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CYANOBACTERIA AS BIOCATALYSTS FOR THE PRODUCTION OF VOLATILE HYDROCARBONS

Verónica Carbonell González

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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ABBREVIATIONS

2-OG	2-oxoglutarate
ACC	1-Aminocyclopropane-1-carboxylic acid
ACCO	ACC oxidase
ACCS	ACC synthase
ACP	Acyl carrier protein
ADO	Aldehyde deformylating oxygenase
AdhE2	Aldehyde reductase/alcohol dehydrogenase
AHL	Acyl-homoserine lactone
ATP	Adenosine Triphospate
CAR	Carboxylic acid reductase
CO_2	Carbon dioxide
CoA	Coenzyme A
Crt	3-hydroxybutyryl-CoA dehydratase
DNA	Deoxyribonucleic acid
EFE	Ethylene-Forming Enzyme
FAAR	Fatty acyl-ACP reductase
Fdx	Ferredoxin
Fpr	NADHPH:ferredoxin/flavodoxin-oxidoreducase
FFA	Free fatty acid
GC	Gas chromatography
GOE	Great Oxygenation Event
Hbd	3-hydroxybutyryl-CoA dehydrogenase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KMBA	2-keto-4-methylthiobutyric acid

LB	Lysogeny broth
LPG	Liquefied petroleum gas
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
ORF	Open reading frame
P5C	1-pyrroline-5-carboxylic acid
petF	Ferrodoxoin
QS	Quorum-sensing
RBS	Ribosome binding site
RNAP	Ribnucleic acid polymerase
SAM	S-adenosyl-L-methionine
Sfp	Phosphonantetheinyl transferase
TCA	Tricarboxylic acid
TB	Terrific broth
TE	Thioesterase
Ter	Trans-enoyl-CoA reductase

ABSTRACT

The genetic engineering of cyanobacteria is seen as a promising strategy to develop future photosynthetic microbial cell factories capable of generating carbon-based compounds from water, CO_2 and solar energy. The synthetic biology approach has already given insight to the strategic re-design of endogenous cellular metabolism, with the diversion of fixed carbon towards specific end products being the main goal. However, such technology is still at research level and several drawbacks need to be overcome before economic viability is achieved. Commonly encountered problems include the genetic instability of heterologous genes, low enzyme efficiency, and a lack of promoters, which are suited to strong and flexible gene expression in cyanobacteria.

In this work, the performance of different promoters in cyanobacteria was evaluated through the heterologous expression of the ethylene-forming enzyme (EFE) from *Pseudomonas syringae.* In this way, EFE, which catalyzes the synthesis of ethylene, was successfully employed as a non-invasive reporter protein. The rate of ethylene accumulation in the headspace of sealed cultures indicated the strength and inducibility of the tested promoters. To study the genetic instability of heterologous pathways, *efe* was also used as a model system. Expression of *efe* in *Synechococcus sp.* PCC 7942 has been reported as being unstable due to gene truncation at CGATG sites, resulting in a loss of ethylene production. Apparently, in later publications, such instability was by-passed via *efe* codon optimization and expression in *Synechocystis* PCC 6803. The need for codon optimization and the avoidance of associated mutational elements was re-evaluated and, demonstrated that these modifications were unnecessary for a sustained and stable production of ethylene. Furthermore, the cause of the genetic instability previously reported in *Synechococcus efe* mutants was identified and, developed stable *efe* mutants for this strain.

To gain new insight to the catalytic performance in enzyme efficiency, the *aldehyde deformylating oxygenase* (ADO) enzyme from cyanobacteria was used. ADO is able to produce C_{n-1} hydrocarbons from their aldehyde precursors. However, it has poor catalytic activity. An *in vivo* method was developed for the comparison of five ADO orthologs in *E. coli*. Results identified ADO-specific differences in regards to catalytic performance and substrate specificity, which may help future ADO engineering strategies and improve the efficiency of hydrocarbon production.

TIIVISTELMÄ

Geneettisesti muokattujen syanobakteerien toivotaan tarjoavan mahdollisuuksia kehitettäessä tulevaisuuden bioteknologisia sovelluksia, joissa mikrobit toimivat fotosynteettisinä solutehtaina, tuottaen haluttuja hiilipohjaisia yhdisteitä auringon energian avulla suoraan ilman hiilidioksidista ja vedestä. Synteettisen biologian menetelmin syanobakteerisolun aineenvaihduntaa on mahdollista muokata kohti haluttuja reaktioteitä tuottosysteemien tehokkuuden ja monimuotoisuuden parantamiseksi, joskin kaupallisten sovellusten kehittämisen esteenä on yhä useita erilaisia biologisia ja teknisiä haasteita. Tyypillisiä ongelmia ovat rakennettujen ekspressiokonstruktien geneettinen epästabiilisuus, kohde-entsyymien liian alhainen katalyyttinen aktiivisuus, sekä dynaamisesti toimivien tuottosysteemien aktiivisuutta kontrolloivien geneettisten säätelyelementtien puute.

Yksi väitöskirjan osa-alueista keskittyi uusien transkriptiota säätelevien geneettisten elementtien, promootterien, karakterisointiin muokatuissa syanobakteerisoluissa. Promootterien kvantitatiiviseen vertailuun käytettiin *Pseudomonas syringae* –bakteerista peräisin olevaa *efe* –geeniä, jonka koodaama entsyymi (EFE; ethylene-forming enzyme) mahdollisti ekspressiotehokkuuden vertailun mittaamalla suljetun kasvatuspullon kaasutilaan muodostuvan etyleenin kertymistä. Vastaavaa *efe*-systeemiä käytettiin myös etyleenin tuottoon suunniteltujen syanobakteerikantojen stabiilisuuden tutkimiseen. Tulokset osoittivat että *efe*-geenin nukleotidisevenssin optimointi ei ole välttämätöntä tuottosysteemin pysyvyydelle *Synechocystis* sp PCC 6803 –kannassa, ja että aikaisemmin julkaistut ongelmat muokatuissa *Synechococcus* sp. PCC 7942 –tuottokannoissa liittyivät ensisijaisesti genomisessa integraatiossa käytettyihin kohdegeeneihin. Väitöskirjatyössä tutkittiin myös syanobakteerien hiilivetybiosynteesiin osallistuvan ADO (aldehyde deformylating oxygenase) –entsyymin ominaisuuksia muokatussa *Escherichia coli* –mallisysteemissä. Luonnossa ADO katalysoi erimittaisten aldehydi-lähtöaineiden konversiota C_{n-1} hiilivedyiksi ja on ollut siksi mielenkiinnon kohteena uusiutuvien polttoaineiden valmistukseen tähtäävässä tutkimuksessa. Työssä kartoitettiin viiden ADO-ortologin eroja haihtuvien lyhytketjuisten alkaanien biosynteesissä. Tulokset osoittavat, että vaikka systeemin yleisenä rajoitteena onkin ADO:n huonot kineettiset ominaisuudet, on eri varianttien välillä selviä entsyymikohtaisia eroja sekä katalyyttisessa tehokkuudessa, että substraattispesifisyydessä.

1. Introduction

1.1 Towards sustainable production of carbon-based chemicals

1.1.1 The need to move from a linear to circular economy

Since the beginning of the 19th century, the development of new technologies boosted economic growth. This growth was due to the use of a new cheap energy source in the form of fossil hydrocarbons. Fossil hydrocarbons are organic compounds rich in carbon-hydrogen bonds. They have been formed over millions of years, beginning as organic matter trapped in the Earth's sedimentary layers. Historically, fossil hydrocarbons have been the cheapest and most efficient energy source available. These qualities allowed the modernization of the transportation sector, the industrial scale product manufacturing [1] and, marked the beginning of the linear economy, on which our current economy is based. The linear economy encompasses three successive principles which are: take, make (use) and waste [2]. Thus, economic growth has been based on the use of non-renewable sources to produce goods that will eventually be accumulated as solid waste in landfill, or released in the atmosphere as greenhouse gases (GHGs) after burning.

Three main problems arise from our dependence on the fossil fuel driven linear economy. Firstly, the combustion of petroleum-derived fuels releases tons of CO_2 into the atmosphere. The catastrophic consequences of this are well known and commonly referred to as climate change and global warming [3]. Secondly, economic growth is directly proportional to waste production and CO_2 accumulation [4]. Finally, the strong dependence of economic growth on a non-renewable energy source carries with it the risk of economic collapse when reserves of the source diminish.

The problems associated with the linear model evidence the urgent need for a rapid transition to a circular economy. The concept of a circular economy is based on CO_2-neutral emissions and limited waste accumulation [5]. In this scenario, the European Union (EU) targets are set for a minimum of 80-95% cuts in emissions compared to 1990 levels by 2050 [6] and there are binding emission targets for transportation vehicles [7]. Moreover, the Renewable Energy Directive (RED 2009/28/EC) promotes the use of energy from renewable sources [8]. Therefore, the development of hydrocarbon substitutes

obtained from renewable sources is one of the key elements in ensuring a smooth transition to a more stable and sustainable economy. In this context, the present thesis focuses on the study of the alternative sustainable production of two volatile hydrocarbons, ethylene and propane. These two fuels are globally important and currently obtained from fossil sources.

Ethylene, a flammable gas, is one of the simplest hydrocarbons. It is an alkene composed of two carbons and four hydrogen atoms (C_2H_4). It is commercially important as a monomer for the manufacture of a wide variety of plastics. Propane is a C3 alkane (C_3H_8) which is the primary constituent of liquefied petroleum gas (LPG). Propane is a colorless and odorless gas with a low boiling point (-42°C) which can easily be liquefied by increasing pressure. This liquefaction facilitates its transportation and storage. Propane is commonly used for heating and cooking, and it is the world´s third most common transportation fuel [9].

Ethylene and propane are volatile hydrocarbons that strongly influence the global economy. Due to their fossil origin, they follow the linear economy model. For example, ethylene is obtained by steam cracking, an energy intensive process, from naphtha (refined product from crude oil) or from ethane (produced from natural gas processing) [10]. In a similar manner, propane is derived from natural gas processing [10], and the adverse consequences for the environment are well known. For instance, to produce one ton of ethylene and propane, approximately 1.5-3 tons [11, 12] and 2.6 tons [13] of CO_2 are released respectively. Furthermore, the carbon contained in the products is subsequently released in the atmosphere as CO_2 upon burning. For example, about 300 000 tons of packaging plastic waste was incinerated in Spain, generating nearly 150 000 tons of CO_2 [12]. In the case of propane, each ton burned in transportation contributes more than 200 tons of CO_2 [14]. Therefore, there is an increased need for developing alternative hydrocarbon production strategies that fit with the principles of the circular economy.

1.1.2 Biomass-based systems to produce hydrocarbon substitutes

Biomass is the organic matter derived from any recently living organism, either from plants, animals, or from their waste residues. All biomass on Earth is derived from atmospheric carbon fixation by autotrophic organisms: plants,

algae and cyanobacteria. Thanks to photosynthetic reactions, these organisms are able to harvest light energy to fix and reduce atmospheric CO_2 into energy rich organic compounds such as carbohydrates, fatty acids and oils [15]. In turn, these energy carriers are then transferred to the so called biomass pyramid, over which loss of energy occurs due to heat and respiration [16]. Biomass is renewable and can be carbon neutral, because its formation and degradation is completed within a short period of time. In fact, only a very small amount of biomass actually escapes the carbon cycle to accumulate and, after millions of years, form nonrenewable fossil hydrocarbons.

Fossil hydrocarbons have a biomass origin as a consequence of ancient photosynthetic activity [17]. This common origin suggests that biomass may be suitable for the production of drop-in fuels with similar chemical properties to fossil fuel counterparts. A drop-in biofuel has the advantage of being compatible with current logistic infrastructures (e.g. distribution pipes and combustion engines) [18, 19]. However, the chemical compositions of most currently available biofuels do in fact differ from their petrochemical partners. Consequently, there are often mechanical compatibility problems (i.e. corrosiveness and lubricity) when biofuels are used as pure blends, being usually restricted as fuel additives, such ethanol, when not used in flex fuel engines. Although these blends help to reduce the GHG emissions from fossil fuels, yet it does not offer a full replacement solution. In this scenario, biomass-based biofuels represented only 4 % of the global road transport fuel in 2016 [20]. In spite of this, biomass provides around 10-14% of world's primary energy consumption and currently occupies the fourth position as a global energy source after its petro-based competitors (coal, petroleum and natural gas) [20, 21].

On an industrial level, there are various routes for conversion of biomass to biofuels. Some of these processing routes are still under development and others are already commercially available. Among the different routes, biochemical, chemical and thermochemical processing are the most relevant examples. Within the biochemical route, biomass can be biologically converted into different end-products using heterotrophic microorganisms as biotechnological hosts. Typically, at the first phase this requires enzymatic biomass breakdown into simple sugars, followed by fermentative processes,

which produce alcohols such as ethanol [22]. In contrast, the chemical route modifies biomass in processes such as transesterification. Transesterification involves the reaction of triglyceride rich biomass with an alcohol (usually methanol) and a catalyst such as sodium hydroxide to produce glycerol and fatty acid methyl ester (FAME), also known as biodiesel [23]. On the other hand, the thermochemical route subjects biomass to high pressure and temperature under the presence of a metal catalyst. In this case, gasification combined with the Fisher-Tropsch (FT) process and the hydrotreatment of vegetable oils (HVO) are most relevant. The FT process is a technology developed in 1923 to produce synthetic fuels from coal and natural gas [24], although it can be adapted to use any source of biomass as a feedstock. The primary requirement is gasification of the biomass to produce syngas, a gas rich in carbon monoxide (CO) and Hydrogen (H_2), which is subsequently processed by FT to produce a hydrocarbon with a similar chemical structure and properties to conventional fossil fuels [24]. When biomass is used, this process is also known as FT-Biomass-to-Liquid (FT-BTL). In the case of the hydrotreatment process, only biomass feedstocks rich in long chain fatty acids are used, such as vegetable oils and animal fats. In this case, the removal of oxygen present in the triglyceride molecules forms hydrocarbons similar to those of petro-based diesel, also known as HVO/HEFA fuels (Hydrogenated Vegetable oils/ Hydroprocessed Esters and Fatty acids) or renewable diesel, which are suitable for current diesel engines [19].

In general, all of the described strategies aim for minimal GHG emissions and waste accumulation. However, challenges do remain, particularly with respect to the incompatibility of most biofuels with global transportation and dispensation infrastructures. This is the case of bio-ethanol and FAME, which are blended with gasoline and diesel respectively to avoid corrosiveness and lubricity problems. Therefore, fossil fuel dependence continues, albeit at a decreasing rate. In addition, the investment costs to safely replace pipelines and motor engines would require decades and surpass trillions of dollars [25]. Consequently, many biofuels may remain inaccessible to much of the global population, which will continue reliance on fossil fuels and contribute to continuous GHG emissions. In contrast, drop-in fuels such FT-BTL and HVO

fuels show promise as pure blends, which do not require extra investment for adaption of current logistics systems.

Despite the fact that biomass production is overall CO_2 neutral, the hydrocarbon products derived from biomass still result in CO_2 emissions [26]. Inefficiencies within the biomass conversion processes also require extra energy to enter into the system. Most often, this additional energy (e.g. for biomass collection, transportation and treatment) is taken from natural gas or other fossil fuel sources [10]. Moreover, levels of GHG emissions are also affected by the nature of the processed biomass. In this case, impacts can be minimized by using waste from agricultural, forestry, industrial or municipal residues [27, 28], although recent studies question the availability of waste sources required to meet current energy demands [29]. In contrast, high impacts of biomass production are associated with the use of intensive agriculture [10, 28, 30, 31]. This is explained by the fact that intensification of the agricultural activity to meet biofuel demands may exacerbate existing problems such as air, soil and water pollution, caused by the use of pesticides, soil degradation and diminution of biodiversity. Additionally, both the pesticides and petroleum fuel required for operation of agricultural processes often carry a carbon footprint not accounted for in Environmental Impacts Assessments (EIA) and Life cycle Assessments [10, 27, 28], which evaluate the environmental effects, such as GHGs emissions, for a given process.

It is also important to note that the interaction between the energy production via biomass and the food industry has been for many years in the forefront of the debate and, therefore, this has certainly affected the balance on the food prices [17]. The reason is that both economical activities – production of food and bio-compounds - compete for the land and water usage, which actually are two highly limited natural sources.

1.1.3 Cyanobacteria as photosynthetic cell factories for the sustainable production of valuable chemicals

Cyanobacteria are a diverse group of gram-negative bacteria that are found in varied environments ranging from the open oceans and fresh water to the extreme conditions of hot springs, deserts and Antarctic areas [32, 33]. The successful distribution of this group is a result of natural evolution and phenotypic adaptability to environmental changes. Genome plasticity, demonstrated most elegantly in the abundant marine cyanobacterium *Prochlorococcus* [34] is a consequence of gene gain and loss through long-term evolution. Over the short term, modifications in gene expression allow the endogenous cellular metabolism to respond rapidly to new environmental conditions [35]. Accordingly, cyanobacteria have evolved into a diverse group of prokaryotes in terms of morphology, genotype, metabolism and ecological distribution [36, 37]. Traditional taxonomy commonly used in botanical nomenclature categorizes these photosynthetic bacteria according to morphological patterns only [37-39]. This classification has later been considered to be superficial and there is still a lack of general consensus regarding nomenclature [36, 40]. An example of this is the cyanobacterial taxonomic system included in Bergey's Manual of Systematic Bacteriology [39, 41]. Although the taxonomic arrangements demonstrate a confluence in terms of evolution from unicellular (groups I-II) to filamentous (groups III-V) cells [33, 42, 43], the growing number of available sequenced genomes, together with new phylogenetic studies, suggest a need for taxonomic revisions based on a combination of phenotypic, ecological, biochemical and molecular methods [37, 40, 44, 45].

According to the available geological records, the emergence of cyanobacteria is estimated at around 2450-2320 Millions of years ago (Ma) [42]. Moreover, their association with the Great Oxygenation Event (GOE), approximately 2300 Ma ago [46], suggests that cyanobacteria were the precursors of aerobic life and of later chloroplast formation [47], thanks to their ability to perform oxygenic photosynthesis [48]. Oxygenic photosynthesis is accomplished by thylakoid membrane embedded protein complexes that mediate light-dependent reactions. This process involves the harnessing of solar energy, which enables the extraction of electrons from water, resulting in the

formation of O_2 as a by-product. Electrons excited by light in Photosystem II (PSII) initiate an electron transport chain and flow from PSII to the Plastoquinone (PQ) pool and then via the cytochrome b_6f complex to Photosystem I (PSI). Electrons need to be excited by light a second time in PSI in order to be able to reduce nicotinamide adenine dinucleotide phosphate ($NADP^+$) and form NADPH. Additionally, adenosine triphosphate (ATP) is generated by ATP-synthase, due to a proton gradient generated across the thylakoid membrane. These two energy carriers, ATP and NADPH, serve the Calvin-Belson cycle for the fixation of CO_2.

Cyanobacteria have attracted attention for their potential use in biotechnological applications for the production of biofuels, bioplastics and other industrial chemicals, in addition to the use of cyanobacteria such spirulina as food supplements and cosmetics. When compared to the biomass conversion systems described earlier, cyanobacteria can offer great advantages. For example, while traditional biomass requires the photosynthetic phase (growth of biomass) and product manufacturing phase (biomass treatment via different processes) at separated stages, cyanobacteria can be designed to allow both phases functioning in the same chassis, thus resulting in an almost direct conversion of CO_2 into end product [17, 52, 53] . In fact, at a global scale they transform and store solar energy at a rate equivalent of around 450 TW [51]. Indeed, 450 TW is about 25 times higher than the total energy consumption of the human population [51]. This direct approach would avoid the extra energy consumption of agricultural activities, biomass transportation and pre-treatment. In addition, cyanobacteria do not need to divert fixed carbon into building complex organic structures like roots, stems, trunks or leaves; hence more energy is available for the direct production of organic molecules with a commercial value. Furthermore, cyanobacteria have fast growth rates and their cultivation does not compete with arable [54] land or drinking water [55]. The direct approach described explains why cyanobacteria are often presented as photosynthetic microbial cell factories [56-58], capable of high-energy conversion efficiency. The concept of photosynthetic microbial cell factories is, however, still an emerging idea and further development of the currently available technology is needed to achieve this goal [17, 59].

In order to develop cyanobacterial cell factories, an interdisciplinary approach such synthetic biology is generally favored over the use of single genetic engineering strategies (e.g. selected gene(s) overexpression/deletion(s)). Synthetic biology is a growing discipline intended to re-design existing biological systems and tailor them to accomplish a new specific function. For that purpose, the initial biological system acts as an envelope where standardized biological parts are introduced and organized as inter-changeable building blocks. Such blocks, which are easily modified according to specific needs, interact with the endogenous metabolism to perform a pre-programmed task [60, 61]. Often, a suitable biological system to be used as a chassis is microbial in nature [52, 62]. This is because microbes offer a minimal cell structure with relatively simple genetic circuits and well characterized metabolic networks. The best studied microbes are the bacteria *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*), and the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) [52].

The study of model organisms like *E. coli* offers a broad range of biological tools. This permits the re-arrangement of genetic circuits to produce a wide range of products, from highly valuable pharmaceutical compounds to bulk chemicals like polymer precursors and biofuels [61-63]. However, these heterotrophic organisms require fixed carbon as substrate, and therefore are unable to meet the scale of global demand in circular economy, despite their important role in waste treatment and recycling also in the future. In contrast, engineering photosynthetic microbes such as cyanobacteria and using a synthetic biology toolbox would be highly sustainable. Since most of the genetic tools established in *E. coli* are incompatible for expression in cyanobacteria, development of a standard cyanobacterial synthetic toolbox is required to maximize the potential of these photosynthetic cells.

Recent advances in the development of the cyanobacterial chassis have allowed more systematic and efficient designs and integration of various heterologous pathways into the native metabolism to produce different types of bio-products. Examples are found in the heterologous production of alcohols [64], alkanes [65], fatty acids [66] and terpenoids [67]. In addition to the target chemical to be produced, cyanobacteria have also been engineered to excrete end products and facilitate separation from the “catalyst”

(cyanobacterial cells). This is the case for recombinant *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*) designed to excrete hydrophilic products such lactic acid and sucrose based on the insertion of heterologous transporters into the membranes [68]. Another strategy is the production of volatile compounds that naturally diffuse from the membrane to the headspace of cultures, like isoprene production in *Synechocystis sp* PCC 6803 (hereafter *Synechocystis*) [69, 70], or ethylene production in both *Synechocystis* [11] and *Synechococcus* [71-73].

In spite of the advances made in the genetic engineering of cyanobacteria, there are still various challenges to develop a photosynthetic microbial cell factory, which is truly economically viable. Yet, the process is high-energy demand and cost-intensive. That would restrict cultivation of cyanobacteria to high valuable products like the case of drug production. Nevertheless, cultivation of cyanobacteria would allow the generation of inexpensive products (like fuels) from only three substrates in a massive manner, if improvement of production levels up to industrial scale with a low cost were achieved. The reasons for low production rates are varied in nature, with low gene expression levels, the genetic instability of heterologous genes, low enzyme efficiency, and/or toxicity of the end products all potentially contributing [73-75].

1.2 Ethylene production *in vivo*

1.2.1 Ethylene biosynthesis in plants and microbes

In nature, ethylene is a phytohormone produced by plants and it regulates many physiological processes including growth, development and plant response to abiotic or biotic stress [76]. In the case of biotic stress, ethylene levels are commonly increased during plant-pathogen interactions to initiate a defense response [77]. Ethylene production by plants is tightly regulated and requires the expression of multiple genes [76, 78]. It is initiated in the Yang cycle via a three-step reaction where methionine serves as a precursor [77] [76]. In the first step, methionine is converted into S-adenosyl-L-methionine (SAM) by SAM synthetase. Subsequently, SAM is transformed into 1-aminocyclopropane 1-carboxylate (ACC) by ACC synthase (ACCS) which serves as a substrate for ACC oxidase (ACCO) in the final step [79-81].

In addition to plants, most microorganisms produce trace amounts of ethylene as a by-product under ammonia limitation, in a process known as ethylenogenesis [82]. In this pathway, ethylene is formed non-enzymatically from the oxidation of 2-keto-4-methylthiobutyric acid (KMBA), a derivative of L-methionine [83], to recover the amino group and use it as a source of nitrogen [84]. Examples of this are found in well studied organisms like *E. coli* [82, 84] and *Cryptococcus albidus* [83, 85]. Nonetheless, enzymatic microbial ethylene production is also found in some pathogenic microbes. This is the case for certain strains of *Pseudomonas syringae* [86] and the fungus *Penicillum digitatum* [87] which use ethylene as a virulence factor during plant infection, altering the plant defense response [86]. In these pathogens, ethylene is produced via the 2-oxoglutarate-dependent pathway, whereby catalysis is performed by a single enzyme, the ethylene-forming enzyme (EFE), and encoded by only one gene (*efe*) [88]. The minimal requirements of EFE driven ethylene production thus represent great potential for biotechnological application. Accordingly, EFE is the main focus of this thesis for bio-ethylene production and the characteristics and expression strategies of various EFEs are described below.

1.2.2 The ethylene-forming enzyme (EFE) and 2-oxoglutarate-dependent pathway

The native production of ethylene has been identified and studied in approximately 230 strains of bacteria. Of these, *P. syringae pv phaseolicola* PK2 (*P. syringae* PK2) has demonstrated the greatest production levels [89]. *P. syringae* PK2 harbors the ethylene-forming enzyme (EFE) which belongs to the superfamily of 2OG-Fe(II)-dependent dioxygenases. This superfamily is known by its ability to incorporate two molecular oxygen atoms into one or more substrates, and the three active site amino acid residues (His-Asp/Glu-His) coordinating the single ferrous iron [90]. Indeed, the EFE H198, Asp191, His 268 conserved amino acids from *P. syringae* PK2 have been proposed as ligands which coordinate the metal in the active site [79, 91, 92]. Identification was deduced from previous studies on site-directed mutagenesis of histidine residues in EFE from *P. syringae* PK2 [93] and sequence alignment with other enzymes belonging to the same superfamily [94]. This is also the case in some

plant ACCOs [79, 91], 2OG-Fe(II) oxygenases from cyanobacteria [92] and other representative EFEs from different strains of *Penicillium* and *Pseudomonas* [91, 94].

The historical characterization of EFE started in 1985 with a partial purification of EFE by Fukuda and co-workers from the fungus P. *digitatum* IFO 9372 [87]. In 1991 a complete purification of EFE from *P.syringae* PK2 was achieved [88], and one year later the nucleotide sequence revealed an open reading frame (ORF) of 1053-bp, coding for a protein of 350 amino acids [95]. Independent *in vitro* analyses using purified enzymes revealed that the enzymes shared similar properties. Both enzymatic systems involved a monomeric protein of 42 kDa that required O_2, 2-oxoglutarate, Fe^{2+} and L-arginine for ethylene formation. The optimum pH and temperature for catalysis were found to be 7-7.5 and 25°C [88] respectively, whereas at 37°C the formation of inclusion bodies was observed [96]. The EFE enzyme has been described to act as a "dual circuit" whereby two reactions take place simultaneously, which produce ethylene and succinate in a molar ratio of 2:1 [97]. In the major reaction, one molecule of ethylene, three of CO_2 and one of water are generated from 2OG and oxygen, with EFE using arginine and ferrous ion as co-factors. In contrast, the minor reaction uses arginine as a substrate, together with 2OG, to form succinate, 1-pyrroline-5-carboxylic acid (P5C), CO_2 and guanidine [79, 91, 92, 97]. Despite this proposed dual-circuit mechanism, these reactions have still not been achieved separately [97].

1.2.3 Heterologous expression of the *ethylene-forming enzyme* gene in cyanobacteria and genetic instability

Since 1992 there has been an increasing interest in expressing and optimizing the EFE pathway in recombinant organisms for biotechnological applications. To this end, *efe* was isolated from the native plasmid pPSP1 of *P. syringae* PK2 and the first heterologous expression was completed in *E. coli* JM109 [95]. For that purpose, Fukuda and co-workers constructed a series of sub-clones harboring *efe* in pUC19-based plasmids, named pEFE01 to pEFE10. That approach resulted in, not only the determination of *efe* nucleotide and amino acid sequences, but also the first *in vivo* measurements of ethylene in the head-space of recombinant *E. coli* cultures [94]. Although initial enzyme activity

was about one-fifth to one-tenth (115 nL C_2H_4 mL^{-1} h^{-1} OD_{610}^{-1}) compared to that of *P. syringae* PK2 [95], further optimization allowed significant increases (about 30 times the native EFE activity) when *efe* was expressed under the control of the lac *E. coli* promoter [96]. Further efforts were then directed at improving production levels by overcoming substrate limitation and through targeted modifications of the central carbon metabolism [98]. In addition to *E. coli*, the heterologous expression of EFE was also attempted in organisms which are traditionally optimized for large-scale industrial production conditions and biomass processing, such as yeasts [99, 100] and fungi [101, 102]. Because of their heterotrophic nature, however, all of these model organisms are unable to directly use the energy from light to fix CO_2 for ethylene formation.

In 1994, the use of the cyanobacterial host *Synechococcus sp.* PCC 7942 R2-SPc, hereafter *Synechococcus*, allowed for the first direct conversion of CO_2 to ethylene [71] (Figure 1). A broad host-range vector pUC303-EFE03 able to replicate in *E. coli* and *Synechococcus* was constructed. The *efe* fragment, flanked by its native promoter and terminator, was cloned from pEFE03, constructed from the set of plasmids developed by Fukuda and mentioned above. This resulted in autotrophic production of ethylene, albeit at low production rates of only about one fifth of the heterotrophic *E. coli* strain, which harbored the same *efe* cassette [71].

Due to the different nature between *E. coli* and cyanobacteria, further efforts targeted the optimization of expression systems in *Synechococcus*. New recombinant *Synechococcus* strains were constructed to express *efe* under the control of different promoters and/or terminators [72]. These efforts resulted in a significant improvement in ethylene production, especially when *efe* was flanked by the cyanobacterial promoter and terminator from *psbAI* (hereafter P_{psbAI} and T_{psbAI}, respectively). P_{psbAI} is a well characterized, strong light inducible promoter in *Synechococcus* [103] and has been used in different studies for the overexpression of various genes [104, 105].

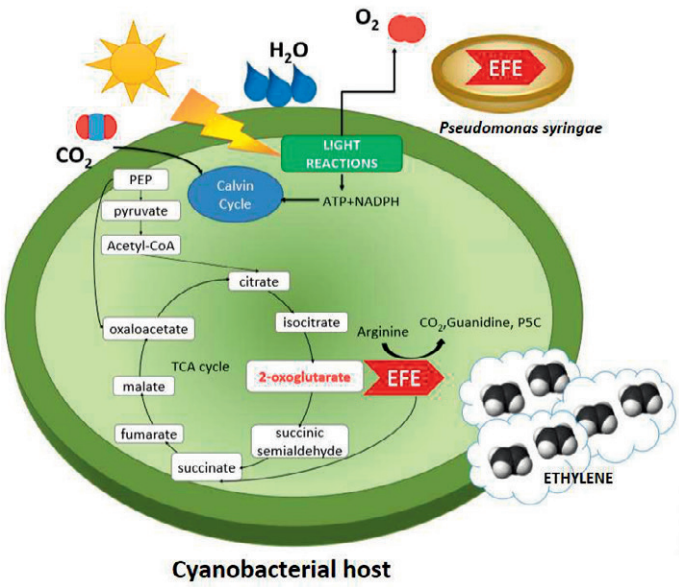

Figure 1. Schematic representation of heterologous expression of the Ethylene-Forming Enzyme (EFE) from *P. syringae* in a cyanobacterial host, which acts as a biosolar-cell factory. Solar energy is harvested by cyanobacterial photosynthesis and water molecules are dissociated to produce oxygen while two energy carriers are formed, ATP and NADPH. These energy carriers are used in the Calvin-Belson cycle to fix inorganic CO_2 and convert it into its organic form via different metabolic pathways. The heterologous expression of EFE from *P. syringae* interacts with the endogenous metabolism via the tricarboxylic acid (TCA) cycle to use 2-oxoglutarate as a substrate, together with arginine. This results in the production of ethylene, succinate, CO_2, guanidine and 1-pyrroline5-carboxylic acid (P5C).

In spite of the observed increases in ethylene production, up to 15 fold with a maximum specific rate of 323 nL C_2H_4 mL^{-1} h^{-1} OD_{730}^{-1}, most *Synechococcus* strains showed a yellowish phenotype indicative of metabolic stress. Moreover, the ethylene production ability was lost after a few generations. Subsequent southern blot analyses indicated the presence of the *psbAI* gene in the plasmid instead of *efe.* The authors described this phenomenon as plasmid instability [72]. The suggested hypothesis was that homologous recombination had taken place between *psbAI* in the chromosome and the plasmid at the P_{psbAI} and T_{psbAI} sites, thus replacing the *efe* gene. This phenomenon only occurred when the lengths of the homologous regions from P_{psbAI} and T_{psbAI} sites in the plasmid where longer than 100 bp.

In order to avoid plasmid instability, the next strategy involved the insertion of *efe* directly at the *psbAI* site in the cyanobacterial chromosome, with the

resulting disruption of the native gene [73]. The generated strain produced ethylene at a high rate, with a maximum level of 451 nL C_2H_4 mL^{-1} h^{-1} OD_{730}^{-1}, but still showed the earlier reported yellowish phenotype and low growth rate, indicating metabolic stress. After four consecutive cultivation batches the ethylene production dropped to zero and the phenotype returned to the characteristic blue-green of healthy cells. Sequence analysis of the strains revealed insertion mutations within three positions of the *efe* open reading frame (ORF), all containing the same nucleotide sequence CGATG. As a possible explanation, it was suggested that these sites may act as a hot-spot for spontaneous mutations activated by a selective pressure, like a 2OG deficiency due to EFE activity [73] or to ethylene toxicity [11]. This phenomenon has been described later as an example of genetic instability of heterologous genes [106]. Thus, genetic instability is defined here as undesirable genetic modifications of heterologous genes that cause a gene disruption and loss of the biotechnological utility.

Almost a decade later, members of the National Renewable Energy Laboratory (NREL) attempted to by-pass the reported genetic instability [11]. Their strategy was based on the following four objectives: (i) optimization of the *efe* sequence avoiding the described mutational "hot spots" without altering the amino acid sequence, (ii) use of the *psbA* promoter from a pea plant to avoid homologous recombination within *psbA* genes in the chromosome, (iii) transformation into a different cyanobacterial species, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), and (iv) insertion into the chromosome at a neutral site (slr0168). Thus, the optimized *efe* gene was named sy-*efe*. The resulting recombinant strain produced ethylene at similar rates as previously published for recombinant *Synechococcus* [73], at almost 400 nL C_2H_4 mL^{-1} h^{-1} OD_{730}^{-1} [11]. Notwithstanding, the strain did not show any sign of instability and ethylene production was continuous over generations. Moreover, the cultures showed a similar blue-green phenotype and growth rate as the wild type.

Such promising results were taken as a starting point for the optimization of production levels. One of the improvements was the chromosomal insertion of a second copy of sy-*efe* at the *psbA2* locus. This strain doubled the ethylene output without affecting growth rate or metabolism, indicating that increased *efe* activity *per se* did not pose any stress to the host. In view of this, growth

conditions and the composition of cultivation media were optimized and tested for productivity at larger scales and with more concentrated cultures. The maximum production peak was around 5700 nL C_2H_4 mL^{-1} h^{-1} with a high cell density of OD_{730} near to 15 for the strain expressing two copies of *efe* [11].

All these efforts raised the question of whether ethylene production is limited by a low flux of total carbon fixation towards the tricarboxylic acid (TCA) cycle [107] with which EFE was integrated. It had been suggested that under photoautrophic growth conditions, the maximum flux of fixed carbon towards ethylene via the TCA cycle would be less than 5% in *Synechocystis* [108]. Yet, further optimization efforts in 2014 by Wei Xiong et al. [108] achieved conversion rates of 10% of fixed carbon to ethylene without affecting the growth rate. This success was achieved by the expression of a single copy of *efe* with an engineered ribosome binding site (RBS) and led to specific ethylene production of 718 nL C_2H_4 mL^{-1} h^{-1} OD_{730}^{-1}, almost 4 times higher than the baseline strain without the engineered RBS [108]. Surprisingly, this genetic modification was enough to redirect 37% of fixed carbon into the TCA cycle, almost 3-fold higher than the amount observed in the WT, indicating the high metabolic flexibility of the *Synechocystis* strain [108].

In light of the findings based on *Synechocystis*, research has progressed to attempt genetic modifications to alter the TCA cycle [98, 109] and the optimization of cultivation techniques in bioreactors [110]. However, the reasons behind the genetic instability of *efe* expression in *Synechococcus* remain unclear. Likewise, it is unknown if the original efe sequence (hereafter *o*-efe), without optimization or avoidance of the suggested mutational hot spots, would also be stable in *Synechocystis* and potentially be a better choice for gene expression.

1.3 Cyanobacteria and aldehyde deformylating oxygenase

1.3.1 Aldehyde deformylating oxygenase

Cyanobacteria are able to produce medium chain hydrocarbons (C_{15}-C_{19}) [111], including alk(a/e)nes, using fatty acid substrates as intermediate precursors [112]. Although their biological function remains unclear, an association of alk(a/e)nes in cold stress tolerance, membrane fluidity, prevention of desiccation and chemical signaling have been proposed [112-115]. Recent studies

suggest that populations of marine cyanobacteria like *Prochlorococcus* and *Synechococcus* produce hundreds of millions of tons of hydrocarbons into the oceans annually, with pentadecane and heptadecane being predominant [115, 116]. Since alkanes are the major constituents of gasoline, this metabolic capacity is of great interest in the context of the biotechnological development of carbon-neutral drop-in fuels. One interesting biotechnological approach is the development of a continuous production system for short-chain volatile alkanes, which are not naturally produced by the cell. In contrast with the native medium-chain alkanes found in cyanobacteria, which are trapped in membranes, volatile alkanes can naturally diffuse across the cells and accumulate in the head-space of the cultures. This approach would allow to see cyanobacteria as biological catalysts, since cells would not need to be harvested and destroyed to collect the alkane product. As cyanobacteria are unable to naturally produce short-chain alkanes, a thorough understanding of the key enzymes from alternative natural pathways is crucial to building and optimizing *de novo* synthetic routes with carbon length tailored to market demands.

Most studied cyanobacteria use the FAAR/ADO route (Figure 2A) for alkane biosynthesis [112]. Within this natural pathway, reactions are carried out by fatty acyl-ACP reductase (FAAR) and aldehyde deformylating oxygenase (ADO) [117, 118]. The process is initiated by the conversion of fatty acyl-ACP by FAAR into an intermediate fatty aldehyde (R-CHO) in a NADPH-dependent reduction reaction [119]. In the subsequent ADO catalyzed oxygenation reaction, the aldehyde intermediate is transformed into C_{n-1} alkanes, and formate is also produced. The ADO enzyme is a monomer soluble protein of 24 kDa that belongs to the nonheme di-iron family of oxygenases [74, 11]. In addition to ADO, the reaction also requires an external protein-based reducing system to supply four electrons. Two of these electrons are needed for the breakdown of the peroxohemiacetal intermediate (as a result of a nucleophilic addition of oxygen to the aldehyde) to form the alkane product. The other two electros allow the formation of water and formate [120] while reducing, and thus reactivating, the di-iron center for further reaction [121].

Although the source of reducing equivalents has not been clearly elucidated in cyanobacteria, recent *in vitro* studies suggest an implication of

cyanobacterial [2Fe-2S] ferredoxin (PetF), which is reduced by Ferredoxin-$NADP^+$ reductase (FNR) consuming NADPH forming an endogenous Fd/FNR/N reducing system [122, 123] . In addition, other non-native external reducing systems have been demonstrated to support ADO activity *in vitro*. Examples of this are the Fd/FNR/N biological system from spinach [119], the *E. coli* NADPH:ferredoxin/flavodoxin-oxidoreducase (*Fpr*) system for the reduction of PetF from *Synechocystis* [129], and chemical donors such as NADH and phenazine methosulfathe (NADH/PMS) [124].

1.3.2 Synthetic pathways for short alkane production using aldehyde deformylating oxygenase

Since the discovery of cyanobacterial ADO enzymes, various synthetic pathways for medium chain alk(a/e)ne (C_{13}-C_{17}) production have been engineered and studied *in vivo* in *E. coli* [62, 119, 125, 126]. Petrol is composed of a mixture of C_4-C_{12} hydrocarbons of alkanes, alkenes and cycloalkanes [126]. Thus *in vitro* studies have been undertaken to demonstrate that different cyanobacterial ADOs (cADOs) are also active with short-chain aldehydes as substrates [128]. Three recent studies have demonstrated the employment of cADOs in synthetic pathways for the *in vivo* propane production in *E. coli* [129, 130, 131]. Here, it is worth noting that only oxygen tolerant enzymes, a primary requirement for compatibility with oxygen producing cyanobacterial hosts, were employed.

In the first study, Kallio and co-workers [129] used the *E. coli* native fatty acid biosynthesis pathway for the production of butyrate from butyryl-ACP (Figure 2B). The expression of the heterologous thioesterase (*Tes4)* from *Bacteroides fragilis*, which shows a preference for C4 substrates, allowed the production of butyrate [129]. Subsequently, a second heterologous enzyme, an ATP-dependent carboxylic acid reductase (CAR) from *Mycobacterium marinum* was co-expressed together with the maturase phosphonantetheinyl transferase (Sfp) from *Bacillus subtilis* [125] to catalyze the conversion of butyrate to butyraldehyde.

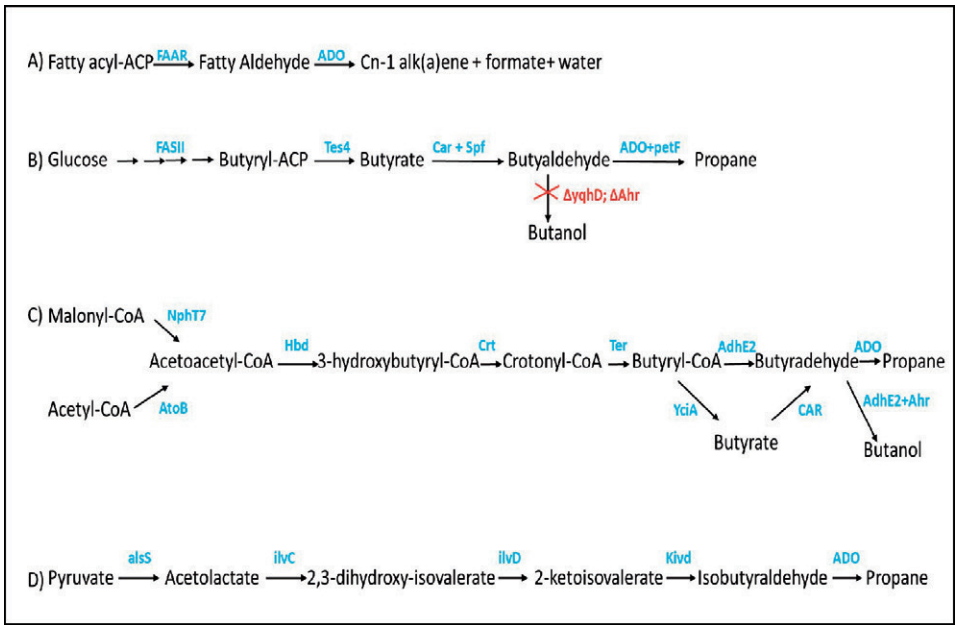

Figure 2. Comparison of the natural ADO pathway for propane production in cyanobacteria [120] (A) with the synthetic pathway assembled *in vivo* in *E. coli* (B-D) based on fatty acid synthesis (FAS) [129] (B), the CoA-dependent butanol pathway [130] (C), and the engineered valine pathway from pyruvate [131] (D). Over-expressed enzymes are shown in blue, endogenous genes deleted to avoid substrate competition are shown in red.

Finally, conversion of the aldehyde precursor was catalyzed by cADO from *Prochlorococcus marinus* (hereafter *P. marinus*), in the presence of oxygen and a novel reducing system. This reducing system consisted of the *E. coli* NADPH:ferredoxin/flavodoxin-oxidoreducase (*Fpr*) system for the reduction of cyanobacterial ferrodoxin PetF from *Synechocystis*. In addition to this novel synthetic metabolic pathway, the study identified some limiting factors that affected the efficiency of propane production. These factors were: (i) the efficiency of fatty acid biosynthesis, which was dependent on aerobic growth conditions, and (ii) competing endogenous reactions, which convert toxic intracellular aldehyde precursors into alcohols. Consequently, improvements in propane production at high O_2 concentrations (80% (v/v)) and a deletion of the endogenous competing pathway for aldehyde detoxification were achieved.

In the second study, Menon et al. [130] reported four different alternative routes to propane production (Figure 2C). These routes start either by the concentration of two molecules of acetyl-coA to form acetoacetyl-coA using the *Ato*B gene from *E. coli* (AtoB route) or by the *nph*T7 gene from *Streptomyces sp.* via the Manolyl-coA (nphT7 route). A common core pathway used selected enzymes from *Clostridium* (3-hydroxybutyryl-CoA dehydrogenase, Hbd, and 3-hydroxybutyryl-CoA dehydratase, Crt) and *Treponema dentricola* (trans-enoyl-CoA reductase, Ter) to form butyryl-CoA in the presence of oxygen. Subsequently, the downstream pathway diverged into two possible routes, the AdhE2 route or TPC7 route. The former route employing bifunctional aldehyde/alcohol dehydrogenase AdhE2 (from *Clostridium*) to mediate two subsequent reactions to convert bytyryl-CoA into butyraldehyde and then, with the co-expression of Ahr, into butanol. At the same time propane was obtained from butyraldehyde by ADO. Since AdhE2 competes with ADO, Menon and co-workers replaced AdhE2 with a thioesterase (TE) YciA from *Haemophilus influenza* to form butyrate from the latter butyril-CoA (TPC7 route). The addition of CAR, as described in the study of Kallio and co-workers [129], provided enough C4 aldehyde substrate to ADO. Accordingly, and similarly to the work of Kallio et al., the competitive native pathways for propane production were also taken into account and deletion of the endogenous *Ahr* and *YphD* genes allowed higher propane production.

In a more recent study, Zhang and co-workers [131] proposed an *E. coli* engineered valine-dependent pathway for the production of isobutyraldehyde, combined with ADO expression for propane biosynthesis (Figure 2D). The engineered pathway consisted of heterologous expression of the *alsS* gene from *Bacillus subtilis* to produce 2-acetolactate from pyruvate. In turn, the conversion to 2,3-dihydroxy-isovalerate and 2-ketoisovalerate were achieved by the overexpression of the *E. coli ilvC* and *ilvD* genes respectively. Finally, the introduction of the *Kivd* gene from *Lactococcus lactis* provided sufficient amounts of isobutyraldehyde for propane formation from ADO.

Table 1. *In vivo* propane production in *E. coli* via three different synthetic pathways (see Figure 2) highlighting some of the key differences between the experimental set-ups.

Pathway	ADO	Propane (mg/L)*	KatE	Additional ADO reduction	IPTG induction	Extra O_2	Ref
B	PM	5	Yes	NADPH:ferredoxin/flavodoxin-oxidoreductase	0.5 mM	Yes	[129]
C	PM	0,25	No	No	0.5 mM	No	[130]
D	PM	0,2	No	No	0.3 mM	No	[131]

* The values represent the highest levels reported in laboratory conditions for each route.

Even though the study of Kallio and co-workers [129] presented higher propane productivity (Table 1), direct comparisons are difficult to make due to the diversity within experimental parameters, the differences between the reaction set-ups and the nature of the engineered routes. For example, higher productivity in the first study may be due to the presence of an effective protein-based reducing system, which provided enough ADO reduction for a greater activity. Additionally, Kallio et al. [129] introduced a catalase (KatE) from *E. coli* to avoid the accumulation of hydrogen peroxide (H_2O_2), an inhibitor of ADO [132]. There is, however, a common consensus that ADO represents the major limiting factor for propane and other alk(a/e)nes production [74, 121, 122, 128, 129].

1.3.3 Aldehyde deformylating oxygenase as the main bottleneck to propane production

Several different research groups have studied the kinetics of the cADOs [74, 120-122, 128, 133]. Although experimental conditions varied (different cADO orthologs, substrates, reducing systems, reaction temperatures and oxygen concentrations), all results suggested that reaction is extremely slow due to the kinetic properties of the cADO enzyme. The highest turnover (k_{cat}) reported was about 16 reactions per minute using n-heptanal as a substrate [118]. However, that value was dramatically lower, less than one min^{-1} [74], in most cases, especially with short aldehyde carbon chains.

These values provide evidence that cADOs represent a true bottleneck for propane and other alk(a/e)ne manufacture, especially when compared with other enzymes upstream of the constructed synthetic metabolic pathways.

For example, one step back in the synthetic routes, CAR enzyme shows a k_{cat} for C4 fatty acids of about 290 reactions per minute [77], which is about 660 fold higher than the value reported for ADO by Zhang and co-workers [83]. In addition to the low k_{cat}, cADOs show poor affinity (high K_M values) towards short-chain aldehydes compared to longer-chain aldehydes [133], which decreases the catalytic efficiency (k_{cat}/K_M) of the reaction. Consequently, protein engineering, together with a reliable comparison between the different cADO orthologues may be a suitable strategy to improve kinetics. This is especially important for the efficient production of short-length alk(a/e)nes, like propane.

Khara and co-workers attempted to improve the K_M values of cADO from *P. marinuns* (strain MIT9319) by modifying the substrate-access tunnel of the enzyme using site-directed mutagenesis [133]. These changes were made with the ultimate intention of favoring the specificity of ADO to short-chain substrates. The strategy was based on the introduction of a steric block at amino acid position 134 or 41 to prevent the access of substrates with a carbon-length higher than C_9 [133]. This resulted in the generation of two ADO variants: A134F, where adenine was replaced by phenylalanine; and V41Y, where valine was replaced by tyrosine. Although the engineered variants showed higher specificity towards short-chain aldehyde substrates, the relative activity of ADO_{V41Y} with $C_{4\text{-}10}$ aldehydes was similar to the wild type. In contrast, increased ADO_{A134F} activity with C_4 and C_5 substrates was reported, resulting in almost two fold higher propane production compared to the wild type. Yet, the turnover of the reactions remained low.

Another example of ADO enzyme engineering is the cADO-L194A variant from *P. marinus* [134]. In this case, the leucine residue at the position 194 was hypothesized to serve as a gateway for substrate entry. However, amino acid substitution by alanine did not show differences in the limitation of substrate access to the active site when compared with the wild type.

In a more recent study, the wild type ADO from *S. elongatus sp.* PCC 7942 was used as a base for the generation of thirteen new variants via site-directed mutagenesis of selected amino acids identified to affect the aldehyde group, hydrophobic tail of the substrate, and the substrate pocket [135]. Among the generated ADO variants, A121F showed enhanced preference towards C4, C6

and C7 substrates. Moreover, the M193Y variant showed an apparent improvement in k_{cat} value (about three fold) with respect to the wild type, when n-heptanal was used as a substrate.

2. Aims

The use of recombinant cyanobacteria as hosts for the efficient production of volatile short chain alka(e)nes like ethylene and propane has been impaired due to a diverse range of challenges. The focus of this thesis is to achieve the microbial production of volatile alka(e)nes, by meeting the following aims:

- The evaluation of the ethylene-forming enzyme (EFE) from *P. syringae* as a non-invasive reporter protein for promoter screening in *Synechocystis sp.* PCC 6803 and *E. coli.*
- The elucidation of the codon optimization effect and avoidance of the associated mutational genetic elements versus the original gene sequence in order to achieve genetic stability for the heterologous expression of *efe.*
- Parallel cyanobacterial expression of the original *efe* (o-*efe*) sequence versus the optimized sequence (sy-*efe*), to distinguish the impacts of codon optimization, mutational hot-spots, and host selection in the genetic stability constructed mutants.
- Development of stable *efe* mutant strains of *Synechococcus sp.* PCC 7942 and identification of the reasons behind the earlier reported genetic instability.
- Development of an *E. coli* based *in vivo* comparison for the evaluation of cADO homologs and the identification of cADO-specific differences in catalytic performance and substrate preference.

3. Materials and methods

3.1 Expression vectors and strains

The expression constructs for ethylene studies in cyanobacterial strains and alkane production in *E. coli* are listed in Table 2. The DNA constructs were generated via standard recombinant molecular biology techniques using commercial enzymes from New England Biolabs band DNA kits from Qiagen. All plasmids were verified by sequencing prior to the transformation to the final expression strain.

Table 2. Expression vectors for ethylene production in cyanobacteria and short alkanes in *E. coli*. In addition to the plasmids containing cADO homologs in *E. coli*, the strains also contained the pET-FPC and pACYC-Fpr plasmids. For more details about the construction of the mutants, see the referenced papers.

Expression vector	Host	Paper
pDF-trc	*Synechocystis*	I
pDF-trc-EFEh	*Synechocystis* / *Synechococcus*	I-II
pDF-lac-EFEh	*Synechocystis*	I
pDF-pet-EFEh	*Synechocystis*	I
pDF-coa-EFEh	*Synechocystis*	I
pDF-smt-EFEh	*Synechocystis*	I
pDF-lac-ACS-ACO	*Synechocystis*	I
pDF-luxRI-EFEh	*Synechocystis*	I
pDF-trc-oEFE	*Synechocystis*	II
pDF-trc-sy-EFE	*Synechocystis*	II
pChr_7942_NSI_o-*efe*_Sp	*Synechococcus*	IV
pChr_7942_NSI_sy-*efe*_Sp	*Synechococcus*	IV
pCDF-ADO (*Synechococcus sp*. RS9917)	BL21(DE3)Δ*yjgB* Δ*yqhD*	III
pCDF-ADO (*Nostoc punctiforme*)	BL21(DE3)Δ*yjgB* Δ*yqhD*	III
pCDF-ADO2 (*Prochlorococcus marinus*)	BL21(DE3)Δ*yjgB* Δ*yqhD*	III
pCDF-ADO2-A134F (*P. marinus*)	BL21(DE3)Δ*yjgB* Δ*yqhD*	III
pCDF-ADO (*Synechocystis*)	BL21(DE3)Δ*yjgB* Δ*yqhD*	III

3.2 Culture conditions

E. coli strains were incubated in LB (Lysogeny Broth) medium at 37°C. Liquid cultures were incubated on a rotary shaker at 150–200 rpm. The growth medium was supplemented with the appropriate antibiotic for the relevant plasmid (see original papers for specific details). *E. coli* strain DH5α was used for plasmid propagation while the strain BL21(DE3)Δ*yjgB* Δ*yqhD* was used for

alkane production (Paper III). An over-night pre-culture was incubated at 37°C in 250 mL Erlenmeyer flasks. The main culture was started from a 1:10 inoculum. Protein expression was induced with 1 mM IPTG at OD_{600} ≈0.5 and incubated at 30°C for 4 hours. After incubation, cells were re-suspended and grown in a rotational shaker at room temperature in reaction buffer (K-phosphate 100 mM, pH 7.5; glucose 25 g/L; corresponding fatty acid, 5 mM) prior to GC-MS analysis.

Cyanobacterial strains were grown in BG-11 medium supplemented with the appropriate antibiotics at 30°C in 1% CO_2 (Paper I and IV) or 3% CO_2 (Paper II) on an orbital shaker at 120 rpm and at a light intensity of 50-100 µmol photons m^{-2} s^{-1}. Induction of EFE protein synthesis was performed depending on the promoter used for each mutant, using either IPTG (1mM for P_{lac} and P_{trc}), $CuSO_4$ (0.5 µM for P_{pet}), $CoCl_2$ (6 µM for P_{coa}), or $ZnCl_2$ (2 µM for P_{smt}) and modified BG-11 lacking the corresponding metal inducer for proper gene repression.

3.3 GC-MS quantitation of volatile hydrocarbons and alcohols *in vivo*

Volatile hydrocarbons and alcohols were monitored by GC-MS using samples taken from the headspace (for hydrocarbons) or liquid phase (alcohols) of sealed culture vials. Samples of cyanobacterial strains were prepared by transferring 1 mL of the cell cultures into sealed 10 mL serum bottles and measurements were taken after 2-4 hours of incubation. In the case of the *E. coli* strains evaluated for ADO expression, 0.5 mL of cells re-suspended in the reaction buffer (see section 3.2 for details) were incubated for 30 min, 1.5 h, 2.4 h, 4 h and 10 h and measurements were performed immediately after each cultivation period. Integrated peak areas were used to calculate the relative ethylene production as nL ethylene/mL culture/h or µmol/L/h for ADO derived hydrocarbons and alcohols. The results were normalized to cell density and a linear calibration curve of the corresponding hydrocarbon/alcohol was made to allow a proper comparison of the obtained abundance signals.

3.4 RNA isolation and cDNA synthesis

Total RNA was extracted from 10 mL of cyanobacterial cultures using the TRIsure™ method (Bioline). One µg of RNA was used for cDNA synthesis using the QuantiTect® Reverse Transcription kit with random primers, according to

the manufacturer's instructions. The obtained cDNA was diluted 5-fold with water and used as template for RTqPCR.

3.5 Real-Time quantitative PCR

Relative *efe* transcript levels were measured using RTqPCR (iQ5 RT-PCR, Bio-Rad). The amplification reactions were carried out using specific primers with 2 µl of the isolated cDNA as the template in a 25 µL total volume (iQ™ SYBR® Green supermix, Bio-Rad). Relative transcript levels were normalized to the reference gene *rnpB*.

3.6 Protein quantification

Purification of His-tagged proteins from *E. coli* lysates was obtained via nickel-affinity chromatography using the His SpinTrap™ kit (GE Healcare) for EFE, or Ni-NTA Columns (Qiagen) for ADO, according to the manufacturer protocols. Protein quantification was performed using SDS-page and Bradford methods.

3.7 Spectrophotometric analysis

An Infinite 200 Pro plate reader (Tecan Ltd) was used to record the absorption spectra (400-750 nm) from 150 µL cyanobacterial samples with the same optical density in 96 well plates.

3.8 Ethylene toxicity test

Synechoccocus was evaluated for its tolerance to ethylene by supplying 99% (v/v) gaseous ethylene (AGA) into 20 mL cell cultures sealed in gas-tight serum bottles (160 mL). Ethylene was supplied directly into the culture media by bubbling for 2 min (≈0.5 bars) with an injection needle through the butyl rubber cap of the sealed bottles. Growth of the cells was followed by monitoring culture OD_{750} for the subsequent 7 days.

4. Overview of results

4.1 Ethylene-forming enzyme

4.1.1 Ethylene-forming enzyme as a reporter protein for promoter screening

Ethylene biosynthesis via the heterologous expression of EFE in *Synechocystis* and *E. coli* was evaluated for its performance as an *in vivo* reporter protein for promoter screening (Paper I). Ethylene naturally diffuses through cell membranes and accumulates in the head-space of the cultures, allowing a non-invasive monitoring system. A primary plasmid was assembled using the origin of replication from pVZ321 (a derivative RSF1010 that allows replication in a wide range of Gram-negative bacteria), the $LacI^q$ repressor, Trc promoter, multiple cloning site and transcription terminators from pTRC99A, and the spectinomycin/streptomycin (Sp^r/Str^r) antibiotic resistance markers from pZE13-MCS. This backbone plasmid, named pDF-trc was designed to work in functional blocks. Every block, consisted of a biological part (for example: promoter, terminator, antibiotic cassette) and was flanked by unique restriction sites. This approach facilitated an easy interchange of genetic elements. These elements were arranged in the following order: Self-replicating region by AvrII-EagI; antibiotic cassette by EagI-BsrGI; the regulator-promoter cassette by BsrGI-(speI)-KnpI sites; *efe* gene by KpnI-PstI; and transcription terminators by PstI-AvrII (Paper I, Fig.1). Accordingly, every derivative of the expression construct was obtained by digestion of the fragments selected to be replaced, with final constructs verified by DNA sequencing.

In order to test the validity of EFE as a reporter protein, seven already characterized promoters were chosen and grouped according to three different induction strategies. The first category was composed of *E. coli* IPTG-dependent promoters (P_{trc} and $P_{A1lacO-1}$). The P_{trc} promoter has been reported to allow well-regulated expression in *E. coli* and *Synechococcus* [136], whereas P_{lac} has shown weak repression in the absence of the IPTG inducer in *Synechocystis* [137]. Thus, $P_{A1lacO-1}$, a P_{lac} derivative, which has been reported to allow stronger repression [138] was included in the study and tested for the first time in *Synechocystis*. Both promoters included the $LacI^q$ repressor and the cassette $LacI^q$-promoter (trc or lac). The second category included the

cyanobacterial metal inducible promoters (P_{petE}, P_{coa} and P_{Smt}, induced by copper, cobalt and zinc respectively). It has been reported that several metal inducible promoters allow a tight control of protein expression [139]. However, the promoters had not been compared with each other for their relative strength. To this end, these promoters were cloned in the pDF vector with their respective regulator elements (*coaR* and *smtB* for cobalt and zinc promoters respectively), except for the copper promoter whose repressor sequence is unknown (Paper I, Fig. 6). The third category included two cell density-dependent synthetic quorum-sensing (QS) promoters, P_{LuxRI} and P_{RhlRI}, from *Vibrio fischeri* and *Pseudomonas aeruginosa*, respectively. At low cell densities these promoters allow a basal expression of *LuxI* (for *V. fischeri*) and *RhlI* (for *P. aeruginosa*). This results in the production of specific acyl-homoserine lactones (AHL), 3-oxo-hexanoyl-AHL and butanoyl-AHL respectively, by AHL-synthase. These signaling molecules are released to the environment and accumulate at increased cell densities. Once a threshold concentration is reached, the AHL molecules bind to the transcriptional regulator LuxR or RhlR and activates downstream genes [140]. These two QS systems, LuxRI and RhlRI, were cloned in a pDF vector upstream of *efe*.

Results presented in paper I revealed that *E. coli* IPTG-dependent promoters produced the highest amounts of ethylene, similar to those reported earlier in cyanobacteria, using the light-inducible promoters P_{psbAI} [72, 73] and P_{psbA1} [11] of *Synechococcus* and *Synechocystis*, respectively. Furthermore, in *E. coli* strains, the expected tight repression of P_{trc} and $P_{A1lacO-1}$ was observed in the absence of IPTG. No repression effects were observed in *Synechocystis* strains expressing *efe* under the control of P_{trc} (Paper I, Fig. 3). In contrast, $P_{A1lacO-1}$ presented an effective repression in low density cultures, showing the highest repression at OD_{750} levels below 0.5 (Paper I, Fig. 5). Cyanobacterial strains harboring the metal inducible promoters showed a tight repression when the respective metal inducer was absent from the cultivation media. The highest cyanobacterial ethylene production rates were demonstrated by the strain under the control of P_{coa}, however values were only 25% of those produced with the *E. coli* promoter variants (Paper I, Fig. 7). In the case of the two synthetic QS systems, only LuxRI was functional in *E. coli* showing similar rates of ethylene production as *E. coli* strains harboring P_{trc} (Paper I, Fig. 9). Unfortunately, when the vector was expressed in *Synechocystis* it showed a

production of ethylene at a rate of only 5% of that produced with the same promoters from the *E. coli* variants, independent of the cell density. The RhlI system did not work in *E. coli* and the expression construct was not tested in cyanobacteria.

4.1.2 Codon optimization of the *ethylene-forming enzyme gene* is not a pre-requisite for the stable production of ethylene in *Synechocystis sp.* PCC 6803

Stable ethylene production was first demonstrated in *Synechocystis* by Ungerer and co-workers [11] via the heterologous expression of a codon-optimized *efe* gene from *P. syringae*. To clarify whether *efe* codon optimization was a pre-requisite for the stable production of ethylene in *Synechocystis*, a plasmid for the expression of the original unoptimized *efe* sequence (o-*efe*) was constructed and compared with two synthetic codon-optimized *efe* variants (sy-*efe*). In order to allow a reliable comparison, o-*efe* was assembled in a pDF-trc plasmid as described in paper I, whereby pDF-trc-EFEh and pDF-trc-sy-EFE (both harboring sy-*efe*, either with or without a 6xHis-Tag respectively) were obtained. The resulting plasmid was named pDF-trc-o-EFE and was confirmed by gene sequencing prior to transformation into *Synechocystis*. Transformants were selected based on spectinomycin and streptomycin resistance and the presence of o-*efe* in the plasmid, which was confirmed by colony PCR.

The three recombinant strains, S6803:o-*efe*, S6803:sy-*efe* and S6803:sy-*efeh*, were cultivated over five consecutive four-day experiments, and ethylene production was measured from the headspace of closed cultures by GC-MS (Paper II). All strains showed stable production of ethylene, with the o-*efe* strain producing about 1.3 and 1.6 times more ethylene than the sy-*efeh* and sy-*efe* strains respectively (Paper II, Fig. 1). Moreover, the S6803:o-*efe* recombinant strain did not show any sign of metabolic burden affecting growth or observable phenotype. Furthermore, transcriptional analysis of the *efe* variants revealed only marginal differences between the three strains, whereby a slightly lower mRNA level was observed for the strain carrying o-*efe* (Paper II, Fig. 2).

4.1.3 Strategy for the stable production of ethylene in *Synechococcus sp.* 7942

In *Synechococcus*, unstable production of ethylene has been associated with the heterologous expression of *efe* from *P. syringae*, occuring when the expression construct contains sequence elements homologous to the *psbAI* site of the chromosome [72, 73]. Due to this adverse effect of *efe* expression from the *psbAI* promoter, an alternative chromosomal integration strategy was evaluated in paper IV. This alternative comprised the construction of a shuttle vector for insertion of *efe* in the Synpcc7942_2498 locus. This site, which codes for a hypothetical protein, SEA0027 [141], has been commonly used as a neutral site for heterologous gene expression in *Synechococcus* and is often referred to as Neutral Site I (NSI) [142-145]. The parent plasmid was built by the assembly of a PCR-amplified *NSI* fragment (1094 bp) into the commercial pUC19 plasmid. Subsequently, two *efe* variants, o-*efe* and sy-*efeh,* were PCR amplified from pDF-trc-oEFE (Paper II) and pDF-trc-EFEh (Paper I) respectively. The fragments carried the Sp^r/Str^r-*LacI*^{q}-P_{trc}-o/sy-*efe*-T_{rrnB} cassette and were inserted into the parent plasmid to generate the final integration constructs *pChr_7942_NSI_o-efe_Sp* and *pChr_7942_NSI_sy-efeh_Sp*. The plasmids were verified by sequencing and transformed into *Synechococcus*. The resulting strains, *Synechococcus:*o-*efe* and *Synechococcus:*sy-*efe,* were selected by their resistance to spectinomycin and streptomycin. Full segregation of the *efe* cassettes into the *NSI* locus was confirmed by PCR (Paper IV, Fig. 1).

The strains were initially evaluated for their capacity to maintain stable production of ethylene during four consecutive batch cultures. Both strains, harboring either the original or optimized *efe* sequence, showed similar ethylene production efficiencies, with a maximum efficiency of 140 µL L^{-1} h^{-1} OD_{750}^{-1} observed (Paper IV, Fig. 2). Further analysis of fresh cultures was performed over a three month period, which revealed the capacity of strains to maintain stable ethylene production (Paper IV, Fig. 6). In addition, the growth rate and the culture color during the extended testing period were similar to the wild-type, indicating that the continuous production of ethylene did not negatively impact the host (Paper IV, Fig. 3 and 4).

4.2 In vivo comparison of five cyanobacterial aldehyde deformylating oxygenase homologs in *E. coli* for the production of short-chain alkanes

Five cyanobacterial ADO orthologs were selected for *in vivo* comparison and evaluation of their catalytic performance for the production of propane, pentane and heptane. Instead of cyanobacteria, *E. coli* was selected as a host due to the previously established and optimized quantitative analytical set-up system [129]. The cADO orthologs consisted of the wild-type variant and modified A134F from *P. marinus*, *Nostoc punctiforme* (*N. punctiforme*), *Synechocystis* and *Synechococcus sp*. RS 9917 (S9917) (Paper III). An amino acid sequence alignment revealed 48-68% sequence identities among cADO homologs. A 75% sequence identity was observed between cADOs from *N. punctiforme* and *Synechocystis*, whereas the cADO from S9917 shared a maximum of 40% sequence identity with the other four orthologs (Paper III, Fig. 2).

Each ADO ortholog was assembled in parallel pCDF-based plasmids for overexpression in *E. coli* BL21(DE3)Δ*yqh*DΔ*ahr*, which contained two additional plasmids that comprised the remaining enzymes of the heterologous hydrocarbon pathway. This pathway consisted of the coexpression of carboxylic acid reductase (CAR) with its maturation factor phosphonantetheinyl transferase (ppt) for the conversion of C4, C6 and C8 of externally supplied fatty acids into their respective aldehydes. Additionally, an efficient reducing system to provide electrons for ADO reaction was assembled by the expression of ferredoxin (petF) and NADPH:ferredoxin/flavodoxin-oxidoreductase (Fpr). The *in vivo* conversion of externally supplied fatty acid precursors was quantified by GC-MS using samples obtained from the headspace of the reaction vials.

The five parallel *E. coli* strains were able to produce heptane, pentane and propane when supplemented with 5 mM octanoic acid, hexanoic acid and butanoic acid, respectively. *Synechocystis* performed best for the pentane production, followed by *P. marinus* strain and the modified A134F strain which demonstrated similar levels to each other, but the variant was able to produce 1.4 fold more heptane. However, A134F was outperformed by *N. punctiforme*,

which produced 1.5 fold more. There were no differences in propane production among the cADO variants, except for S9917. In contrast, S9917 ADO produced the lowest alkane amounts compared to the other homologs (Paper III, Fig. 3). Moreover, the extracted His-tagged ADO enzymes at four time points of the protein expression were quantified by Bradford and SDS-PAGE revealing that the enzymes were stable. However, the S9917 ADO protein abundance was significantly lower (about 11-20% less), correlating with its inferior hydrocarbon production (Paper III, Fig. 5).

5. Discussion

5.1 Use of ethylene-forming enzyme as a quantitative reporter in cyanobacteria

Regulation of heterologous gene expression is one of the main considerations when engineering microbial production hosts for biotechnological applications. Turning the expression systems on and off is typically accomplished by promoters, which are DNA regions that control transcription. The fine control of any heterologous gene transcription becomes especially crucial when there is a potential risk of metabolic burden, or that toxic products will be generated. The ideal promoter would allow strong and modulated gene expression after its activation by a non-toxic inducer. Yet, it should be fully repressed in the absence of the inducer. Various promoters have already been characterized in *E. coli* and cyanobacteria [137, 146]. In most cases, fluorescence proteins have been used as a broad-host reporter protein for gene expression, which is indicated by fluorescence intensity. However, in cyanobacteria, a measurement background correction is often needed to remove interferences caused by endogenous pigments. Nevertheless, this strategy has previously allowed a successful promoter comparison in cyanobacteria [137].

In paper I, the EFE driven biosynthesis of ethylene was presented as an alternative non-invasive reporter system for monitoring the performance of engineered expression systems, including promoters, in cyanobacteria. A broad-host range expression plasmid backbone was constructed to allow the assembly of six different promoters upstream of *efe* for parallel expression in *Synechocystis*. The strength and induction/repression capacities of the promoters were used as comparative parameters by measuring the rate of ethylene accumulation in the head-space of the cultures. The varying ethylene levels among the different *efe* strains allowed a suitable promoter comparison. Consequently, in later studies *efe* was successfully used as a quantitative reporter to evaluate its translation efficiency within the genetic context of RBS sequences in cyanobacteria [164].

The *E. coli* IPTG-dependent promoters offered the highest ethylene production levels, although they show different repression behavior in the

absence of the IPTG inducer. While in *E. coli* P_{trc} was effectively repressed, it operated constitutively in *Synechocystis*. This could be due to structural differences in cyanobacterial RNA polymerase that could affect the interaction with the orthologous *E. coli* promoter [147]. Another reason could be the different intracellular levels of the LacI repressor among these species. Although the LacI amounts were not evaluated in paper I, a later publication [147] reported that the *E. coli* NEB5α and DH5αZ1 strains contained about 5 and 16 times more LacI per cell respectively, than *Synechocystis* cells. This could indicate a lower transcription or translation efficiency of the orthologous LacI repressor in cyanobacteria. In contrast, $P_{1AlacO-1}$ was effectively repressed in both *E. coli* and *Synechocystis*. The main difference between P_{trc} and $P_{1AlacO-1}$ is the presence of a second lacO operator between the -35 and -10 boxes. Although a single lac operator allows the binding of the LacI repressor to block RNA polymerase, a second operator can enhance repression via DNA-looping, as a consequence of LacI-tetramer binding [147]. However, the repression efficiency in cyanobacteria decreased at higher cell densities. Thus indicating a possible endogenous sugar accumulation, such as allolactose binding $LacI^q$.

Metal inducible promoters demonstrated inferior ethylene production levels compared to lac-derived promoters. The highest rate of production was about 25% of that obtained with lac-derived systems. Despite this, P_{coa} stood out as having excellent repression efficiency in the absence of cobalt ions. In the work presented in this thesis, metal concentrations employed were below half inhibitory concentration levels (IC_{50}). Higher concentrations, which would be required to enhance expression levels, would likely induce metabolic stress [148]. Toxic concentrations of metals can increase protein denaturation, oxidative stress and can affect the photosynthetic apparatus by damaging some of its components, such as the light harvesting-like proteins [148].

The synthetic QS promoters, which are dependent on cell density, were evaluated for their potential as self-regulated systems. QS promoters were investigated to avoid the requirement of expensive (and potentially toxic) chemicals for inducing gene expression. The LuxRI system was functional in *E. coli* and demonstrated potential for heterologous application. However, in *Synechocystis*, only low levels of ethylene production, independent of cell density, were detected. Further studies will be needed to identify what limits

the successful application of the LuxR1 system in cyanobacteria. The basal ethylene production levels observed in *Synechocystis* indicate that at least the first step of the expression strategy was functional and that constitutive expression of *luxI* for AHL-synthase formation [149], and *efe* for ethylene production were achieved. The failure of auto-induction may have been due to deficiencies in AHL auto inducer formation, required to boost *luxI* and *efe expression* at high cell densities. Possible reasons include a low transcription/translation of *luxI* or folding-associated problems, a lack of available substrate for AHL formation, AHL hydrolysis at high pH levels [150], or cyanobacterial acylase activity as a possible quorum quenching mechanism to degrade AHLs produced by other species [151]. In the case of the RhII system, it did not work in *E. coli* and thus it was not tested in *Synechocystis.* In *P. aeruginosa*, the regulation of the RhII system is not fully understood and its induction may also depend on interaction with other types of AHLs produced in different regulon systems, such as LasRI, which is associated with biofilm formation [150]. Thus, co-expression of the LasRI regulon together with LuxRI in *E. coli* could encourage successful promoter activation and allow the expression of downstream genes at increased cell densities.

5.2 Genetic instability of *ethylene-forming enzyme* gene in cyanobacteria

5.2.1 Codon optimization vs. original sequence

Genetically stable microbial platforms are typically required for ensuring the success of any biotechnological application. Genetic stability is defined here as the resistance of the heterologous gene(s) to change over the time in a microbial host. The heterologous expression of *efe* from *Pseudomonas syringae pv. phaseolicola* in *Synechococcus sp.* PCC 7942 has been reported to be unstable [72, 73]. This is as a result of gene truncation at the GCATG sequences repeated throughout the ORF [73]. This unstable expression has been reported to be overcome using gene codon optimization (avoiding the CGATG genetic elements associated with gene disruption) in an alternative cyanobacterial host, *Synechocystis sp.* PCC 6803 [11].

In order to distinguish the impact of codon optimization from the impact of a change in host, work presented in this thesis was based on the parallel

expression of the original *efe* (o-*efe*) sequence versus the codon optimized synthetic sequence (sy-*efe*) in an extrachromosomal self-replicating vector transformed in both *Synechocystis* (Paper II) and *Synechococcus* (Paper IV). In this way, the importance of host strain was demonstrated to be of little influence on the genetic stability of the heterologous gene. While slightly higher in *Synechocystis*, similar ethylene production levels were observed for both strains, without lowered growth rate or any other sign of metabolic burden. Indeed, transformants of both strains remained stable, even over prolonged cultivation of up to three months.

The synthetic *efe* gene harbors three main modifications via synonymous nucleotide substitution on which the amino acid sequence remains intact: (i) the avoidance of the associated genetic elements for gene disruption; (ii) addition of 6xCAC nucleotide sequence after the start codon for the insertion of six histidine residues at the N-terminal of EFE; and (iii) codon optimization for the preferred codon usage in cyanobacteria.

The genetic elements on which mutations concentrate are also known as mutational hot-spots [152]. Besides Takahama's studies, there is no other evidence in the literature for a high frequency of mutations being associated with the CGATG sequence. However, this sequence is found around three times in the ORF of different *efe* homologs encoding 2-OG-dependent ethylene/succinate-forming enzymes (see Table 3). Although the o-*efe* variant used in this thesis (the same as in Takahama's work) did not suffer from genetic instability, it is worth noting that the experimental methods and conditions were different. Accordingly, one possible future approach to assess the impact of these genetic elements would be the comparison of different *efe* orthologs (see Table 3) and sy-efe under the exact laboratory conditions which induced o-*efe* disruption at GCATG sites.

The second modification of sy-*efe* was made with the intention of facilitating protein purification. However, the addition of purification tags may have an effect on protein expression [153]. One such example of this is enhanced protein expression observed due to mRNA stabilization gained by the addition of a 6xHis-tag at the N-terminus of enzymes. Such stabilization avoids hairpin loop formation in the translation initiation region and facilitates ribosome binding to the mRNA [153]. This could explain why, in paper I, *efe* transcript and

ethylene production levels of his-tagged sy-*efe* (sy-*efe*) were slightly higher than sy-*efe* without a his-tag. However, this advantage is not sufficient to exceed ethylene production levels observed in o-*efe*.

Table 3. Microorganisms with genes encoding 2-OG-depedent ethylene/succinate-forming enzymes harboring CGATG elements.

Organism	Lineage	*efe* bp	CGATG repeats	Gene identifier
Pseudomonas syringae pv. phaseolicola (o-efe)	Gram negative bacteria	1071	3	AF101058.1
Pseudomonas syringae pv. glycinea strain ICMP2189	Gram negative bacteria	1053	3	EF175870.1
Pseudomonas syringae pv. sesami	Gram negative bacteria	2971	4	AB025277.1
Pseudomonas syringae pv. cannabina	Gram negative bacteria	1117	3	AF101059.1
Pseudomonas syringae pv. pisi	Gram negative bacteria	1095	4	AF101061.1
Ralstonia solanacearum GMI1000	Gram negative bacteria	1038	2	NC_003296.1
Isaria fumosorosea ARSEF 2679	Fungi	1428	2	NW_017387303.1
Penicillium digitatum Pd1	Fungi	1246	5	NW_014574621.1
Trichoderma gamsii strain T6085	Fungi	1381	3	NW_017386876.1
Pochonia chlamydosporia 170	Fungi	1292	1	NW_017263808.1
Togninia minima UCRPA7	Fungi	1046	2	NW_006921229.1
Neofusicoccum parvum UCRNP2	Fungi	1154	1	NW_006887962.1
Pyrenophora tritici-repentis Pt-1C-BFP	Fungi	1239	3	NW_001939247.1

The third modification of sy-*efe* was codon optimization. During mRNA translation, each codon is recognized by the anticodon of specific tRNAs charged with a corresponding amino acid. Different codons can encode the same amino acid [154]; however, the preference for a particular codon varies between different organisms and reflects the available tRNA pool within the cells [155]. In this sense, the most frequently used codons in one organism may be rarely used in another, thus affecting the rate at which a heterologous

protein is expressed [156]. Consequently, codon optimization has been shown to increase the heterologous expression of proteins. One frequently used approach for codon optimization is the synonymous substitution of rare codons by those most frequently used in the highly expressed genes of the host [156, 157]. The presence of rare codons has been suggested to play a role in mRNA stabilization, modulation of translation speed and protein folding [156-158]. Codon optimization at the beginning of a gene (around 16 codons) has been demonstrated to improve stability of mRNA and to facilitate the initiation of translation. However, optimization further along the gene sequence may increase translation speed, which could impair co-translational protein folding [156]. In this thesis, sy-*efe* was optimized by the substitution of each rare codon with the most abundantly used codon in *Synechocystis*. However, these modifications were not effective in improving protein expression, indicating that the presence of rare codons in the primary *efe* sequence was not a limiting factor for efficient EFE translation in *Synechocystis*.

5.2.2 The role of the *PsbA* gene family and its relationship with the reported genetic instability of the *ethylene-forming enzyme* gene in *Synechococcus*

Until now, the heterologous expression of *efe* in *Synechococcus* has been reported to be unstable. One common feature of previously employed expression strategies was the use of promoter and terminator sequences from endogenous *psbAI* of *Synechococcus* [72, 73]. Due to the apparent adverse effect of the *psbAI* site on *efe* expression, I evaluated an alternative *efe* expression strategy based on chromosomal integration at the Synpcc7942_2498 locus (Paper IV). This site has been commonly used as a neutral site for heterologous gene expression in *Synechococcus* and is often referred as Neutral Site I (NSI) [142-144]. Stable ethylene production was demonstrated in *Synechococcus*, even over extended cultivation. This result highlighted the importance of chromosomal integration site selection for biotechnological application.

The reason behind the previously observed genetic instability of *efe* at the *psbAI* site may be associated with the tight regulation of the *psbA* gene family under changing environmental conditions. In *Synechococcus*, there are three

psbA genes that code for two different PSII reaction center D1 proteins [142]. Under normal growth light conditions, the *psbAI* gene encoding the D1:1 form represents 94% of the total D1 protein in the thylakoid membrane [159]. Under high light intensity, or other stress conditions, there is a shift in gene expression and *psbAI* is repressed, while *psbAII* and *psbAIII* are activated. These two latter genes code for the D1:2 form, which is more resistant to photodamage.

In earlier studies, *Synechococcus* recombinant strains where *efe* replaced the *psbAI* gene were cultivated at low light intensities (below 50 µmol photons m^{-2} s^{-1}) [73], whereby *psbAII* and *psbAIII* are repressed. This alters the normal function of PSII leading to photoinhibition and the activation of protection mechanisms, such as carotenoid synthesis to combat oxidative stress [160]. Thus, resulting in the yellow phonotype reported by Sakai and Takahama [72, 73]. Since a rapid repair of PSII is essential for cell survival under changing environmental conditions [161], the host may activate *psbAII* and *psbAIII* expression to produce D1:2 at low light intensity. In addition to the PSII-repair cycle, cyanobacteria also possess DNA-repair mechanisms which can restore the function of light-damaged genes [162]. Although the reason for *efe* gene truncation remains unknown, an association with DNA-repair mechanisms may be expected. It would be interesting to re-evaluate the unstable strain and monitor possible variations in the DNA repair of genes to gain new insights into the mechanistic process that has been shown to lead to heterologous gene disruption [73].

Contrary to *Synechococcus* observations, *efe* expression at the *psbA1* site of the *Synechocystis* chromosome resulted in stable ethylene production. This can be explained by the different regulatory mechanisms controlling the expression of the *psbA* gene family [161]. Whilst in *Synechococcus psbAI* is predominant under normal growth conditions, in *Synechocystis psbA1* is mostly silent. Indeed, expression of *psbA1* has only been recorded in *Synechocystis* under anaerobic conditions, while the expression of *psbA2* and *psbA3* dominate under standard conditions.

Additionally, it is important to consider the limitations of heterologous gene expression at the chromosome due to the low-copy number. Nevertheless, one interesting alternative in *Synechocystis* would be the use of the

endogenous plasmid pCA2.4 as a target for heterologous gene expression, as it has been shown to increase the gene copy number up to seven times higher than that of a chromosomal integration site [165].

5.3 Aldehyde deformylating oxygenase and the microbial production of volatile alkanes

Microbial production of short chain volatile hydrocarbons is an interesting strategy to allow the development of continuous biological production platforms. While long carbon chain products usually require the break-down of the cells for product harvest, short volatile hydrocarbons can naturally diffuse through membranes and accumulate in the head-space of cultures. In this context, the cyanobacterial *aldehyde deformylating oxygenase* (cADO) enzyme has been integrated in different heterologous hydrocarbon pathways and expressed in *E. coli* to catalyze the last step-reaction required to convert aldehyde precursors into C_{n-1} alkanes [62, 119, 125, 126]. Although these expression platforms were successful in producing alkanes of different lengths, production levels remained very low, in the range of 5-600 mg/L [62, 126, 127, 129, 130, 132]. In all cases, cADO was suggested as a limiting factor. This was due to observations of low turnover and a poor affinity for aldehyde substrates, particularly shorter chain aldehydes [132, 135].

Making use of the natural diversity of cyanobacteria, paper III describes a novel *in vivo* comparison system for the identification and evaluation of differences in the catalytic performance and substrate affinity of specific ADOs. A previously reported analytical system for evaluating ADO dependent propane production served as the base for the construction of five parallel pathways in *E. coli* [129]. Each pathway involved the overexpression of a carboxylic acid reductase (CAR) for reduction of supplemented fatty acids (C4, C6 and C8) to corresponding aldehydes, and an ADO homolog from different cyanobacteria (*N. punctiforme, Synechocystis, P.marinus, P.marinus* A134F, and *Synechococcus*), to evaluate the production efficiencies of propane, pentane and heptane. In addition, the system included a ferredoxin (PetF from *Synechocystis*) and ferredoxin-oxidoreductase (Fpr from *E. coli*) to ensure an efficient supply of electrons for ADO activity. The role of Fpr in this redox system was crucial in allowing a reliable comparison between the ADO

orthologs. Previous studies have demonstrated that Fpr co-expression with petF is able to increase propane production up to 8 fold [163]. The presence of Fpr thus prevented a reduction in ADO activity and allowed observations of differences in performance that were actually ADO-specific.

The approach employed provided reproducible data and allowed for a reliable statistical analysis of ADO-specific differences. Accordingly, the ADO from *N. punctiforme* demonstrated the best overall performance with the highest initial heptane and pentane production rates. Surprisingly, the *P. marinus* ADO A134F variant specifically engineered and reported to increase substrate preference towards short carbon-chains [133] revealed only slightly higher heptane production than the wild type enzyme. Another interesting result was the ability of the comparative expression method to detect the inferior performance of ADO reported earlier for *Synechococcus* [117]. Indeed, the protein content for the *Synechococcus* enzyme was the lowest of all ADOs and correlated with low production levels of the evaluated alkanes (C3-C7). Further sequence analysis identified conserved amino acid substitutions located on the outer surface of the *Synechococcus* ADO enzyme (Paper III). These substitutions likely affect the quaternary structure and enhance *in vivo* enzyme degradation. Therefore, the lower alkane production levels could be explained by an inferior intracellular protein concentration rather than differences in kinetic parameters.

In terms of substrate affinity (K_M), the general pattern observed for all ADO variants indicated a higher preference towards longer carbon chain aldehydes and slower initial reaction rates for shorter substrates. Together with decreasing K_{cat} values [133], this pattern reflects the results of other studies which have demonstrated lower catalytic efficiency (K_M/K_{cat}) for short-carbon chain precursors. However, two exceptions were detected whereby heptane production was lower than that of shorter chain products. These exceptions were for the *Synechococcus* and *Synechocystis* ADO variants. *Synechococcus* demonstrated higher production capacities for both propane and pentane, and *Synechocystis* for pentane. Such exceptions indicate the potential for further development and optimization of these systems for the continuous production of volatile, short-chain hydrocarbons.

6. Concluding remarks

The use of cyanobacteria as photo-catalysts, where biomass is not consumed but instead the cells are used as a chassis for the expression of designed metabolic pathways, is an attractive approach for the production of volatile hydrocarbons directly from CO_2 and water. However, this approach is still at the basic research phase, with further development required to establish the economic feasibility of proposed photo-biological platforms. In this context, the work presented in this thesis advances the current knowledge of the microbial production of short chain, volatile alka(e)nes. These compounds are easily removed from cells due to their natural diffusion through the cell membranes and accumulation in the gas phase of the cultures, allowing thus, an immediate separation of the end-product from the biocatalytic process after synthesis.

In particular, the present thesis has contributed:

- An evaluation of EFE as a non-invasive reporter protein suitable for the further development of a cyanobacterial synthetic biology toolbox.
- An investigation into the performance of different promoters for EFE expression in cyanobacteria and cataloging of differences in promoter strength and regulatory capacities.
- A re-evaluation of the previously reported requirement of codon optimization of *efe* and the avoidance of associated mutational genetic elements for stable expression in cyanobacteria.
- The elucidation of the reason behind the reported genetic instability of *efe* in *Synechococcus sp.* PCC 7942.
- The development of stable *efe* strains in *Synechococcus*, avoiding chromosomal disruption of the *psbAI* gene.
- The development of an *E. coli* based *in vivo* method for the comparative evaluation of cADO homologs.
- The identification of ADO-specific characteristics affecting catalytic performance and substrate preference.

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