher concentrations. With this strategy, alkanes could be produced both from pure acetic acid and from the acetic acid produced by the acetogen *A. woodii*. In our experiments, the alkane production was more efficient when pure acetic acid was used as the feed when compared to the medium from the acetogenic cultivation. One explanation is that the used medium also contained the biomass of *A. woodii*, leading to relatively lower carbon-to-nitrogen ratio, potentially negatively effecting the alkane production.

We have previously studied wax ester (WE) production from acetate in *A. baylyi* [12]. WEs are produced from the same intermediates that are used in the alkane production pathway (fatty aldehyde and fatty acyl-CoA), and thus it is interesting to compare the efficiency of WE and alkane production. The WE titer from acetate (ca. 90 mg/L, [12]) was significantly higher than the alkane titer in the present study (< 1 mg/L), suggesting that the precursor supply is not limiting the alkane production even in the relatively low-carbon conditions. This implies that future research should focus on improving the last step (conversion of fatty aldehydes to alkanes) in the alkane production pathway. For example, the activity of ADO may be improved by the coexpression of an electron transfer system [46].

Acetate is a platform chemical that can be produced from CO_2 by various microbial processes, such as microbial electrosynthesis or syngas fermentation. Throughout this study we utilized acetate as the carbon source for alkane production to allow the connection of the alkane-producing module to an upstream acetate-producing module. As a proof of principle, in the final stage of this study, we connected the alkane production with the upstream acetate production from CO_2 by the acetogen *A. woodii*. The two-stage production system converges around acetate, and enables the construction of versatile processes by plugging different acetate-producing and -consuming modules in the system. For example, the final product can be modified by engineering the production host. We have previously studied the wax ester production from acetate produced by the acetogens [12], while other studies have reported the production of triacylglycerides [13], alcohols [47], or *n*-butanol, polyhydroxybutyrate, and isoprenoids [48]. In this study, aliphatic long-chain alkane production from acetate was demonstrated, further expanding the product range to hydrocarbons, enabling the production of drop-in replacements for liquid fuels.

Even though the production of heptadecane from CO_2 was demonstrated in this proof-of-concept study, the low titers and productivities achieved underline the challenges related to the production of long-chain hydrocarbons. The highest microbial alkane production titers have been reported with engineered *E. coli*, exceeding 2 g/L [49]. However, these titers have been achieved with more favorable carbon sources, such as glucose. Acetate produced by acetogens could serve as a more sustainable carbon source, but alkane production from acetate has not been previously studied. *A. baylyi* is proposed as a potent host for the hydrocarbon production from acetate due to its relatively high growth rate on acetate, the tendency for storage lipid accumulation, and its amenability to genetic engineering [12, 29, 32, 38]. In this study, the first steps in improving heterologous alkane production in *A. baylyi* were taken. However, to realize efficient alkane production from CO_2, significant improvement in the alkane production efficiency along with bioprocess optimization is required. The previous study describing alkane production from CO_2 and H_2, using engineered bacterium *Cupriavidus neacator*, reported the production of 4.4 mg/L alkanes, highlighting the difficulty of obtaining high titers with cumbersome substrates [50].

The modular nature of the two-stage production system brings the additional advantage that both the CO_2 fixation and alkane production can be optimized independently of each other. For example, significantly higher acetate production rates have been obtained with *A. woodii* with optimized reactor design [39] or metabolic engineering [51]. In this work, we focused on the alkane production from acetate by *A. baylyi*, but the overall productivity and titer of the system could be enhanced by optimizing both modules. As an easy-to-engineer chassis, *A. baylyi* can potentially be utilized for the production of a range of compounds, further emphasizing the modular nature of the system.

Conclusions

In this study, we engineered *A. baylyi* ADP1 for the non-native production of alkanes from acetate. The performance of nine different synthetic pathways was compared with a twin-layer biosensor capable of detecting both the intermediate (fatty aldehyde) and the end product (alkane) of the pathway. The utilization of the twin-layer biosensor enabled the balancing of the pathway to avoid the accumulation of the intermediate fatty aldehyde, which led to improved alkane production. Even though the alkane titers remained low, the successful application of the biosensor in improving the heterologous alkane biosynthesis encourages further research.

This was the first demonstration of alkane production from acetate, and enabled the connection of the alkane-producing module to an upstream acetate production from CO_2 and H_2 by acetogenic bacteria, thereby leading to the net production of long-chain alkane from CO_2 and H_2.

Methods

Bacterial strains and genetic modifications

The previously described alkane biosensor (ASI) constructed in *A. baylyi* ADP1 (DSM 24139) [28] was used for the construction of alkane-producing strains. For molecular cloning and plasmid maintenance, *Escherichia coli* XL1-Blue (Stratagene, USA) were used. *Acetobacterium woodii* (DSM 1030) was ordered from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

For the expression of the alkane biosynthetic genes, a previously described expression cassette was used [35]. The cassette contains an IPTG-inducible T5/*lac* promoter, followed by the *lacI* gene and the Biobrick RFC10-accepting cloning site (*Eco*RI–*Xba*I–*Spe*I–*Pst*I) (Fig. 1b). The cassette also contains a spectinomycin resistance marker gene under its own promoter, and flanking sites to facilitate the integration of the cassette to the prophage region of *A. baylyi* [52]. *Acr1* (ACIAD3383) was amplified from the genome of *A. baylyi* ADP1 and *Ramo* (an open reading frame encoding for a putative short-chain dehydrogenase WP_022976613.1) from the genome of *Nevskia ramosa* (DSM 11499) with primers tl17 and tl18 and tl33 and tl34, respectively (Table 1). A*ar (S. elongatus aar,* WP_011242364.1*), seado* (*S. elongatus ado*, WP_011378104.1), *pmado* (*P. marinus ado*, WP_011130600.1) and *cer1* (*Arabidopsis thaliana*, NP_001184890.1) were codon-optimized and purchased from Genscript (USA) with appropriate restriction sites and ribosomal binding sites (Biobrick part BBa_0034). The nucleotide sequences of the genes are provided in the Additional file 1. These ADO and AAR analogs were sequentially cloned to the expression cassette using the Biobrick RFC10 protocol to produce nine different combinations. For the RBS modulation, the *aar* gene was PCR-amplified with the primers containing the appropriate RBS sequences (Table 1), and cloned to the *seado*-containing expression cassette. The expression cassettes were transformed to the alkane biosensor strain by natural transformation as described previously [32], and colonies were selected with 50 μg/L spectinomycin. All genetic modifications were confirmed with PCR and sequencing.

Medium and culture conditions

Cloning and routine maintenance of *A. baylyi* and *E. coli* were performed in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl). In alkane production experiments, *A. baylyi* were cultivated in modified minimal salts medium MA/9. The medium contained (per liter): 5.52 g Na_2HPO_4*$2H_2O$, 3.4 g KH_2PO_4, 1 g NH_4Cl, 2 g casein amino acids, 0.008 g nitrilotriacetic acid, 2.0 mg $FeCl_3$, 11.1 mg $CaCl_2$, and 240 mg $MgSO_4$. Acetic acid was used as the carbon source in concentrations indicated in the results section. IPTG (isopropyl β-D-1-thiogalactopyranoside) was used for the induction of the alkane biosynthesis genes in concentrations indicated in "Results" section. Spectinomycin (50 μg/mL) was used for selection.

For the real-time in vivo luminescence and fluorescence measurements, the cells were cultivated in 96-well plate (Greiner Bio-one, Austria) in Spark microplate reader (Tecan, Switzerland). The cells were inoculated from overnight culture to an initial optical density of approximately 0.05 and cultivated in 200 μL of medium. The plate was placed inside a humidity cassette (Tecan) to prevent evaporation. The cultivation and measurement were performed at 30 °C. Optical density (600 nm), luminescence and fluorescence (excitation 485 ± 10 nm, emission 510 ± 5 nm) signals were measured every 30 min, and the plate was shaken for 5 min twice an hour

Table 1 Primers used in the study

Name	Sequence 5′ → 3′	Description
tl17	TGGAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGATATCAATCAGGGAAAAACGCG	acr1 forward, biobrick
tl18	GTTTCTTCCTGCAGCGGCCGCTACTAGTATTATTACCAGTGTTCGCCTGGG	acr1 reverse, biobrick
tl33	CGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGAATTACTTCGTCACCGG	ramo forward, XbaI
tl34	GGCCGCTACTAGTATTATTACCAATGCTCGCCCTTG	ramo reverse, SpeI
0030_Aar	TGGAATTCGCGGCCGCTTCTAGAGATTAAAGAGGAGAAATACTAGATGTTTGGTTTAATTGGCCA	aar forward, BBa0030, biobrick
0031_Aar	TGGAATTCGCGGCCGCTTCTAGAGTCACACAGGAAACCTACTAGATGTTTGGTTTAATTGGCCA	aar forward, BBa0031, biobrick
0032_Aar	TGGAATTCGCGGCCGCTTCTAGAGTCACACAGGAAAGTACTAGATGTTTGGTTTAATTGGCCA	aar forward, BBa0033, biobrick
Aar_rev	CTGCAGCGGCCGCTACTAGTATTATTAAATTGCTAAAGCCAGCG	aar reverse, biobrick

(108 rpm, 2.5 mm amplitude). The relative fluorescence values were obtained by dividing the fluorescence reading with the optical density. The cumulative luminescence values were calculated by adding all the luminescence readings together.

Acetobacterium woodii was grown in DSM 135 medium containing (per liter): 1 g NH_4Cl, 0.33 g KH_2PO_4, 0.45 g K_2HPO_4, 0.1 g $MgSO_4 \times 7H_2O$, 2 g yeast extract, 10 g $NaHCO_3$, 10 g D-fructose, 0.5 g L-cysteine-HCl, 30 mg nitrilotriacetic acid, 60 mg $MgSO_4 \times 7H_2O$, 10 mg $MnSO_4 \times H_2O$, 20 mg NaCl, 2 mg $FeSO_4 \times 7H_2O$, 3.6 mg $CoSO_4 \times 7H_2O$, 2 mg $CaCl_2 \times 2H_2O$, 3.6 mg $ZnSO_4 \times 7H_2O$, 0.2 mg $CuSO_4 \times 5H_2O$, 0.4 mg $KAl(SO_4)_2 \times 12H_2O$, 0.2 mg H_3BO_3, 0.2 mg $Na_2MoO_4 \times 2H_2O$, 0.6 mg $NiCl_2 \times 6H_2O$, 6 µg $Na_2SeO_3 \times 5H_2O$, 8 µg $Na_2WO_4 \times 2H_2O$, 20 µg biotin, 20 µg folic acid, 100 µg pyridoxine–HCl, 50 µg Thiamine–HCl $\times 2H_2O$, 50 µg riboflavin, 50 µg nicotinic acid, 50 µg D-Ca-pantothenate, 1 µg vitamin B12, 50 µg *p*-aminobenzoic acid, and 50 µg lipoic acid. For autotrophic growth with CO_2, fructose was omitted, the concentration of yeast extract increased to 4 g/L and $NaHCO_3$ decreased to 5 g/L [39].

Bioreactor

The bioreactor cultivations were carried out in 1-L vessel (Sartorius Biostat B plus Twin System, Germany) with a working volume of 600 mL (*A. woodii*) or 300–800 mL (*A. baylyi*), as indicated in the results section. pH was controlled to 7.0 by automated addition of 1 M H_3PO_4 or 5 M KOH. Temperature was kept at 30 °C and stirring speed at 300 rpm. For the *A. baylyi* cultivations, air was supplied at 1 L/min, and partial oxygen pressure controlled to 20% saturation by supply of oxygen. For *A. woodii* cultivations, the reactor was operated at anaerobic conditions, and a mixture of H_2 and CO_2 (80%/20%) was purged through the system at 1 L/min.

Analytical methods

Acetate concentrations were measured with high-performance liquid chromatography using LC-20AC prominence liquid chromatograph (Shimadzu, Japan) and Rezex RHM-Monosaccharide H+ (8%) column (Phenomenex, USA) with 5 mM H_2SO_4 as the mobile phase.

Hydrocarbon extraction and analysis was performed as described earlier [28]. Briefly, cells from 10 mL of the culture were collected by centrifugation, and lipids extracted with methanol–chloroform extraction. The chloroform phase was analyzed by gas chromatography–mass spectrometry (GC–MS). The GC–MS analysis was performed with Agilent Technologies 6890N/5975B system, column HP-5MS 30 m × 0.25 mm with 0.25 µm film thickness, He flow 4.7 mL/min, 1 µL splitless injection, oven program: 55 °C hold 5 min, 55–280 °C 20°/min ramp, 280 °C hold 3 min. Scan 50–500 m/z, 1.68 scan/s. Heptadecane peaks were identified and quantified based on external standard (Sigma-Aldrich, USA).

Additional file

Additional file 1. Nucleotide sequences.

Authors' contributions

TL, SS, and VS designed the study. TL performed the molecular, microbiological, and analytical work, analyzed the data, and wrote the manuscript. HV participated in initial strain construction and characterization. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Additional File 1: Nucleotide sequences

Production of alkanes from CO2 by engineered bacteria

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The nucleotide sequences of the alkane biosynthesis genes utilized in the study are provided below.

seado (*S. elongatus ado*, WP_011378104.1)

ATGCCACAACTGGAAGCGTCGTTGGAACTGGATTTTCAATCGGAAAGCTATAAAGATGCGTATTCACGT
ATCAATGCCATCGTCATTGAAGGTGAACAGGAAGCATTTGATAACTATAACCGTTTGGCAGAAATGCTG
CCGGATCAGCGCGATGAACTGCATAAATTAGCGAAAATGGAACAACGTCACATGAAAGGTTTTATGGCC
TGTGGCAAAAATTTGAGCGTAACCCCAGATATGGGTTTTGCGCAGAAATTTTTCGAACGCTTGCATGAA
AACTTTAAAGCAGCAGCAGCAGAAGGCAAAGTGGTTACATGTCTGTTGATCCAAAGTCTGATCATCGAA
TGCTTTGCAATCGCAGCGTATAATATTTATATCCCTGTTGCCGATGCTTTTGCACGTAAAATTACTGAAGG
TGTAGTCCGTGATGAATATCTGCATCGCAATTTTGGCGAAGAATGGTTAAAAGCTAACTTTGATGCGAGT
AAAGCCGAACTGGAAGAAGCCAATCGTCAAAACTTGCCGCTGGTGTGGTTAATGTTGAATGAAGTTGCT
GATGATGCACGTGAATTAGGCATGGAACGCGAATCTTTGGTGGAAGATTTTATGATTGCGTATGGCGAA
GCCCTGGAAAACATTGGCTTTACAACCCGTGAAATCATGCGTATGAGTGCGTATGGTCTGGCAGCAGTT
TAA

pmado (*P. marinus ado*, WP_011130600.1)

ATGCCTACCTTAGAAATGCCGGTAGCAGCGGTCTTGGATTCAACTGTAGGTAGTTCTGAAGCACTGCCG
GATTTTACGAGCGATCGTTATAAAGATGCTTATTCACGCATTAATGCGATTGTGATCGAAGGTGAACAG
GAAGCGCATGATAACTATATTGCCATCGGCACCCTGTTACCAGATCATGTCGAAGAATTAAAACGTTTGG
CGAAAATGGAAATGCGCCATAAGAAAGGTTTTACAGCCTGTGGTAAAAATCTGGGCGTGGAAGCAGAT
ATGGATTTTGCACGTGAATTTTTCGCCCCACTGCGCGATAACTTTCAGACTGCGTTAGGTCAAGGCAAAA
CCCCTACATGCTTGCTGATTCAGGCACTGTTGATCGAAGCATTTGCGATTTCGGCATATCATACGTATATC
CCTGTTAGTGATCCATTTGCCCGTAAAATTACCGAAGGTGTGGTTAAAGATGAATATACACATCTGAACT
ATGGCGAAGCCTGGCTGAAAGCTAACTTAGAATCGTGTCGTGAAGAACTGTTAGAAGCTAATCGCGAA
AACTTGCCTCTGATTCGTCGCATGTTGGATCAGGTGGCAGGTGATGCCGCTGTTTTACAAATGGATAAA
GAAGATTTGATCGAAGATTTTCTGATTGCGTATCAGGAATCTCTGACAGAAATCGGCTTTAATACTCGTG
AAATTACGCGCATGGCAGCAGCAGCACTGGTGAGCTAA

cer1 (*Arabidopsis thaliana*, NP_001184890.1)

ATGGCAACCAAACCTGGCGTGTTAACTGATTGGCCTTGGACGCCGTTGGGTTCATTTAAATATATTGTGA
TCGCTCCGTGGGCAGTTCATTCGACCTATCGTTTTGTTACAGATGATCCTGAAAAACGCGATTTAGGTTA
TTTTCTGGTATTTCCGTTTCTGTTGTTTCGTATTCTGCATAATCAGGTCTGGATCTCTTTAAGCCGCTATTA
TACCAGTTCTGGCAAACGTCGCATTGTGGATAAAGGTATCGATTTTAACCAGGTTGATCGTGAAACAAAT
TGGGATGATCAAATCCTGTTTAACGGTGTGCTGTTTTATATTGGCATCAATTTGCTGCCAGAAGCTAAAC
AACTGCCTTGGTGGCGTACCGATGGCGTGCTGATGGCAGCGTTAATTCATACAGGTCCTGTTGAATTTTT
GTATTATTGGCTGCATAAAGCATTACATCATCATTTTCTGTATTCTCGTTATCATAGCCATCATCATAGTAG
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CAATCCCATTGTTGACCACACTGTTAACTAAAACGGCGAGTATTATCTCTTTTGCCGGCTATATCATCTAT
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TATCATTTACGCATCGGCTTGGCGTCGTTTGCCAGTTATCCATTTGCGTATCGTTGGTTTATGCGCTTGCT
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CAGATATGAAAGTACGTCTGGTCGATGGTAGTCGTCTGGCAGCAGCAGTGGTCATTAACAGCGTACCTA
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TTCCGTTTTCTCAGTTTCCACTGAAACAATTACGTCGCGATTGTATTTATCATACCACACCTGCGTTGATCG
TACCGAAAAGTCTGGTGAACGTTCATTCTTGCGAAAATTGGCTGCCACGTAAAGCTATGAGTGCAACTC
GCGTTGCTGGTATTTTGCATGCACTGGAAGGCTGGGAAATGCATGAATGTGGTACGTCACTGTTATTGT
CGGATCTGGATCAGGTTTGGGAAGCCTGCTTATCTCATGGTTTTCAACCACTGTTATTGCCTCATCATTAA

aar (*S. elongatus aar,* WP_011242364.1)

ATGTTTGGTTTAATTGGCCATTTGACCTCGCTGGAACAGGCACGTGATGTTAGTCGTCGCATGGGCTATG
ATGAATATGCGGATCAGGGTCTGGAATTTTGGAGTTCTGCCCCGCCACAAATTGTGGATGAAATCACCG
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CAGGTCGAAGCCGCTGCAAAAACTCTGGGTATTGATATCACTCAAGCGACGGTTGCCGTGGTTGGTGCT
ACGGGCGATATTGGTTCGGCAGTATGCCGTTGGTTGGATCTGAAATTAGGTGTCGGCGATTTGATTCTG
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CCAGCAACATTAAAACAACCTTGCGTGTTGATTGATGGTGGCTATCCAAAAAATTTGGGTAGCAAAGTG
CAAGGTGAAGGCATTTATGTTCTGAACGGTGGCGTGGTTGAACATTGTTTTGATATTGATTGGCAGATC
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GAATTTGAAGGCTGGCATACCAATTTTTCATGGGGTCGTAACCAGATTACAATCGAAAAAATGGAAGCG
ATCGGCGAAGCCTCGGTGCGTCATGGTTTTCAACCGCTGGCTTTAGCAATTTAA

acr1 *(A. baylyi ACIAD3383)*

ATGATATCAATCAGGGAAAAACGCGTGAACAAAAAACTTGAAGCTCTCTTCCGAGAGAATGTAAAAGGT
AAAGTGGCTTTGATCACTGGTGCATCTAGTGGAATCGGTTTGACGATTGCAAAAAGAATTGCTGCGGCA
GGTGCTCATGTATTATTGGTTGCCCGAACCCAAGAAACACTGGAAGAAGTGAAAGCTGCAATTGAACAG
CAAGGGGGACAGGCCTCTATTTTTCCTTGTGACCTGACTGACATGAATGCGATTGACCAGTTATCACAAC
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CAATGATCGCACCCACCAAAATTTATAAATACGTGCCCACGCTTTCCCCAGAAGAAGCCGCAGATCTCAT
TGTCTACGCCATTGTGAAACGTCCAAAACGTATTGCGACGCACTTGGGTCGTCTGGCGTCAATTACCTAT

GCCATCGCACCAGACATCAATAATATTCTGATGTCGATTGGATTTAACCTATTCCCAAGCTCAACGGCTG
CACTGGGTGAACAGGAAAAATTGAATCTGCTACAACGTGCCTATGCCCGCTTGTTCCCAGGCGAACACT
GGTAA

ramo *(Nevskia ramosa* WP_022976613.1)

ATGAATTACTTCGTCACCGGCGCCACCGGTTTCATCGGCAAGCATCTGATCGAGCGCCTGCTGGCGCGC
CCGGATGCCACCATCCACGTGCTGGTCCGCGCATCTTCGGAAGACAAGTTCGCCGCCTTGCAGGAGCGC
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CTCGGCCTATGTCGCCTCCAAGGCCGCGCTCGATGCCTTCACCCGCTGCCTGTCGGCCGAAGTGAAAGG

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