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Suvi Santala

Developing Synthetic Biology Tools and Model Chassis:
Production of Bioenergy and High-Value Molecules

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Abstract

One of the aims of synthetic biology is the sustainable production of high-value compounds and bioenergy molecules. Synthetic biologists exploit fundamental engineering principles, such as DNA component standardization, modular genetic circuits, and *de novo* design, to create novel production pathways and products. A well-characterized host cell serves as the chassis for the system construction; generally, the model bacterium *Escherichia coli* is applied. However, the metabolism and characteristics of *E. coli* are not ideal for all applications. Furthermore, many *E. coli* based systems are patent protected which restricts the use in forthcoming application.

Acinetobacter baylyi ADP1 is a potential alternative host for synthetic biology. The metabolism and genetics of the strain are well-understood, and the engineering of its genome is technically straight-forward. The versatile and unusual metabolic pathways, including those producing long chain hydrocarbons, can be rerouted, modified, and integrated into novel ones. I exploited *A. baylyi* ADP1 as a model host for the production of high-value hydrocarbons, triacylglycerols and wax esters. I employed metabolic engineering, novel molecular monitoring tools, and synthetic pathway design to improve the production, and to demonstrate the utility of ADP1 as a synthetic biology host. In particular, the production of triacylglycerols was improved over 5-folds by targeted gene deletions which resulted in redirected carbon flux towards the product and elimination of competitive pathways.

The long-chain hydrocarbon metabolism, including alcohol and wax ester biosynthesis, is not yet fully understood. These pathways are regulated through several mechanisms sensitive to specific environmental conditions and the cellular states. However, the lack of robust and straight-forward analysis tools has restricted the studies of lipid metabolism and production kinetics. I developed a simple *in vivo* tool for the investigation of the long chain hydrocarbon metabolism in real-time. The tool is based on a light-producing reporter enzyme, bacterial luciferase. The enzyme utilizes a specific intermediate of the hydrocarbon synthesis pathway as a substrate for bioluminescence production. Initially, the tool was applied for monitoring the wax ester metabolism of *A. baylyi* ADP1. Subsequently, I modified the monitoring tool for studying the degradation of alkanes. The studies suggest that the tool can be applied for production optimization in different hosts and for a variety of products. I also reconstructed the wax ester synthesis pathway of *A. baylyi* ADP1 by replacing a natural

key enzyme with an alternative well-characterized component, enabling a regulated production of unnatural wax esters.

Bioprocess control and scale-up of production systems are challenging. Multispecies cultures are suggested to improve the robustness and performance of bacterial production processes. I exploited the metabolic versatility of *A. baylyi* ADP1 to construct a rationally engineered synthetic coculture with *E. coli*. The designed coculture exhibited improved biomass and recombinant protein production compared to the pure culture of *E. coli*.

To conclude, I have shown that the strain ADP1 is a suitable host for synthetic biology applications, especially for long-chain hydrocarbon production, the development of novel tools for metabolic studies, and for exploiting the existing unusual metabolic networks of the cell. Thus, further studies of the remaining challenges related to ADP1 bioprocess and as-of-yet uncharacterized cell mechanisms, are warranted.

Tiivistelmä

Synteettinen biologia on tieteenala, joka yhdistää insinööritieteet, informaatioteknologian, ja molekulaarisen bioteknologian. Synteettisessä biologiassa hyödynnetään standardoituja biologisia elementtejä, kuten hyvin tunnettuja DNA-komponentteja ja niistä koostuvia geneettisiä piirejä, joiden avulla voidaan systemaattisesta suunnitella ja rakentaa biologisia laitteita. Hyvin tunnetut työkalut mahdollistavat tiedon nopean lisääntymisen ja alan nopean kehityksen. Tämän tieteenalan teknologioiden avulla voidaan tuottaa teollisuuden kannalta arvokkaita molekyylejä, kuten bioenergiakomponentteja ja uusia älykkäitä lääkkeitä. Biologiset laitteet rakennetaan eläviin isäntäsoluihin, jotka toimivat systeemin biologisena kehyksenä ja ovat siten myös toimivuuden kannalta merkittävässä asemassa.

Tunnetuin ja eniten käytetty isäntäsolu on *Escherichia coli* -bakteeri. Tämä bakteeri ei kuitenkaan ole ominaisuuksiensa puolesta optimaalisin vaihtoehto kaikkiin sovelluksiin, eikä sen aineenvaihdunta tarjoa mahdollisuutta tutkia kaikkia merkittäviä biokemiallisia reittejä. Aineenvaihduntareittien tuntemus on välttämätöntä, kun rakennetaan ja optimoidaan uusia tai muokattuja reittejä tärkeiden molekyylien tuottamiseksi.

Acinetobacter baylyi ADP1 -bakteerikanta on yksi potentiaalisista, vaihtoehtoisista isäntäsoluista synteettisen biologian sovelluksiin. Kyseisen bakteerin genomi ja metabolia tunnetaan hyvin, ja sen geneettinen muokkaus on helppoa ja suoraviivaista. Lisäksi solun aineenvaihdunta on erittäin mielenkiintoinen; kannan luontainen kyky tuottaa pitkäketjuisia hiilivetyjä, kuten biopolttoainetuotantoon soveltuvia triglyseridejä ja vahaestereitä, tarjoaa hedelmällisen lähtökohdan aineenvaihdunnan tutkimiseen ja muokkaamiseen.

Väitöskirjassani osoitan, että *A. baylyi* ADP1 -kantaa voidaan hyödyntää synteettisen biologian isäntäsoluna ja mallisysteeminä. Paransin tutkimuksessani bakteerikannan triglyseridituotantoa metaboliamuokkauksen keinoin: Aineenvaihdunnan mallintamisen perusteella identifioitiin geenejä, joiden poistaminen vaikuttaa suotuisasti triglyseridien tuottoon. Poistamalla tietty geeniyhdistelmä voitiin eliminoida solunsisäisiä kilpailevia reittejä ja ohjata hiilivuo kohti tuotetta. Muokkauksen tuloksena triglyseridituotanto parantui noin viisinkertaisesti.

Pitkäketjuisten hiilivetyjen aineenvaihduntareitit eivät ole vielä hyvin tunnettuja. Tämä johtuu osittain siitä, että tutkimukseen tarvittavia yksinkertaisia ja dynaamisia työkaluja

ja menetelmiä ei ole ollut saatavilla. Tutkimuksessani kehitin uuden molekulaarisen työkalun, jonka avulla pitkäketjuisten hiiliyhdisteiden tuottoa voidaan monitoroida solun sisällä reaaliaikaisesti. Työkalu perustuu bakteerilusiferaasientsyymiin, joka tunnistaa spesifisesti ko. aineenvaihduntareittien välituotteen, pitkäketjuisen aldehydin, ja reagoi sen kanssa tuottaen näkyvää mitattavaa valoa eli bioluminesenssia. Työkalun toimivuus osoitettiin tutkimalla *A. baylyi* ADP1 -kannan vahaesterimetaboliaa, mutta sitä voidaan soveltaa myös muihin organismeihin ja tuotteisiin. Hyödynsin työkalua myös mukauttamalla sen detektoimaan alkaaneja ja diesel-peräisiä yhdisteitä sekä näiden yhdisteiden hajotusta.

Rekonstruoin tutkimuksessani myös ADP1-kannan vahaesterituottoreitin: yksi reitin avainentsyymeistä korvattiin hyvin tunnetulla DNA-komponentilla, jota käytettiin täysin uudessa tarkoituksessa. Reitin uudelleensuunnittelun ja -rakentamisen tuloksena pystyttiin tuottamaan kontrolloidusti synteettisiä vahaestereitä, jotka eroavat ominaisuuksiltaan ADP1:n luonnollisista vahaestereistä.

Yksi synteettisen biologian haasteista on rakennettujen systeemien toimivuus ja stabiilius suuren mittakaavan prosesseissa. Prosessit, joihin osallistuu useita yhteistyössä toimivia bakteerikantoja, ovat mahdollisesti vakaampia, sillä oikeanlaiset populaatioyhdistelmät edistävät suotuisten olosuhteiden säilyttämistä ja prosessin suorituskykyä. Tutkimuksessani osoitan, että ADP1-kantaa voidaan hyödyntää myös täysin uudella tavalla *E. coli* -pohjaisissa yhteiskasvatuksissa; geneettisen muokkauksen tuloksena luotiin synteettinen, keinotekoisesti symbioottinen yhteiskasvatus, jossa biomassan ja rekombinanttisen proteiinin tuotto parani verrattuna *E. coli* -puhdasviljelmään.

Yhteenvetona totean, että *A. baylyi* ADP1 soveltuu synteettisen biologian isäntäorganismiksi erityisesti osa-alueilla, jotka liittyvät pitkäketjuisten hiiliyhdisteiden tuottamiseen sekä tutkimiseen ja jotka hyödyntävät solun omia aineenvaihduntareittejä. Kannan bioprosessin kehittäminen sekä toistaiseksi tuntemattomien mekanismien karakterisointi asettavat haasteita, mutta bakteerin moninaiset ominaisuudet ja potentiaali puoltavat sen jatkokehittämistä synteettisen biologian sovelluksiin.

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Nokia, December 2014

Sirpi Santala

Contents

List of Publications

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

I. Santala, S., Efimova, E., Kivinen, V., Larjo, A., Aho, T., Karp, M. & Santala, V. (2011) Improved Triacylglycerol Production in *Acinetobacter baylyi* ADP1 by Metabolic Engineering. *Microbial Cell Factories* **10**:36.

II. Santala, S., Efimova, E., Karp, M. & Santala, V. (2011) Real-Time Monitoring of Intracellular Wax Ester Metabolism. *Microbial Cell Factories* **10**:75.

III. Santala, S., Karp, M. & Santala, V. (2012) Monitoring Alkane Degradation by Single Biobrick Integration to an Optimal Cellular Framework. *ACS Synthetic Biology* **1**(2):60-4

IV. Santala, S., Efimova, E., Koskinen, P., Karp, M & Santala, V. (2014) Rewiring the wax ester production pathway of *Acinetobacter baylyi* ADP1. *ACS Synthetic Biology* **3** (3):145-51

V. Santala, S., Karp, M. & Santala, V. (2014) Rationally Engineered Synthetic Coculture for Improved Biomass and Product Formation. *PLoS ONE* 9(12): e113786.

The author (as S. Myllyntausta) has also contributed to the following patent as an inventor and an author:

Aho, T., Karp, M. Kivinen, V., Koskinen, P. Larjo, A. Myllyntausta, S. Santala, V. (2012) Patent US 20120151833 A1 Improvement of lipid production.

Author contribution

I. Suvi Santala wrote the paper and is the corresponding author. She planned and conducted the wet lab experiments and interpreted the results. V. Santala participated in designing the experiments and interpretation of the results. V. Kivinen, A. Larjo, and T. Aho performed the dry-lab experiments. E. Efimova contributed to lipid analytics.

II. Suvi Santala wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results. V. Santala participated in designing the experiments. E. Efimova contributed to lipid analytics.

III. Suvi Santala wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results. V. Santala participated in designing the experiments and interpretation of the results.

IV. Suvi Santala wrote the paper and is the corresponding author. She and V. Santala planned and conducted the experimental work and interpreted the results. E. Efimova and P. Koskinen conducted the lipid analytics.

V. Suvi Santala wrote the paper and is the corresponding author. She and V. Santala planned and conducted the experimental work and interpreted the results.

All the work was performed under the supervision of Prof. Matti Karp and Adj. Prof. Ville Santala.

Abbreviations

ACP	Acyl carrier protein
ADO	Aldehyde-deformylating oxygenase
ADP1	*Acinetobacter baylyi* ADP1
AHL	Acyl-homoserine lactone
CoA	Coenzyme A
CGP	Cyanophycin granule peptide
CDS	Coding sequence
CDW	Cell dry weight
cm	Chloramphenicol
EPS	Exopolysaccharide
FA	Fatty acid
FAEE	Fatty acid ethyl ester
FAld	Fatty aldehyde
FAME	Fatty acid methyl ester
FAR	Fatty acid reductase
FFA	Free fatty acid
FMN(H_2)	Flavinmononucleotide (reduced from)
GC-FID	Gas chromatography – Flame ionization detector
GC-MS	Gas chromatography – Mass spectrometer
GFP	Green fluorescent protein

HPLC-GPC	High-performance liquid chromatography - Gel permeation chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIS	In Vitro Imaging Station
kan	Kanamycin
MAGE	Multiplex Automated Genome Engineering
MCS	Multiple cloning site
NMR	Nuclear magnetic resonance spectroscopy
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NEB	New England Biolabs
Lux	Bacterial luciferase enzyme complex
OD_{600}	Optical density (at 600 nm wavelength)
RBS	Ribosome binding site
sp. (spp.)	species (pl.)
SPE	Solid phase extraction
TAG	Triacylglycerol
tet	Tetracycline
TLC	Thin layer chromatography
WE	Wax ester
WS/DGAT	acyl-CoA:fatty alcohol acyltransferase (wax ester synthase) / acyl-CoA:diacylglycerol acyltransferase

1 Introduction

Synthetic biology is a new emerging discipline combining life sciences, information technologies, and engineering. Synthetic biology seeks rational and sustainable solutions for improving human health, promoting energy self-sufficiency, producing important commodities, generating novel molecules and products with new features, and addressing environmental and agricultural issues. The most acute targets include reducing and preventing food shortage, developing novel drugs for complex diseases, and compensating for the depletion of fossil energy sources with green energy technologies.

The fundamental philosophy of synthetic biology lies in redesigning biology, applying standard engineering principles, methods, and organisms. This ideology redefines biological systems and particularly the organisms, emphasizing their properties in terms of design, programmability, and modularity, rather than according to the taxonomical or microbiological characteristics or status. The new engineering principles and methodologies of synthetic biology have led to a tremendous increase in complexity and novelty of biocompounds and pathways, compared to typical products obtained by means of conventional genetic engineering, such as single proteins or small metabolites. However, increased complexity requires more comprehensive design and computation. Instead of the extensive and consuming work of trial and error, synthetic biology aims at providing tailored and well-characterized working platforms for construction of newly designed cells performing determined tasks. In addition, robust amenable monitoring tools and functional cellular working platforms are required to fulfill the increasing demands of the designed biological systems.

This thesis reviews some of the most important technologies, engineering principles, achievements, and challenges of synthetic biology, the main focus being on prokaryotic systems. Chapter 2 gives a general overview of what synthetic biology is, whereas Chapters 3 and 4 focus on more specific research topics, bioenergy production and multicellular systems. As a reflection of the potential deficiencies and gaps of the field emerged by the current literature, Chapter 5 outlines the hypotheses and objectives of my study. Chapters 6 and 7 summarize the methods and results presented in the original papers I-V and discuss the research outcomes and future prospects in the context of the current state of synthetic biology research.

2 Synthetic biology revolution

The discovery of restriction enzymes in the 1970's gave birth to recombinant DNA technology and molecular cloning, ushering in a discipline of modern biotechnology. An early success in the field is the production of recombinant human insulin in engineered *Escherichia coli*. These new technologies led to dramatic development in engineering microbial cells for producing important commodities for pharmaceutical and chemical industries, such as novel drugs, vitamins, antibodies, and fine chemicals.

Roughly a decade later, the development of DNA sequencing techniques allowed the first complete genome sequence of an organism to be announced in 1995 (*Haemophilus influenzae*) (Fleischmann et al. 1995), followed by the first drafts of human genome in 2000 (Venter et al. 2001). The rise of 'scale-up' systems biology brought computer scientists and biologists together, expanding the possibilities to combine experimental and computational data (Westerhoff and Palsson 2004; Lanza et al. 2012). At the same time, the term synthetic biology became established (Endy 2005), emphasizing the urge for rational engineering, control, and programmability of newly designed cells; the traits lacking from conventional genetic engineering. During the past decade, the field and scope of synthetic biology has grown massively and made its breakthrough recognized largely by scientific communities as well as governmental and industrial players.

The idea of computational design and construction of regulatory circuits performing desired functions became one of the central concepts of synthetic biology. The first synthetic toggle switch (Figure 2.1) was constructed in 2000, performing two-state transcriptional regulation for expression of fluorescent protein (Gardner et al. 2000).

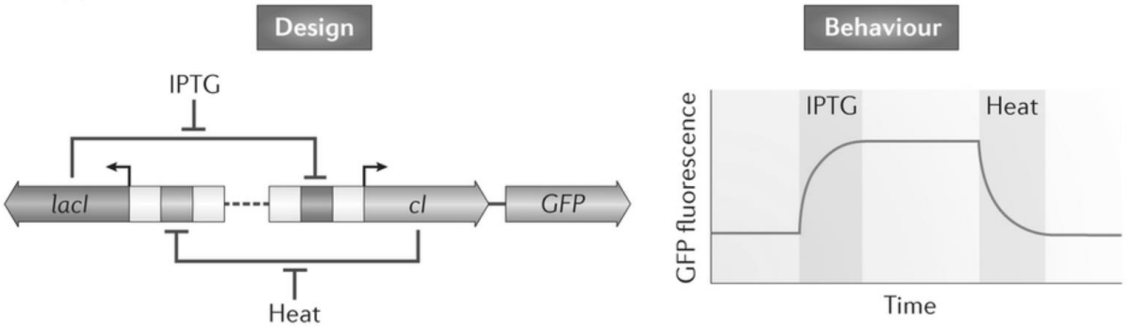

FIGURE 2.1. The toggle switch constructed for on/off gene expression via dual-repressor system. In the circuit, only one of the two repressor genes is active at a given time, resulting in a stable transcriptional state defined by environmental stimulus (IPTG, Heat). Modified from (Gardner et al. 2000).

Growing interest in engineered genetic switches analogous to electrical circuits has resulted in development of more sophisticated auto-regulatory feedback modules and oscillators, and devices displaying Boolean logic gate behavior (see chapter 2.1.1). The first circuits based on cell-cell communication were published shortly after, giving impulse to study and engineer synthetic microbial cocultures (Bulter et al. 2004).

An interdisciplinary community of synthetic biologists had rapidly evolved, consisting of molecular biologists, chemists, computer scientists, and engineers. The first official meeting for Synthetic Biology (SB1.0) was held in 2004 at the Massachusetts Institute of Technology (MIT), USA. The same year another notable event – soon becoming a tradition – iGEM (International Genetically Engineered Machine) competition took place (http://igem.org/). The rapidly developing techniques produced a tremendous amount of new data, which soon led to an open-access philosophy within the community. As an example of the communal approach, The Registry of Standard Biological Parts was established in 2003, providing standard DNA components and devices for academic researchers free of charge (http://parts.igem.org/). At the moment, more than 15 000 parts are registered.

Whole-genome engineering was taken to the next level when a complete genome of *Mycoplasma genitalium* was synthesized by scientists of J. Craig Venter Institute (Gibson et al. 2008). Subsequently, Venter and colleagues created a viable synthetic cell with artificial genome of a size 1.1 Mbp, exploiting chemical synthesis and novel DNA assembly techniques (Gibson et al. 2010). After the first decade of the millennium, the scientists had taken the first steps toward the ultimate goal, a completely programmable cell with desired functions and characteristics.

2.1 Engineering principles

Synthetic biology is all about design, rationalizing the complexity of natural systems by applying the key concepts of engineering. The main principles of synthetic biology involve standardization, specification, compatibility, modularity, and simplicity. Systematic design is described as a continuous cycle including a computer aided modeling, the implementation of the biological system, and testing and validation, finally leading to detailed specifications of the system (Baldwin et al. 2012). Standardized biocomponents can be assembled to create synthetic devices performing defined functions and devices comprise larger systems conducting complex tasks (Figure 2.2).

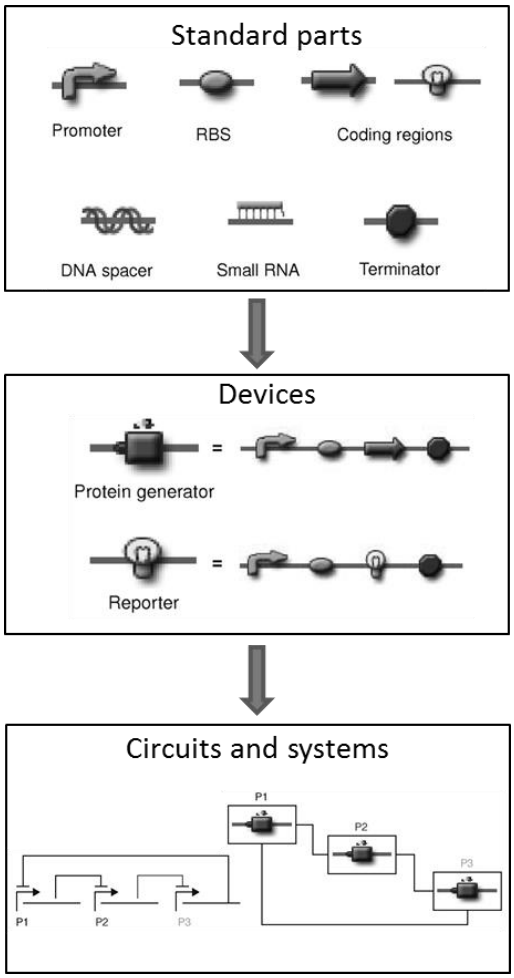

FIGURE 2.2. The hierarchy of creating synthetic biology circuits and systems from standard parts. Modified from (Marchisio and Stelling 2009)

2.1.1 Standard parts

Bioparts are pieces of DNA encoding designed biological functions. The first approach to facilitate a straight-forward engineering of biological systems was the creation of the BioBrick standard (Shetty et al. 2008). The well-characterized and compatible DNA components comprise a vast collection of a variety of reporters, enzyme coding genes, regulatory elements, degradation tags, multienzyme complexes, and ready-made pathways to ease the cellular engineering. The package also includes tailored protocols for BioBrick^{TM} cloning, based on standard vectors and restriction sites. The number of deposited individual bioparts, or ”DNA components”, along with committed laboratories increase continuously. The Registry of Standard Parts serves as a reservoir for the defined DNA components, enabling the construction of genetic devices and systems of increasing complexity (iGEM.org).

For part standardization, a comprehensive characterization with defined system specifications is carried out to produce a technical ‘datasheet’. The datasheet contains details such as the part number, static performance, a dynamic response, the used chassis, part compatibility, and reliability (Figure 2.3). The datasheet provides a general description and summary of characteristics of the part or device, enabling a straight-forward reuse of the component. (Canton et al. 2008) However, as a time-consuming protocol, the *in vivo* part characterization remains a bottleneck in rational and predictable engineering. An alternative part standardization approach has been introduced, completely based on *in vitro* characterization of the DNA regulatory elements exploiting *E. coli* cell-free extract (Chappell et al. 2013).

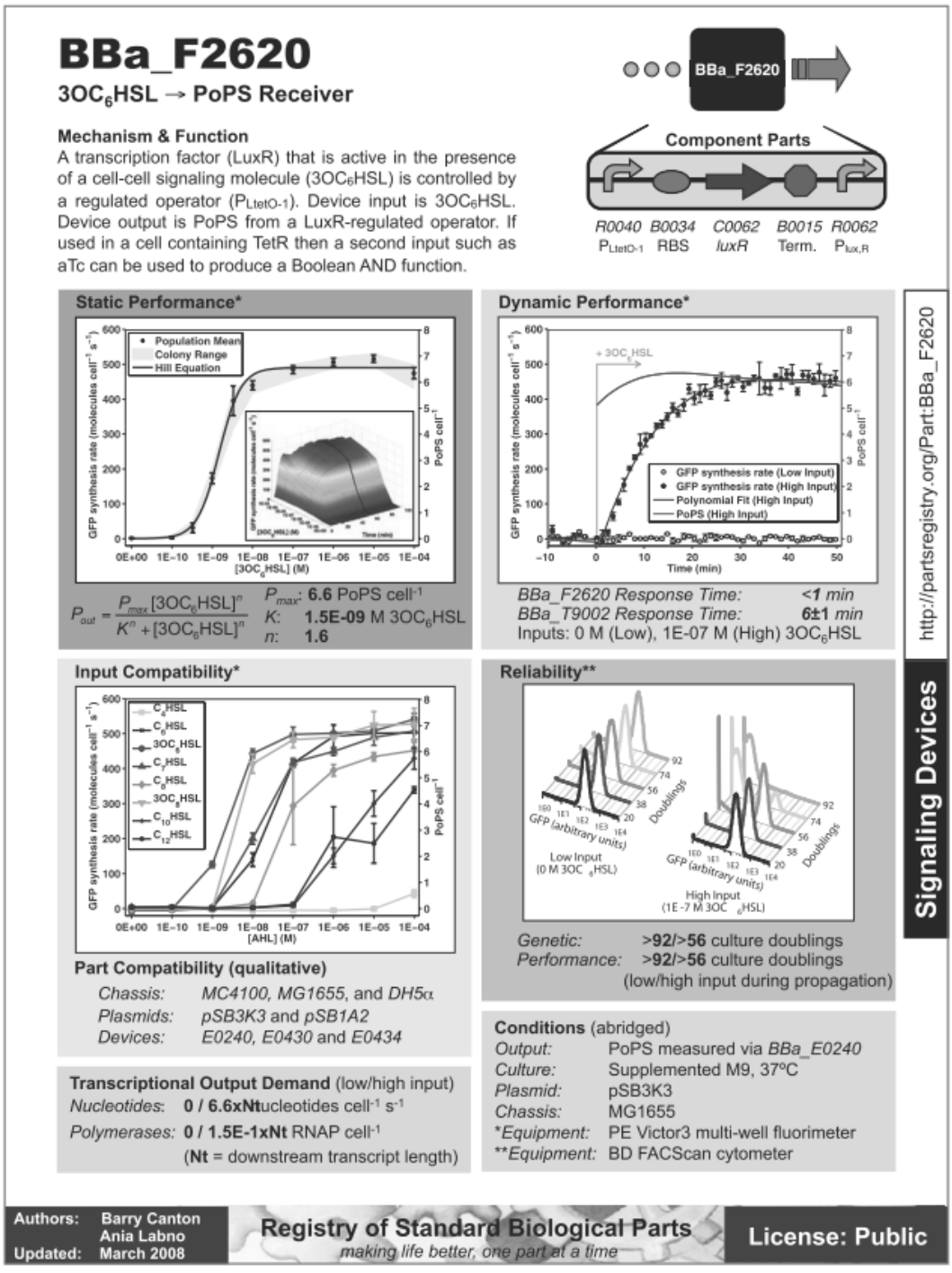

FIGURE 2.3. An example of a technical datasheet for a standard biological part (Canton et al. 2008). Open access.

Despite the attempts to control and instruct the part characterization, the concept of Canton et al. seem idealistic; the open access policy and the vast number of different

depositors have resulted in significant fluctuations in the quality and functionality of the deposited parts and devices in the registry. In addition, predicting the compatibility of different parts and especially the part function in different conditions and hosts has turned out to be very problematic.

Recent advances in *de novo* DNA synthesis technologies have enabled a precise design and realization of modified and optimized genes for reasonable prices. Synthetically tailored genes are especially convenient for protein engineering (Fowler et al. 2010; Kim et al. 2013), orthogonal gene expression systems (Rhodius et al. 2013), and customized pathways with synthetic control elements (Temme et al. 2012). Another approach to create new DNA components is 'part mining' using metagenomic libraries as source for the resynthesis of novel bioparts (Stanton et al. 2014). Altogether, if synthetic biologists seek standard parts with maximal orthogonality and predictability, *de novo* designed and synthesized bioparts may be the only practical way to increase the reliability of the part-based systems.

2.1.2 Synthetic gene circuits

Synthetic genetic circuits are functional entities performing defined tasks (Sprinzak and Elowitz 2005; Brophy and Voigt 2014). Circuit design is preferably assisted by computational tools (Clancy and Voigt 2010; MacDonald et al. 2011; Rodrigo and Jaramillo 2013) and well-characterized parts serve as building blocks for circuit modules (Weiss et al. 2003; Voigt 2006; Mutalik et al. 2013). The increasing complexity of bottom-up engineered gene networks requires a rational approach to design and predict the circuit behavior (Mukherji and van Oudenaarden 2009).

Synthetic regulation is essential, since many natural genes and gene clusters are silent unless induced by a specific molecule or conditions that can be inconvenient or unknown (Frasch et al. 2013). Circuits can be regulated at either transcriptional or post-transcriptional level. In digital transcriptional circuits, input and output promoters define the expression state to be simply either ON or OFF, and the circuit performance can be monitored using reporters such as fluorescent proteins (Wang et al. 2011). Digital circuits can be built based on logic gates with AND, NAND, OR, NOT, or NOR gates according to Boolean logic (Figure 2.4). In principle, Boolean logic gates consist of two or more input signals and return a single output, namely "true" or "false". Dynamic circuits, such as oscillators, are more difficult to screen and monitor, and thus mainly proof-of-principle systems have been described (Elowitz and Leibler 2000; Stricker et al. 2008). Promoter architectures acting as circuit regulators typically involve DNA binding proteins such as LacI, LuxR, TetR or AraC or combinatorial approaches

exploiting them (Cox et al. 2007), but also RNA molecules (Lucks et al. 2011), metabolites or even changes in environmental stimulus (Levskaya et al. 2005; Tabor et al. 2011) can serve as transcription regulators.

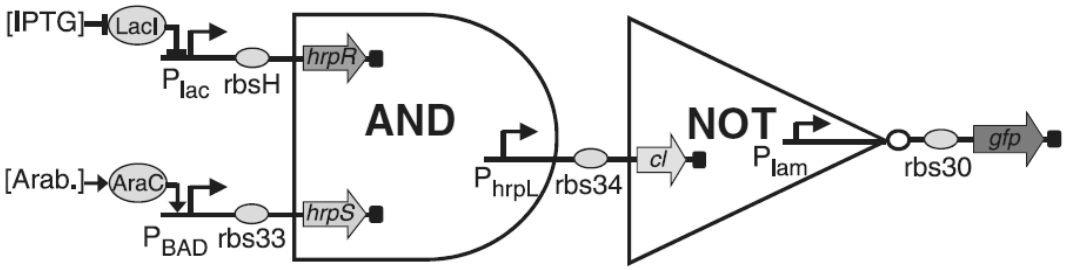

FIGURE 2.4. An example of an orthogonal logic NAND gate constructed in *E. coli*. Reporter protein (GFP) production is ON unless both external signals (IPTG and Arab.) are given. The dynamic range can be fine-tuned using modified RBS (rbs34, rbs30). Modified from (Wang et al. 2011).

Post-transcriptional circuits typically involve interactions between non-coding RNAs and DNAs, proteins, or small molecules (Isaacs et al. 2004). RNAs are naturally modular multifunctional molecules possessing unique sequence-specific characteristics at both structural and functional levels, thus serving as a useful platform for the design and evolution of novel type of regulatory, control, and sensor devices (Liang et al. 2011; Isaacs 2012; Mutalik et al. 2012).

Regulatory devices functioning through protein-protein interactions and allosteric regulatory systems enable direct and dynamic spatio-temporal regulation of a protein function in cells (Grunberg and Serrano 2010; Olson and Tabor 2012). Post-transcriptional regulation potentially puts less stress and burden on cells, which can be crucial in larger circuit designs.

Genetic circuits hold huge potential for future applications in the fields of biomedicine and biotechnology (Lu et al. 2009). Ideally, circuits could be used for programming cells displaying precisely timed regulatory systems sensitive to specific signals, molecules, or environmental changes. Connected circuits constitute larger genetic programs, and the most complex recently reported circuits have involved up to 11 regulatory proteins and 38 additional genetic parts (Moon et al. 2012). However, the described synthetic systems are still limited in complexity compared to natural systems. In order to build up more complex circuits with broader dynamical range several major challenges must be overcome. For example, more efficient and precise design tools must be developed for obtaining correctly balanced systems. In addition, more robust monitoring tools with a wider range of suitable reporters are required to screen for circuits with optimized

performance. Also, a better understanding about factors affecting the performance of a circuit and individual components within the context is required, and advances in technologies for building up larger circuits involving several devices and components are needed (Brophy and Voigt 2014). Moreover, even well-designed and tuned circuits often suffer from instability and loss-of-function in long term use (Sleight et al. 2010a).

Genetic circuits are typically very sensitive to the cellular and environmental context. Cross-talk between exogenous and endogenous cellular systems can decrease the predictability and robustness of circuits and individual parts in cells (Cardinale and Arkin 2012). Thus orthogonal, i.e. isolated expression systems uncoupled from cellular regulation are generally a more preferable approach. Orthogonal expression can be defined either at cellular level as a host independent expression system diminishing any interaction between exogenous and endogenous reactions, or at circuit level, implicating an independent transcriptional regulation of different gates, devices, or modules in parallel. For example, an orthogonal gene expression pathway in *E. coli* based on specific transcription-translation machinery recognizing only defined sequences in DNA and mRNA was previously introduced (An and Chin 2009). Several other tools for orthogonal regulation have been also developed and introduced (Rao 2012). For complex circuits, however, the number of well-known uncorrelated transcription factors is currently insufficiently low, limiting the circuit size. Part mining (Stanton et al. 2014), design and construction of novel regulatory elements, and evolution of existing transcription factors (Kamionka et al. 2004) are applied for facilitating the construction of orthogonal circuits consisting of a large number of elements.

During the last decade, a wide-ranging set of different circuit designs were introduced. However, fundamental limitations still exist, thus preventing the final breakthrough and full-fledged exploitation of the synthetic programs. For example, constructing a functional and predictable circuit is still largely conducted by trial and error, which in practice means the screening of tens, hundreds, or even thousands of differentially constructed circuit candidates. The screening is dependent on convenient assay methods or sophisticated flow cytometry instrumentation exhibiting high-throughput cell sorting, as for partly limiting the circuit range and function. Moreover, the current systems often suffer from “a proof-of-principle syndrome”; the scale-up of circuits is still insufficient as the circuits operate correctly only at optimized conditions and in a defined cell environment. Other problems restricting the circuit robustness include a potential toxicity to cells, metabolic loading, inaccurate modeling, and lack of analysis and design tools.

A representative example of the challenges in circuit design is the rebuilding of the nitrogen fixation gene cluster in *Klebsiella oxytoca* (Temme et al. 2012). The cluster containing 20 genes in seven different operons was “refactored”. In the process, all the known and hidden natural regulatory elements, noncoding DNAs, and nonessential genes were removed. The genes were reorganized into new operons that function under the regulation of synthetic elements. The resulting synthetic cluster contained 89 individual genetic parts. The maximal nitrogenase activity exhibited by the refactored system was approximately 7 % of that of the wild type system, and only 2 % when expressed in a non-native host, namely *E. coli* (Temme et al. 2012). More previously, the modularity of the system was exploited in creating genetic permutations to further investigate and optimize the cluster functionality (Smanski et al. 2014). More than a hundred different variants of each operon were combinatiorally assembled and analysed, and the information was applied in further design cycles. Eventually, a nitrogenase activity of 57 % of the wild type system in *K. oxytoca* could be achieved. This variant recovered 7 % activity in *E. coli*, whereas a variant specifically optimized for *E. coli* yielded nearly 20 % activity. The study demonstrates the complexity of redesigning highly evolved natural systems and the difficulty of maintaining and determining the functionality of corresponding synthetic systems, especially if non-native hosts are used. Nevertheless, only two hosts were tested in the described study; thus it would be very interesting to investigate, how the activity range of the original refactored design would have changed in a broader range of different cellular environments. In another words, could choosing the “right” host in some cases compensate for the heavy optimization process?

In opposite to building up circuits from scratch, integrated circuits directly exploit the host machinery and metabolism to carry out the functions (Nandagopal and Elowitz 2011). Integration can occur at different levels from partially autonomous synthetic circuits to rewired or completely integrated pathways (Figure 2.5). Integrated synthetic circuits can improve functionality, allow more complex design, and broaden the usability of single bioparts in new contexts.

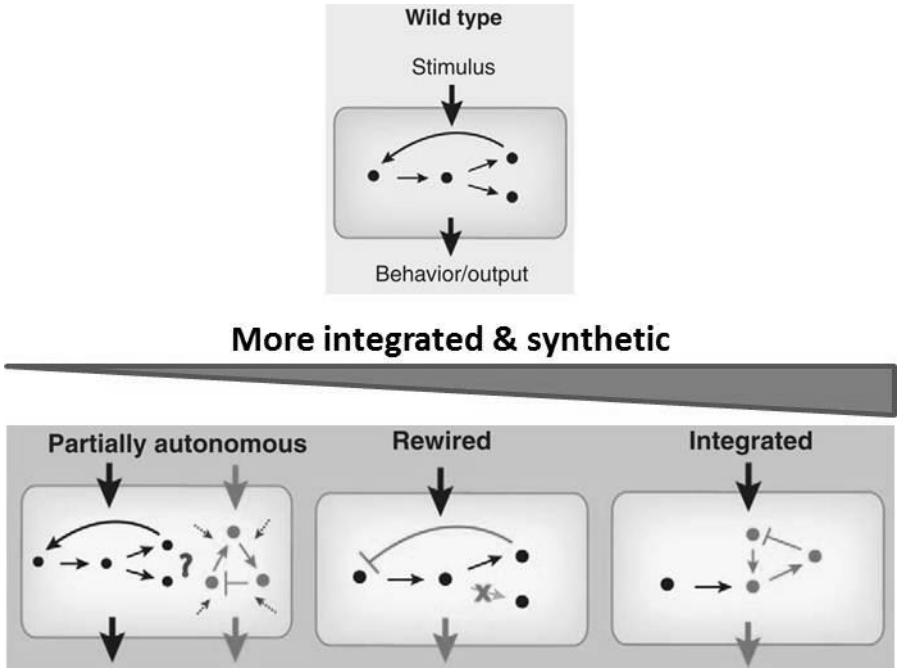

FIGURE 2.5. Integration of synthetic pathways to cellular environment. Modified from (Nandagopal and Elowitz 2011).

2.1.3 Overview of recent DNA assembly and genome engineering methods

An increasing number of novel methods for a rapid, reliable, and simple assembly of DNA components, and comprehensive genome engineering were introduced during the past decade. A dramatic drop in *de novo* DNA synthesis prices has changed the focus of molecular cloning from DNA restriction/ligation based protocols towards a more comprehensive design of seamless gene cassettes, complete pathways and even genomes. Figure 2.6 presents the frequency of use of recent DNA assembly methods in the field of synthetic biology in 2013.

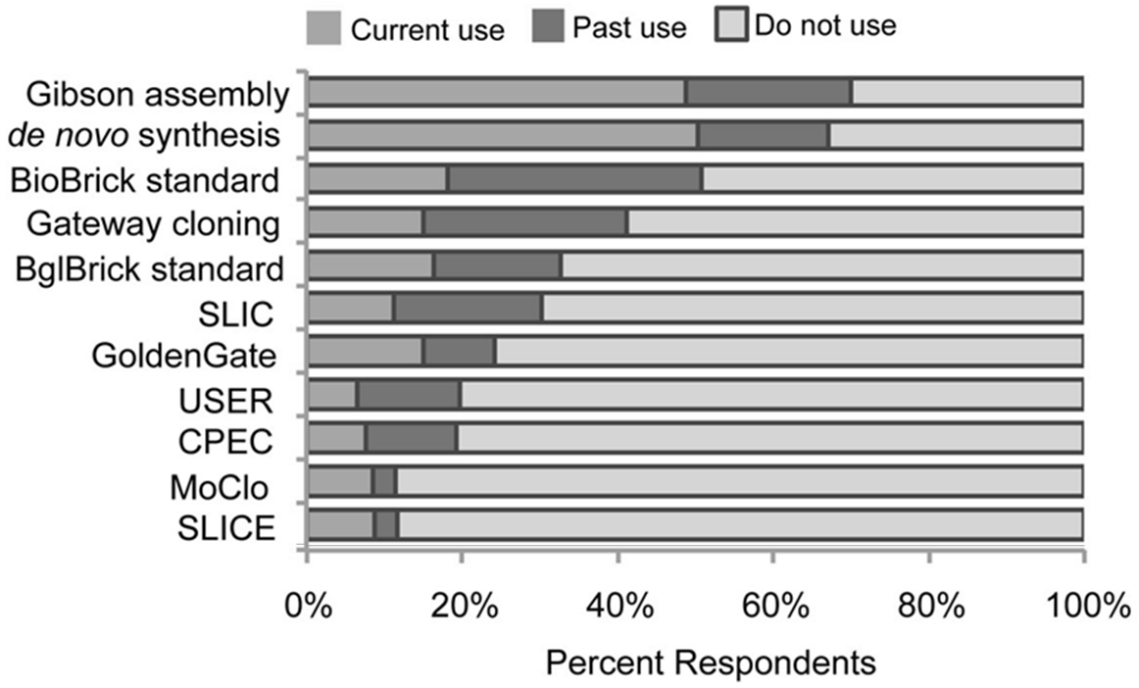

FIGURE 2.6. The frequency of use of recent DNA assembly techniques in 2013. Modified from (Kahl and Endy 2013).

Even though not being the most modern and convenient method for a rapid assembly of standard parts, the BioBrick™ cloning is still widely in use and can be granted as a forerunner to the upcoming approaches. It is based on specific restriction sites (namely *EcoR*I-*Not*I-*Xba*I-(-)-*Spe*I-*Not*I-*Pst*I) present in all standard vectors and BioBricks, enabling a sequential addition of several parts to the same vector. As an advantage, the method does not require DNA amplification or design/use of oligonucleotides when available parts and vectors are exploited. However, for practical reasons the size and complexity of the insertion is quite limited, and the step-by-step addition of parts is time-consuming, and thus not significantly different from conventional molecular cloning.

Probably the most revolutionary and today the most widely used DNA assembly method, Gibson assembly, was introduced in 2009 (Gibson et al. 2009). The method is based on overlapping sequencing in amplified target DNA fragments which are joined together by T5 exonuclease, DNA polymerase, and heat-labile ligase in a one-step isothermal reaction (Figure 2.7). The method requires specific synthesized oligonucleotides (or genes) for each insert fragment. This fast and straight-forward method is especially convenient for cloning several components simultaneously and for very large DNA fragments.

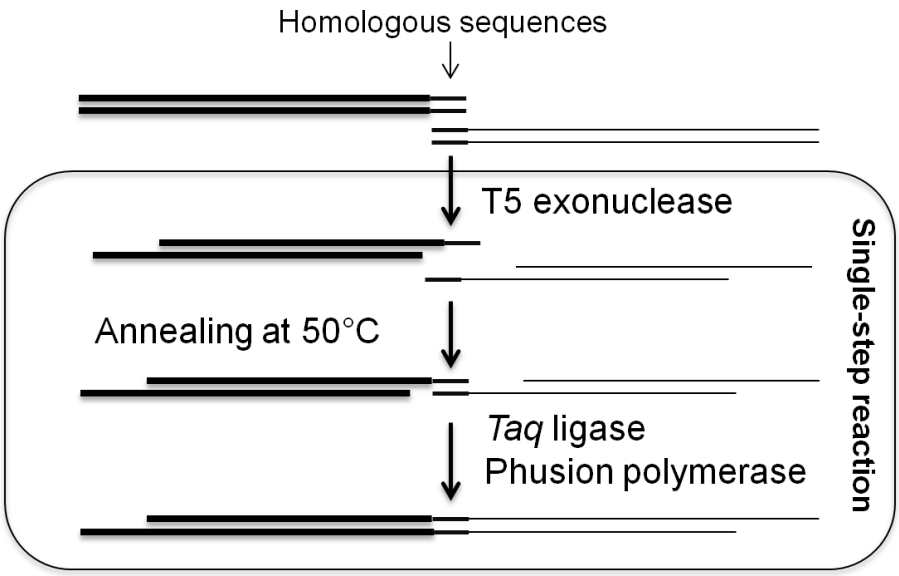

FIGURE 2.7.The Gibson Assembly method. The one-step reaction is carried out isothermally in 50 C exploiting a 5' exonuclease, a DNA polymerase and a DNA ligase (here; T5 exonuclease, Phusion polymerase and *Taq* ligase, respectively). Target DNA fragments (synthesized or amplified by PCR) share an overlapping sequences that are treated with the exonuclease to create overhangs in the 5' ends. Once the complementary 3' overhangs anneal, the DNA polymerase and ligase fill and seal the gap, while the heat-labile exonuclease becomes inactivated.

Other notable *in vitro* DNA assembly methods include In-Fusion (Clontech) (Sleight et al. 2010b), SLIC (Sequence and Ligation Independent Cloning) (Li and Elledge 2007), CPEC (Circular Polymerase Extension Cloning) (Quan and Tian 2014), GoldenGate (Engler and Marillonnet 2013), and USER (Uracil-Specific Excision Reagent; NEB) (Nour-Eldin et al. 2010). Recently, a biotechnology company DNA2.0 introduced a new promising method, Electra Vector System IP-free® cloning (http://www.prweb.com/releases/2013/6/prweb10802605.htm). The developers promise "a simple, PCR-free, one-tube universal cloning process that can be performed in a five-minute bench-top reaction with the fidelity of a restriction-based cloning system". The method is based on a commercial reaction mixture and standard vectors. Most importantly, the use of the method is not restricted by intellectual property issues, enabling the utilization of the method also in industrial and commercial applications without a license.

In general, *in vitro* assembly methods are faster, more stable, and easier to use compared to *in vivo* methods. At the moment, the bottleneck of *in vitro* methods is the amplification step, which is more prone to errors than cellular replication, and not

generally suitable for amplification of fragments as large as genome-size. For example, by exploiting the efficient DNA uptake and recombination machinery of *S. cerevisiae* whole genomes (to date up to 1.8 Mb (Karas et al. 2013)) can be constructed and amplified in the yeast cell (Gibson 2011; Benders 2012).

In addition, efficient tools for whole-genome engineering have been developed. MAGE (Multiplex Automated Genome Engineering) is an *in vivo* method for editing and evolving the host genome (Wang et al. 2009), thus far mostly applied in *E. coli*. By MAGE, broad sequence diversity can be generated at many targeted genome locations in a large population of cells at high efficiency. Modifications in the genome are achieved by repeatedly introducing the designed fragments of synthetic DNA (oligos) targeted at the lagging strand of the replication fork in DNA replication, thus resulting in allelic replacement. The recombination is mediated by a bacteriophage λ-Red ssDNA-binding protein β. The technologies have enabled the introduction of "genome rewriting", demonstrated recently in *E. coli:* all the stop codons TAG were replaced with TAA, giving insights to possibilities for expanded biological functions, protein diversity, and viral resistance in genetically recoded organisms (Lajoie et al. 2013). Recently, a derived MAGE method, yeast oligo-mediated genome engineering (YOGE), has been introduced to *S. cerevisiae* (DiCarlo et al. 2013). For higher organisms, a revolutionary CRISPR-Cas9 (Cong et al. 2013) system based on a natural immune response to short RNAs has proven its power, and holds potential for future gene/genome therapeutics.

2.1.4 Synthetic biology and metabolic engineering

One major approach to realize synthetic biology is metabolic engineering. The more specific goal of metabolic engineering is to develop methods for designing, analyzing, and optimizing metabolic networks, typically with the objective of finding targets for engineering the cell factories (Bailey 1991; Nielsen et al. 2014). The directed and specific modifications of metabolic pathways are introduced to cells for an improved synthesis of products. Improving the host cell can involve strategies for broadening the substrate range, improving product/substrate tolerance, improving productivity or yield, or accelerating the cell growth rate. The systems biology driven approach exploits the computational analysis of metabolic models and simulations to calculate and redirect fluxes within the cell. To date, probably the most notable achievement in the field of metabolic engineering is the reconstruction of a synthesis pathway for the production of an anti-malaria drug precursor (Martin et al. 2003). As a result of years of optimization, the process was further developed for the commercial production of Artemisinin in metabolically engineered yeast (Paddon et al. 2013).

Metabolic engineering includes a comprehensive engineering of the essential pathways for converting the substrates to products. Foundational elements encompass the determination of pathway fluxes of both synthetic and native routes, genome-scale modeling for identifying optimal gene expression profiles and gene modulation targets, as well as the kinetic and thermodynamic analysis of pathways for identification of bottlenecks (Stephanopoulos 2012).

While metabolic engineering is concentrated on manipulating and combining natural biochemical pathways, synthetic biology aims at reprogramming cellular behavior and creating advanced modular systems for novel products as of yet nonexistent in nature. However, synthetic biology and metabolic engineering are highly synergistic disciplines, as presented in Figure 2.8, and on the edge of comprising a comprehensive toolbox with efficient methodologies, tools, and intellectual scientific information.

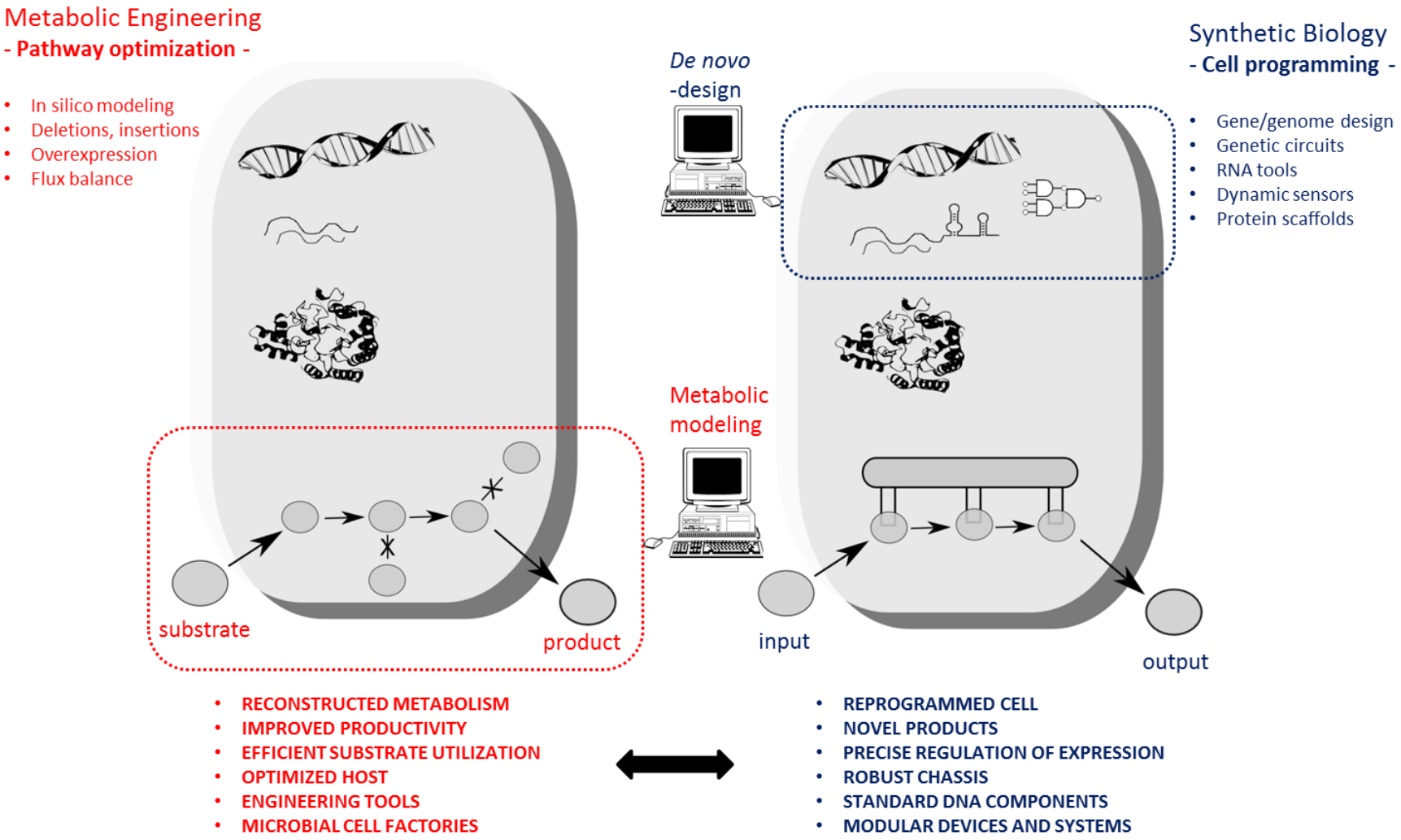

FIGURE 2.8. The interface of metabolic engineering and synthetic biology.

2.2 Chassis

2.2.1 Well-defined organisms as cellular frameworks

In order to obtain an unambiguous response of a standardized component, and to understand the function of natural and non-natural circuits in a cellular environment, a host cell in synthetic biology must provide a specified, tractable, predictable, and well-defined working platform. Basically, the host cell, i.e. chassis, serves as a framework for the installation of man-made biological devices. However, even though the number of complex program designs is increasing, only limited information is available for defining the chassis.

Designing the genome, genes, and the integration of components into the host are crucial elements in the generation of functional and comprehensive biological systems. However, even the simplest natural pathways comprise a network of thousands of interactions at both transcriptional and post-translational levels. Thus orthogonal expression, as discussed in Chapter 2.1, is one of the major challenges in maintaining the fabricated system analogous to the original design. Whole-genome engineering and streamlining of the host, briefly discussed in the next subchapter, increase the level of orthogonality and thus the predictability of non-native cellular processes. On the other hand, the chassis can serve as a fruitful platform for constructing complex pathways with less effort of fabrication, and an intentional integration of non-natural components to the host metabolism can broaden the possibilities to exploit individual parts in novel ways.

2.2.2 From minimal genomes to synthetic cells

Minimal genomes help us better understand and predict cellular systems. The fundamental problem behind the construction of ultimately reduced genomes lies in the definition of ‘minimal genome’, which inevitably is specified by the environmental conditions, defined level of cell functionality and fitness, and the ability to perform specific tasks. The most notable attempts to establish minimalistic cells by a top-down approach include the engineering of *M. genitalium* (Glass et al. 2006) and *E. coli* (Posfai et al. 2006; Hirokawa et al. 2013) genomes.

Nowadays, genome reduction, or synthetic genomics, is more considered as a tool for an increased functionality of a cell rather than aiming at as a small genome as possible. As one of the goals of synthetic biology is to increase the level of robustness

and functionality of a host cell, genome reduction is seen as an iterative method of finding the optimal set of essential genes to facilitate the desired functions of a programmed cell (Danchin 2012; Leprince et al. 2012b). Streamlining the host genome reduces the unnecessary or counterproductive reactions, and simplifies the interactions between cellular components. Genome reduction thus promotes the redesign of an optimal chassis enabling 'plug-and-play' engineering and takes a step closer towards an ultimately synthetic and artificial cell.

At present, the design principles consider maintaining cellular properties such as fitness near to the one of the wild type strains, and more stable, flexible, and evolvable production platform with less redundancy. More specifically, the genome streamlining is concentrated on removing introns (in eukaryotes), tRNA genes, regulatory elements, transposons, and DNA repeat sequences. Novel genome engineering tools, such as MAGE and inducible evolution system SCRaMbLE (synthetic chromosome rearrangement and modification by loxP-mediated evolution) are employed (Dymond et al. 2011). An international on-going project called Sc2.0 is currently working on building up the first synthetic yeast genome by a bottom-up approach (Annaluru et al. 2014) (http://syntheticyeast.org/sc2-0/). The project aims at increasing fundamental knowledge on for example chromosome properties, genome structure and organization, the function of RNA splicing and small RNAs, and distinction between prokaryotes and eukaryotes. Furthermore, the resulting 'synthetic yeast' would possess unlimited possibilities for practical use in the field of synthetic biology. Advances in genome design and construction will allow us to fabricate minimal cells that can serve both as high-capacity test-beds for fundamental genomic studies and as a chassis for the installation of programmed circuits.

2.2.3 Alternative hosts for synthetic biology

The choice for an optimal chassis is dependent on multiple factors. Straight-forward genome engineering and efficient regulatory structure are evident requirements for a cell platform, but also other biophysical characteristics, such as metabolic resources, exploitable pathways, and robustness in challenging bioprocesses are essential (Foley and Shuler 2010; Fisher et al. 2014).

The most conventional work-horse of all time is beyond dispute *E. coli*, exploited both as a model strain for prokaryotic systems and in commercial applications for production of a variety of important biocompounds such as recombinant proteins (Huang et al. 2012), commodity chemicals (Yim et al. 2011; Chen et al. 2013), and drug molecules (Martin et al. 2003). The cumulative and comprehensive knowledge regarding *E. coli*

genome (Blattner et al. 1997; Baba et al. 2006) among other –ome levels information (Han and Lee 2006; Ishii et al. 2007) and bioprocess technologies (Lee 1996) has ensured the status as a cellular framework also for synthetic biology.

E. coli serves as a convenient host platform, but as the scope of synthetic biology continuously expanding, domestication of other potential bacteria could provide certain advantages with regard e.g. to broader metabolic landscape, catalytic activity, and tolerance to chemicals and products. To cite an article of Nikel et al. (2014): “is this organism [*E. coli*] really the only bacterium that can be used in both fundamental synthetic biology and applied biotechnology?” To explore this, the following subchapters introduce some alternative bacterial hosts and describe their most important characteristics in terms of synthetic biology, the main focus being on *Acinetobacter baylyi* ADP1. For comparison with *E. coli*, some key features of the host candidates are collected in Table 2.1.

TABLE 2.1. Comparison between the model hosts *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas putida* , and *Acinetobacter baylyi* ADP1.

	***E. coli* (K12)**	***B. subtilis* (168)**	***P. putida* (KT2440)**	***A. baylyi* (ADP1)**
Natural environment	gastrointestinal tracts, water env.	soil, water env., plant rhizosphere gastrointestinal tracts	soil, plant rhizosphere	soil, water environment, human skin
Genome size	4.6 Mbp, 4288 CDS	4.2 Mbp, 4100 CDS	6.2 Mbp, 5420 CDS	3.6 Mbp, 3325 CDS
Genomic complexity	Lot of repeat sequences, genes are scattered all over the genome	25 % duplicate genes	Lot of repeat sequences, genes scattered	no repeats, genes oriented as clusters and 'catabolic islands'
Metabolic model	available	available	available	available
Genes; reactions	1445; 2286	1103; 1437	900; 1071	774; 875
Databases	EcoliWiki, EcoCyc	SubtiWiki, BsubCyc	Pseudomonas Genome Database	AcinetoScope (in MicroScope)
Generation time[a]	40 min.	95 min.	100 min.	35 min.
Temperature range for efficient growth	30-38 °C	25-35 °C	18-30 °C	20-38 °C
Substrate utilization				
Substrate range	narrow; simple sugars	wide; simple and complex carbohydrates, peptides	wide; sugars, organic acids, arom. comp., long chain hydrocarbons, alcohols etc.	wide; sugars, organic acids, arom. comp. long chain hydrocarbons, alcohols etc.
typical aer./anaer. byproducts	CO_2 / acetate	CO_2 / lactate, acetate	CO_2 / -	CO_2 / -
Natural products	ethanol, hydrogen	antimicr. compounds, 2,3-butanediol	polyhydroxyalkanoates, antimicr. comp., biosurfactants	Triacylglycerols, wax esters, cyanophysin, biosurfactants
Pathogenicity to humans	wild type strains	none; potentially probiotic	none	none
Generally regarded as safe (GRAS)	approved	approved	approved	N/A
Antibiotic sensitivity[b]	sensitive to common antibiotics	sensitive to common antibiotics	limited sensitivity	sensitive to common antibiotics
Genetic tools	widely available	available	available	available
Promoters	e.g. T5, T7, Lac, tet, BAD	e.g. T5, T7, Lac, tet, BAD (as in *E. coli*)	Lac, tet, BAD	T5, T7, Lac, BAD
Transformability	electroporation, calcium chloride treat.	natural competence, electroporation	electroporation	natural competence, electroporation
Tolerance to toxic compounds[c]	weak	good	very good	good
Foundational research	extensive	extensive	well established	well established in defined fields
Existing applications, e.g.	numerous[1,3]	numerous[1]	several[2,3]	few[3]
Commercial availability regarding patents	heavily patented, a true issue	widely patented	patents exist	very few patents, not an issue

[a]Minimal medium, glucose or succinate (ADP1) as a sole carbon source

[b] e.g. ampicillin, tetracycline, kanamycin, chloramphenicol

[c] e.g. aromatic compounds, solvents, halogens, heavy metals, hydrocarbons, alcohols

[1] recombinant protein production

[2] bioremediation

[3] biosensors

2.2.3.1 Bacillus subtilis

Bacillus subtilis is the best characterized and the most widely exploited host of Gram-positive bacteria (Dubnau 1982). The bacterium is both utilized as a model host for fundamental research and a work horse in biotechnological processes. Due to its superior bioprocess characteristics, that is, the lack of toxic byproducts, high production yields (up to 20-25 g/l), and the facility for efficient secretion of the products, *B. subtilis* has been broadly utilized in the production of recombinant proteins, antibiotics, and vitamins (Hao et al. 2013; van Dijl and Hecker 2013).

Being a facultative aerobe and a biofilm and spore-forming bacterium, *B. subtilis* can resist harsh environmental stress and nutrient deprivation for long periods. Moreover, the bacteria possess a complex motility and chemotaxis system. The bacteria can also produce a variety of secondary metabolites including fungal and bacterial inhibitors, providing a competitive advantage in natural environments (Stein 2005). *B. subtilis* can utilize a variety of carbohydrates and peptides as a carbon source and is capable of nitrate assimilation. The widely used laboratory strain *B. subtilis* 168 is auxotrophic for tryptophan.

B. subtilis 168 has been long exploited in molecular genetic, proteomic and biofilm studies (Lemon et al. 2008; Becher et al. 2011; Commichau et al. 2013). The genome of the strain was sequenced in 1997 (Kunst et al. 1997), followed by a construction and more recently enhanced metabolic model (Henry et al. 2009). In addition, a comprehensive database for *B. subtilis* genomic and metabolic information has been recently established (Michna et al. 2014).

The laboratory strain can be induced for natural competence (Hamoen et al. 2003), which promotes genetic engineering. Genome engineering tools (Kumpfmuller et al. 2013) and expression vectors (Nguyen et al. 2005), some being BioBrick compatible (Radeck et al. 2013), are widely available for the strain. Furthermore, a genome reduction approach has been applied to *B. subtilis* to increase the host robustness (Westers et al. 2003; Ara et al. 2007); subsequently it was also demonstrated that streamlining the genome resulted in improved biomass and protein productivity (Morimoto et al. 2008; Manabe et al. 2011). Recently, Tanaka et al. determined nonessential regions in the *B. subtilis* 168 genome by successfully deleting 146 individual regions covering ~76 % of the genome (Tanaka et al. 2013) and information was exploited in further improvement of the model predictions regarding the cell viability. These studies demonstrate the potentiality of the strain for synthetic biology applications and pave the way for a minimal *B. subtilis* cell factory.

2.2.3.2 Pseudomonas putida

The genus *Pseudomonas* comprises a vast number of Gram-negative, aerobic bacterial species involving both pathogenic and non-pathogenic strains (Palleroni 2010). Common characteristics include the ability to adapt to different nutritional and physicochemical environments, the capability to survive stress, and the ability to synthesize bioactive compounds (Silby et al. 2011). The laboratory strain *P. putida* is a non-pathogenic soil bacterium possessing broad catabolic diversity for the utilization of various aliphatic, aromatic, and heterocyclic compounds, organic acids, alcohols and other complex hydrocarbons as carbon sources (Jimenez et al. 2002).

P. putida has been widely exploited as a model bacterium in fundamental studies regarding environmental bacteria. The potential of the strain to efficiently degrade and convert toxic organic wastes and petroleum-based compounds to harmless or value-added compounds has led to extensive studies and bioremediation applications in the field of environmental biotechnology (Poblete-Castro et al. 2012).

Apart from being exploited in bioremediation and biocatalysis applications, *P. putida* has potential for the production of industrially relevant compounds. The strain naturally produces polyhydroalkanoate (PHA), biocompatible and biodegradable polymer exploited in biomaterial industries and tissue engineering (Tripathi et al. 2013). Moreover, *Pseudomonas* strains have been broadly exploited in *de novo* synthesis and bioconversion of chiralic compounds and other important chemicals (Poblete-Castro et al. 2012).

The metabolic characteristics of *P. putida* promote its use in industrial scale processes; simple growth requirements, the versatile carbon metabolism, and efficient machinery for product tolerance and cofactor regeneration rate serve as a base for a promising cell factory for various applications. Moreover, *P. putida* KT2440 genome sequence (Nelson et al. 2002) and construction of a metabolic model (Nogales et al. 2008) have promoted the strain usability in biotechnology. For example, high butanol tolerance (Ruhl et al. 2009) and recombinant expression of alcohol producing genes from *C. acetobutylicum* have enabled the production of butanol in titres 120 mg/l (Nielsen et al. 2009). Also, the substrate range has been further extended for the utilization of pentose sugars by metabolic engineering (Meijnen et al. 2008). In general, tools for gene and genome engineering in *P. putida* are sufficiently available (de Lorenzo et al. 1990; Silva-Rocha et al. 2013). Genome streamlining, i.e. the removal of unnecessary parts of the chromosome, have been also applied to *P. putida* (Leprince et al. 2012a).

Recently, the *P. putida* strain has been increasingly brought up in the context of synthetic biology (Nikel et al. 2014). For example, in a previous study the TOL toluene degradation pathway of *Pseudomonas* was exploited in constructing a multicellular logic gate based on cell-cell communication and metabolic wiring (Silva-Rocha and de Lorenzo 2014). In the system, toluene served as in input for a sender strain converting the compound to benzoate, the output molecule. Benzoate was sensed by a receiver cell which responded to this input by producing visible light as a measurable output signal.

2.2.3.3 *Acinetobacter baylyi* ADP1

Acinetobacter baylyi ADP1 (here: ADP1), previously referred as BD413, is a Gram-negative, non-motile, strictly aerobic laboratory strain. The strain was derived from a heavily encapsulated ubiquitous soil bacterium *Acinetobacter baylyi* BD4 by a single-step mutation (Taylor and Juni 1961; Barbe et al. 2004). *Acinetobacter* spp. typically produce extracellular polysaccharides (EPS) to form a protecting capsule and to facilitate substrate uptake, but in contrast to BD4, the derived strain ADP1 possesses only a “mini-capsule” (Kaplan and Rosenberg 1982). The strain ADP1 is nutritionally versatile, possessing catabolic features similar to taxonomically close relatives *P. aeruginosa* and *P. putida* (Barbe et al. 2004). The strain does not, however, carry any virulence or pathogenicity factors.

The genome of ADP1 consists of one circular chromosome containing 3.6 million base pairs with GC-content of 40.3 %. There are 3325 coding sequences of which 3197 are annotated as protein coding genes. About 20 % of ADP1 genes are associated to catabolic functions. Most of the catabolism related genes are organized in five clusters or ‘catabolic islands’, with operons tens of thousands base pairs long. (Young et al. 2005) The genome possessing the exceptional orientation of genes serves as a highly convenient platform for genome editing. The strain ADP1 is closely related to *E. coli*, allowing the integration of existing knowledge about the genetics and metabolism. ADP1 exhibits most of the beneficial features of *E. coli* but there are also relevant differences that promote ADP1 as a potential host for synthetic biology.

Acinetobacter strains are frequently found in a variety of growth environments with quickly changing conditions. This can be seen in the strain characteristics regarding catabolic diversity, wide growth temperature range, efficient substrate utilization, tolerance to toxic compounds, and production of storage compounds, such as cyanophycin granule peptide (CGP), triacylglycerols (TAG), and wax esters (WE) (Kalscheuer and Steinbüchel 2003; Elbahloul et al. 2005).

Acinetobacter strains efficiently utilize a wide range of polar and non-polar hydrocarbons such as aliphatic alcohols, carbohydrates, long-chain fatty acids, glycols and polyols, aromatic and halogenated compounds, amino acids, alkanes, and small organic acids as a sole carbon and energy source. *A. baylyi* strains lack the gene for pyruvate kinase, as well as for glucokinase, hexokinase and a glucose transporter phosphotransferase system (PTS), which are important enzymes in a carbohydrate metabolism (Barbe et al. 2004). Therefore ADP1 cannot directly phosphorylate glucose, and a glucose molecule is oxidized to gluconate on the outer surface of the inner membrane by an electron carrier associated to glucose dehydrogenase, pyrroloquinoline quinine (PQQ). Notably, due to exceptional glucose metabolism following a modified Entner-Duodoroff pathway (Entner and Doudoroff 1952), ADP1 grows generally better on carbon sources that enter the main metabolic pathways through citric acid cycle (such as acetic acid) than on carbon sources that are processed in glycolysis (Barbe et al. 2004; Young et al. 2005).

To briefly mention other important catabolic pathways, the degradation of aromatic compounds is mediated by the multistep β–ketoadipate pathway, similar to pseudomonads (Young et al. 2005; Williams and Kay 2008). Nine essential enzymes are involved in the conversion of aromatic compounds to protocatechuate, and further to β-ketoadiapate, and finally TCA cycle intermediates (Ornston 1966). Also, the utilization of alkanes is a wide spread trait among *Acinetobacter* species. In ADP1, the degradation is dependent on several genes including constitutively transcribed *rubAB* and *xcpR.* The terminal alkane hydroxylase *alkM* and the regulator *alkR* are inducible and found to be essential when grown on alkanes (Geissdorfer et al. 1995; Ratajczak et al. 1998; Ishige et al. 2000).

With regard to valuable biocompounds, the most interesting pathways of ADP1 involve the synthesis of fatty acid (FA) derived long chain hydrocarbons, WEs and TAGs (Figure 2.10). TAGs are non-polar and hydrophobic glycerol triesters with three FAs, whereas WEs are oxoesters of long-chain primary fatty alcohols and long-chain FAs. Both molecules serve primarily as carbon storages and are mobilized under carbon limiting conditions, but they can also function against dehydration (Wältermann and Steinbüchel 2005).

The key step in TAG synthesis is the esterification of a long chain FA with a diacylglycerol molecule, a common precursor to bacterial phospholipid synthesis. The esterification is carried out by a membrane-bound bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase enzyme WS/DGAT (*atfA*, ACIAD0832) (Kalscheuer and Steinbüchel 2003). TAGs are mainly produced in a stationary growth phase under

nitrogen limiting conditions, and they stored as intracellular lipid inclusions (Wältermann et al. 2005).

Wax esters possess more dynamic nature compared to TAGs (Fixter et al. 1986) and they can occur as intracellular inclusions of various shapes (Ishige et al. 2002). In the natural WE synthesis pathway of ADP1, a NADPH dependent fatty acyl-CoA reductase Acr1 (Reiser and Somerville 1997) converts a fatty-acyl CoA molecule to a corresponding fatty aldehyde, followed by a conversion of fatty aldehyde to fatty alcohol by a yet uncharacterized alcohol dehydrogenase/aldehyde reductase(s). In the final step, the fatty alcohol molecule is esterified with a fatty acyl-CoA molecule by the well-characterized bifunctional enzyme WS/DGAT, resulting in the formation of a wax ester molecule. The natural WEs in ADP1 predominantly consist of monounsaturated C16 or C18 carbon chains when the cells are grown on glucose. However, the utilization of alkanes or alkanols as a substrate results in a significant accumulation of WEs (Ishige et al. 2002), and the alkyl chain lengths are determined by the used substrate.

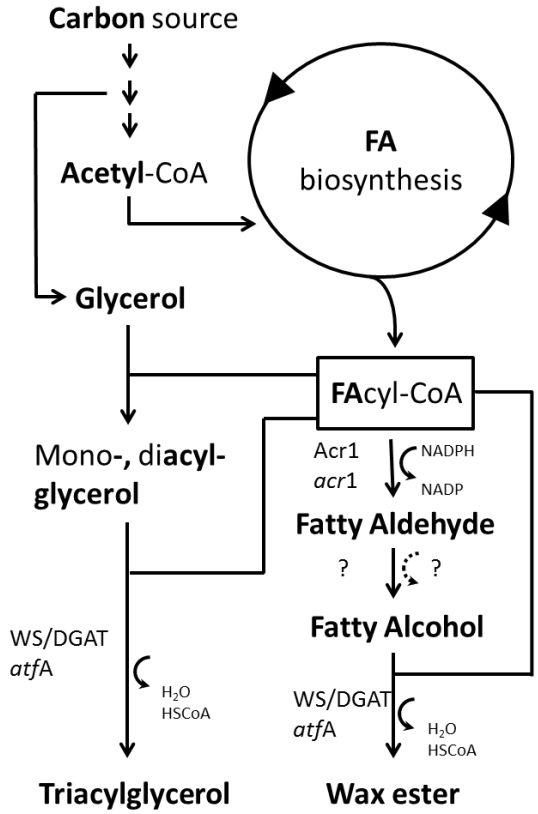

FIGURE 2.10. The biosynthetic pathways related to neutral lipid production of *A. baylyi* ADP1.

Another interesting biomolecule produced by *Acinetobacter* strains is cyanophycin granule peptide (CGP). The molecule is a branched polypeptide consisting of aspartate backbone and arginine residues exploitable e.g. in polyacrylics synthesis. The natural production of CGP is triggered by a phosphate starvation and excess provision of arginine (Elbahloul et al. 2005), and the synthesis is catalyzed by cyanophycin synthetase (CphA) (Krehenbrink et al. 2002). The enhanced production of CGP has been demonstrated in engineered ADP1 (Elbahloul and Steinbüchel 2006); the deletion of the arginine regulatory protein (*argR*) and the arginine succinyltransferase (*astA*), or the overexpression of *phoB* of phosphate regulon system increased the CGP production by up to 8.6 fold.

The most impressive work done with ADP1 thus far constitutes a comprehensive analysis on ADP1 genome, transcriptome, and metabolome levels. The multiomics approach has involved the construction of a metabolic model (Durot et al. 2008) encompassing 875 reactions, 701 distinct metabolites, and 774 genes. In addition, a complete collection of a single gene knock-out mutant library was constructed (de Berardinis et al. 2008), followed by the experimental annotation of genes (Genoscope 2009). Recently, an extensive analysis of ADP1 transcriptome and metabolome levels in response to different perturbations was carried out (Stuani et al. 2014).

Most interestingly, the strain ADP1 is naturally transformable (Palmen and Hellingwerf 1997), enabling straight-forward gene and genome engineering. Transformable *Acinetobacter* strains do not discriminate between homologous and heterologous DNA or display any sequence specificity at the stage of binding and uptake. Linear and plasmid DNA are brought into the cells by the same uptake system, followed by DNA incorporation to the chromosome by homologous recombination, or plasmid recircularization (Palmen et al. 1993). Thus single or multiple gene deletions and insertions using synthetic gene fragments or gene cassettes can be carried out in a high-throughput manner using an automated system (Figure 2.11). However, compared to for example the widely exploited λ red recombinase –mediated chromosomal incorporation and replacement (Datsenko and Wanner 2000), the recombination machinery of ADP1 requires relatively long homologous sequences (optimally >500 bp (Simpson et al. 2007)) for the genome target site, thus slightly complicating the construction of the genome engineering tools. In addition, relatively large amounts of DNA are required for transformations at sufficient rate. Therefore, increasing the efficiency of natural transformation and homologous recombination represent one important engineering target in developing the strain ADP1 as a chassis.

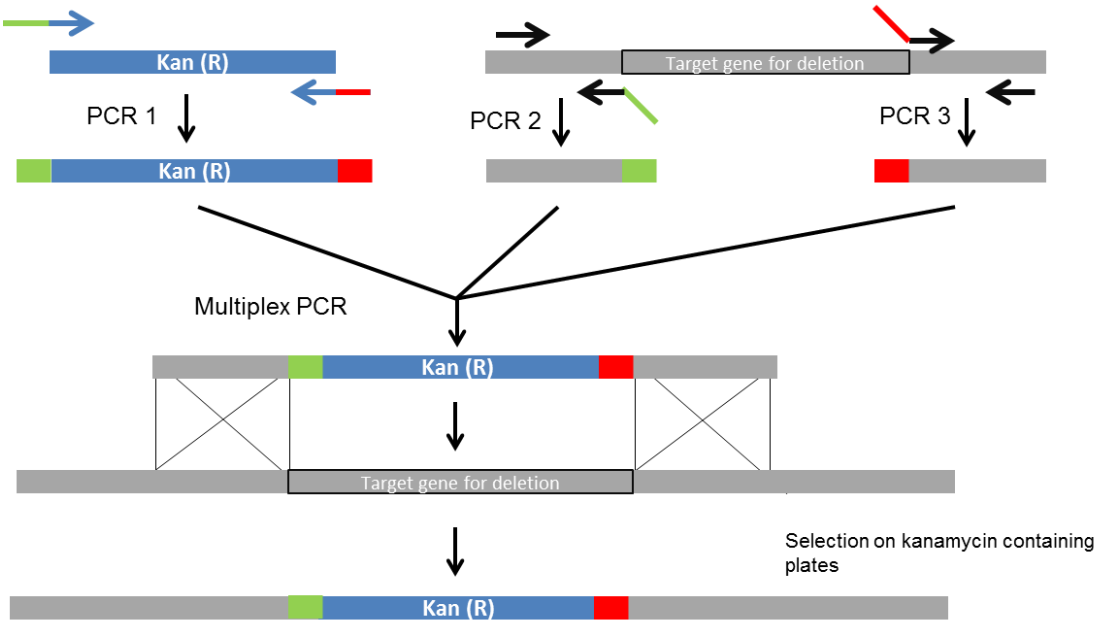

FIGURE 2.11. The method for a site-specific gene knock-out in *A. baylyi* ADP1 using a synthetic gene cassette.

To further facilitate the use of ADP1 in metabolic engineering and synthetic biology, efficiently replicating expression vectors for the strain have been recently described (Bryksin and Matsumura 2010; Murin et al. 2012). The vectors are also compatible with the BioBrick™ standard. The study by Murin et al. also demonstrates that the commonly used promoters, such as T5, T7, and BAD, are functional in ADP1.

Despite the attractive characteristics of ADP1 and the recently increased use as a model strain in fundamental genomic and metabolic studies (Metzgar et al. 2004; de Berardinis et al. 2009; Elliott and Neidle 2011; Zhang et al. 2012b), only a few true application platforms have been introduced, apart from exploiting the individual enzymes of ADP1 (see Chapter 3; (Stöveken and Steinbüchel 2008)). Table 2.2 presents some described approaches to exploit *Acinetobacter* strains in biotechnology. Due to the versatile catabolic machinery of ADP1, the field is largely focused on biosensor and bioremediation applications in environmental bioengineering. However, industrially relevant biomolecules such as bioemulsifiers, lipases, and CGP that are naturally produced by *Acinetobacter* strains have also drawn interest. As *P. putida*, ADP1 holds potential for whole-cell biocatalysis and bioconversion processes.

TABLE 2.2. Examples of *Acinetobacter* based applications.

Application or Product	Field	Strain	Engineering	Reference
Detection of contaminants and xenobiotics from soil and water environments by whole cell biosensors	Environmental biotech. / Bioremediation	ADP1	Expression of *lux* operon under specific promoter	(Zhang et al. 2012a) (Abd-El-Haleem et al. 2006) (Abd-El-Haleem et al. 2002) (Wang et al. 2014b)
Cyanophycin	Biotechnology		Inactivation of *argR*, *astA,* overexpression of *phoB*	(Elbahloul and Steinbüchel 2006)
Crude oil removal from soil	Bioremediation	A3		(Hanson et al. 1997)
Bioemulsan	Biotechnology	RAG-1 (Several)		(Shabtai 1990; Shabtai and Wang 1990) (Gutnick et al. 1989)
Modified emulsan			Protein engineering	(Dams-Kozlowska and Kaplan 2007)
Modified emulsan			Transposon mutations	(Johri et al. 2002)
Bio-Pd catalysts				(Baldi et al. 2011)
Emulsan / adjuvant	Biomedicine			(Panilaitis et al. 2002)
Wax esters	Biotechnology	M-1		(Ishige et al. 2002)
Lipases	Biotechnology	(Several)		(Snellman and Colwell 2004)

3 Synthetic biology for sustainable bioenergy

In the times of consistently growing energy demand and increasing insecurity related to fossil fuels supply and environmental concerns, synthetic biology aims at fighting the challenges with novel microbial platforms for sustainable bioenergy production. Transportation fuels comprise a major share of the consumed energy, and biologically produced advanced biofuels are suggested to replace the fossil counterparts and food-crop based first generation biofuels. The synthetic biology approach enables the production of customized drop-in liquid fuels with defined characteristics, not restricted to the properties of natural products. Despite the existing and optimized processes for bioethanol production, advanced biofuels (i.e. long chain (C≥4) alcohols, alkanes, FA alkyl esters, terpenes) have drawn a lot of attention due to their incomparable properties, higher energy content, and compatibility with existing engine systems and infrastructure.

Atmospheric carbon dioxide and solar energy are stored in different forms of biomass. Microbes have the capability to convert the biomass into high-energy compounds exploitable in biofuels. Metabolic engineering and synthetic biology focus on enhancing the production systems to be more robust in terms of product quality, quantity, and sustainability. Optimally, custom-made fuel components compatible with the existing infrastructure could be produced from cheap and sustainable non-food substrates, such as agricultural or forest waste, or energy crops (Figure 3.1). In addition to constructing the actual metabolic pathways in cells, novel strategies for improved product titers involve a comprehensive omics –level analyses and sensor systems. The strategies promote the identification of the bottle necks, the alleviation of product toxicity, and the construction of protein scaffolds to facilitate optimal metabolic fluxes.

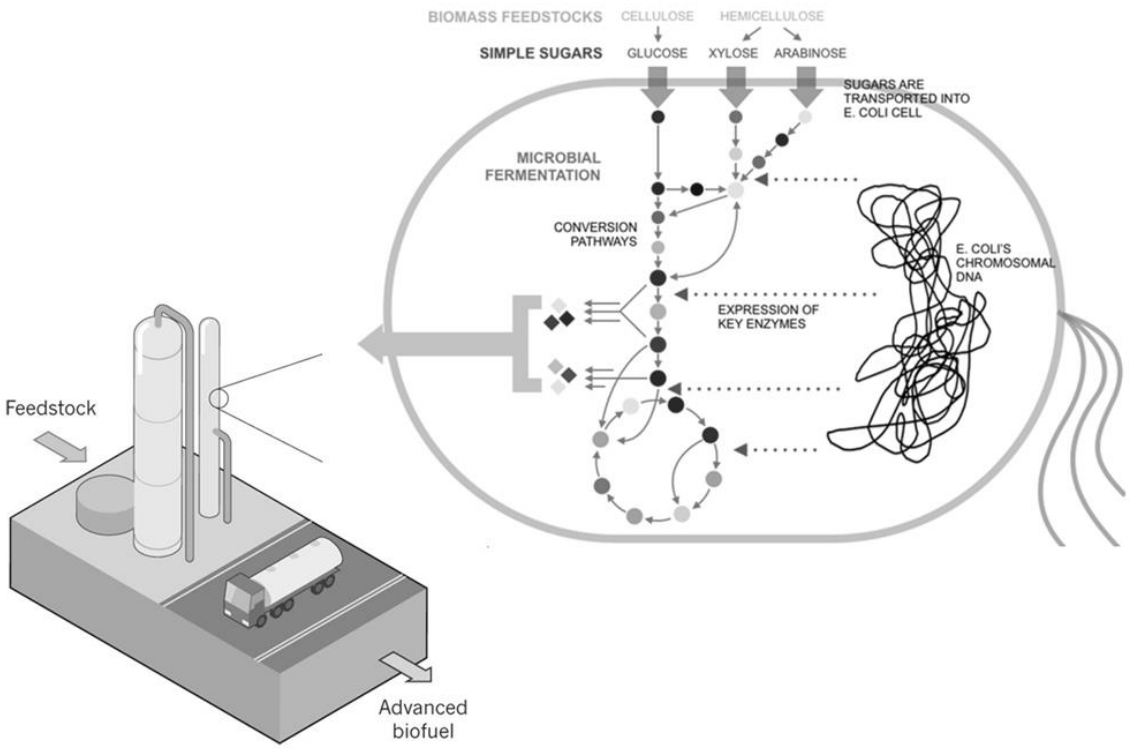

FIGURE 3.1. Sustainable production of advanced biofuels by the bacterium *E. coli.* Modified from (Peralta-Yahya et al. 2012) and (Kung et al. 2012)

Despite the significant improvements achieved with the new strategies for redesigning and engineering cell factories, there are still unsolved issues related to process scale-up and economy, insufficient product titers, the inhibitory effects of products and intermediates, the efficient utilization of cellulosic substrates, and constraints set by cell metabolism. In the following section, some of the major advances in the field of engineered bacterial production of advanced biofuels are described. Although the focus is on prokaryotic systems, it is noteworthy that several eukaryotic microbes, such as oleaginous yeasts *Yarrowia lipolytica* and *Cryptococcus* spp. (Beopoulos et al. 2009; Ageitos et al. 2011), and metabolically engineered *S. cerevisiae* (Runguphan and Keasling 2014; Zhou et al. 2014), represent important hosts in the production of bioenergy molecules.

3.1 Production of advanced biofuels

3.1.1 Alcohols

For short and medium-chain alcohol production, either fermentative or non-fermentative pathways can be exploited. In a typical approach, acetyl-CoA dependent fermentative pathways (Figure 3.2) employing enzyme activities from *Clostridium* species have been used to produce isopropanol (Hanai et al. 2007; Inokuma et al. 2010) and 1-butanol (Atsumi et al. 2008a) in an engineered *E. coli*. The titers for 1-butanol have been further enhanced by metabolic engineering, applying several gene knock-outs for eliminating competitive pathways (Atsumi et al. 2008a) and further pathway optimization by replacing individual enzymes (Bond-Watts et al. 2011). By these approaches, titers up to 4.65 g/l have been achieved (Bond-Watts et al. 2011). Other reported recombinant prokaryotic hosts for the production of 1-butanol include *P. putida* and *B. subtilis* (Nielsen et al. 2009), justified by better product tolerance compared to *E. coli*. Photosynthetic carbon fixing cyanobacteria have been also employed (Machado and Atsumi 2012). Recently, a synthetic pathway for butanol production with improved oxygen tolerance was established by employing an acyl carrier protein (ACP) dependent pathway instead of the acyl-CoA dependent fermentative pathway; the expression of a thioesterase from *Bacteroides fragilis* and an aldehyde reductase *ahr* from *E. coli* resulted in a butanol titer of ∼300 mg/l (Pasztor et al. 2014).

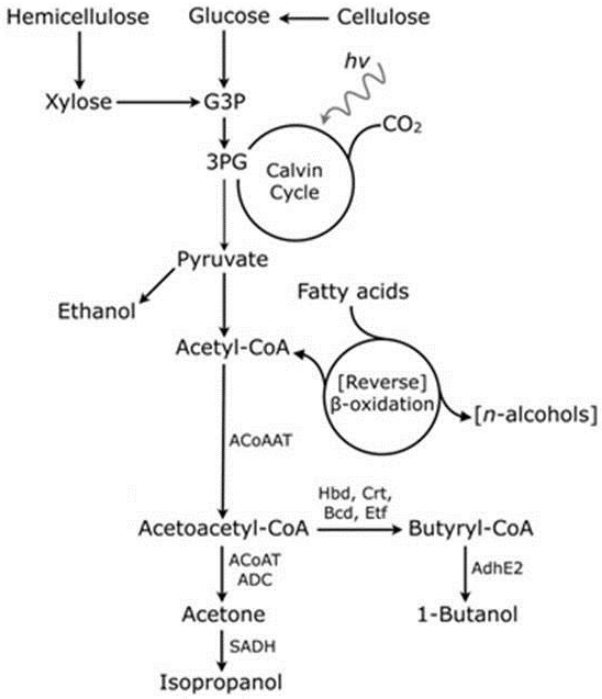

FIGURE 3.2.A schematic overview of possible fermentative pathways for alcohol production. The pathways include reactions from both autotrophic and heterotrophic organisms. Modified from (Lamsen and Atsumi 2012).

In 2008, Atsumi et al. introduced a synthetic non-fermentative pathway for the production of branched higher alcohols such as isobutanol and 2-methyl-1-butanol. Only traces of these compounds are naturally produced by bacteria. The pathway is taking advantage of the NADPH dependent amino acid biosynthesis pathway producing 2-ketoacids, which are further converted to alcohols through decarboxylation and reduction reactions by 2-keto acid decarboxylase KDC (e.g. *kivD* gene from *Lactococcus lactis*) and an alcohol dehydrogenase (e.g. ADH2 from *S. cerevisiae*), respectively (Atsumi et al. 2008b) (Figure 3.3). The pathway can be readily expressed in a variety of hosts, and toxic intermediates are not produced. A substantial number of studies exploiting the ketoacid pathway for the production of alcohols, chemicals, and other biocompounds have been reported (Jambunathan and Zhang 2014).

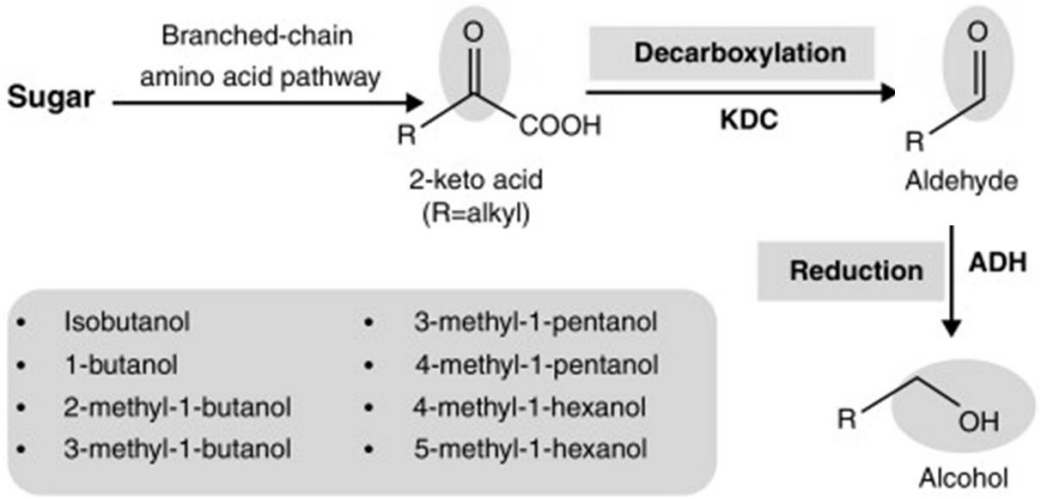

FIGURE 3.3. The aminoacid biosynthesis related ketoacid pathway for non-fermentative production of aliphatic and branched alcohols. KDC - 2-keto acid decarboxylase, ADH – Alcohol dehydrogenase. Modified from (Jambunathan and Zhang 2014).

Another approach to produce higher alcohols is to exploit reverse fatty acid β-oxidation pathway, normally assigned for the degradation of acyl-CoAs in aerobic conditions. In semi-aerobic and anaerobic conditions with artificially regulated β-oxidation and alcohol producing pathway genes it has been possible to produce 1-butanol and longer *n*-alcohols (Dellomonaco et al. 2011; Clomburg et al. 2012; Gulevich et al. 2012).

3.1.2 Isoprenoid fuels

To briefly introduce promising new candidates for advanced biofuel production, isoprenoids, i.e. terpenes are a functionally and structurally diverse group of hydrocarbons, typically serving as relevant precursors for medical industries. Some isoprenoid products, such as pinene and farnesene, can be potentially used as gasoline, diesel, or jet fuel. Isoprenoids are produced by an acetyl-CoA originated mevalonate (MEV) pathway or by a 1-deoxy-D-xylulose-5-phosphate (DXP) pathway beginning with glyceraldehyde-3-phosphate and pyruvate, resulting in universal precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), respectively. These molecules can be further processed by classes of terpene synthases and cyclases to form a variety of mono- or polycyclic or aliphatic compounds. Farnesene and saturated farnesane have been produced in engineered *E. coli* from farnesyl pyrophosphate intermediate by introducing a codon optimized β-farnesene synthase from *Artemisia annua.* The production process was scaled up by

Amyris Biotechnologies, Inc. for the mass production of farnesene as a fuel additive. (Renninger and McPhee 2008)

3.1.3 Fatty acid derived compounds

Fatty acids (FA) have high energy content and properties convenient for fuel purposes, but due to their ionic nature they cannot be directly exploited as biofuels. Fatty acids and ac(et)yl-CoAs are however important precursors for several interesting molecules, such as FA alkyl esters, alkanes, fatty alcohols, and triacylglycerols (TAG). Fatty acid and acyl-CoA synthesis pathways have been extensively studied and engineered, especially in *E. coli*, to facilitate the production of advanced biofuels (Handke et al. 2011; Lennen and Pfleger 2012; Xu et al. 2013).

TAGs constitute of three fatty acids esterified with a glycerol backbone, and they are considered as an appropriate feedstock for a biodiesel synthesis process. Even though the TAG molecules are not directly exploitable as drop-in in liquid fuels, they are compatible with existing production processes and infrastructure; TAGs can be derived to a mixture of esters constituting of long chain fatty acids and short chain alcohols, namely fatty acid alkyl esters, such as FA methyl esters (FAME) or ethyl esters (FAEE) suitable for traffic fuel. TAGs are neutral lipids and natural carbon and energy storages in animals, plants and in a number of bacteria such as *Streptomyces*, *Nocardia*, *Acinetobacter*, and *Rhodococcus* species (Alvarez and Steinbüchel 2002). *Rhodococcus opacus* cells, for example, can naturally accumulate up to 80 % TAG of cell dry weight in nitrogen limiting conditions (Alvarez et al. 1996). In a study of Kurosawa et al. (2010), a titer of 77.6 g/l TAGs could be obtained in a batch bioprocess of *R. opacus* containing high glucose concentration and critical C/N ratio of 17.8 (Kurosawa et al. 2010). More recently, the same group demonstrated a more sustainable approach to TAG production with *R. opacus* engineered with *Streptomyces* DNA library for using high concentrations of xylose as a substrate (Kurosawa et al. 2013).

In a synthetic biology point of view, more readily engineered cell systems enable broader substrate and product range and regulated production, and thus serve as a more convenient approach for biodiesel production. The emphasis has been on the direct production of drop-in FA based fuels, for example FAEEs, exploiting the newly described acyl-coenzyme A, diacylglycerol acyltransferase WS/DGAT (*atfA*) from *A. baylyi* ADP1 (Kalscheuer and Steinbüchel 2003; Kalscheuer et al. 2004; Kalscheuer et al. 2006a). Fatty acid synthesis is strongly regulated, feedback inhibited, and dependent on acetyl-CoA supply, for which the engineering of the production of FA

based compounds is challenging. The earliest example of direct production of FAEEs (Kalscheuer et al. 2006a) in *E. coli* was achieved by the external supply of fatty acid substrate, followed by a pilot-scale production of FAEEs in an optimized bioprocess (Elbahloul and Steinbuchel 2010). In the study of Steen et al. (2010) no substrate addition was required; an improved carbon flux towards FA and acyl-CoA syntheses and eventually FAEE production was obtained by metabolic engineering. The modifications included the overexpression of modified cytosolic thioesterase 'TesA lacking the leader sequence for periplasmic expression, the elimination of the β-oxidation cycle by *fadE* deletion, and overexpression of acyl-CoA ligases and *fadD*, facilitating the activation of FFAs to acyl-CoA. For FFAs, titers of 1.2 g/l could be obtained (Steen et al. 2010). Production of the alcohol counterpart (ethanol) was established by expression of pyruvate decarboxylase *pdc* and alcohol dehydrogenase *adhB* from *Zymomonas mobilis.* In the final step of the *in vivo* esterification of fatty acyl-CoA and short chain alcohol, the above mentioned bifunctional and highly unspecific acyltransferase WS/DGAT was exploited, eventually resulting in titer of 674 mg/l FAEEs. In addition, the expression of an endoxylanase catalytic domain (Xyn10B) from *Clostridium stercorarium* and a xylanase (Xsa) from *Bacteroides ovatus* enabled a consolidated process of utilization of hemicellulosic substrate and production of biofuel. In the next step, the production of biofuel components directly from switch grass was demonstrated (Bokinsky et al. 2011). Shortly after, the FAEE titer could be increased to 1.5 g/l with a sophisticated regulator/sensor system (see Chapter 3.2) responsive to FA and acyl-CoA levels in the cell (Zhang et al. 2012c). In the study by Choi and Lee (2013), FAEEs were produced by expressing a mutated alcohol dehydrogenase (*adhE*) from *E. coli* and the wax ester synthase WS/DGAT from ADP1, resulting in the titer of 480 mg/l C10-C14 FAEEs.

Fatty aldehydes, fatty alcohols, and wax esters are products of different stages of a single pathway derived from FAs (Figure 3.4). These long chain hydrocarbons are considered as high-value molecules (appr. 1500 $/t) exploited mainly in fine chemical, cosmetics, medicine, and food industries. Due to their properties, they are also convenient for bioenergy production. Fatty aldehydes and alcohols are produced from FA or fatty acyl-CoA/ACP substrates through reduction reactions by fatty acid/aldehyde reductases (FAR) (Table 3.1). Fatty alcohols with variable chain lengths have been produced in heterologous *E. coli* by altering the thioesterases used, i.e. BTE from *Umbellularia californica* or 'TesA from *E. coli,* and the reductase counterpart, a bifunctional FA-CoA reductase from *Simmondsia chinensis* or Acr1 from *A. baylyi* ADP1, leading to an alternative synthesis of C12/14 or C16/18 fatty alcohols (Zheng et al. 2012b). More recently, significant amounts of C12-18 alcohols (1.725 g/l) were

produced in a fermentation process of engineered *E. coli* expressing a bifunctional acyl-CoA/aldehyde reductase from *Marinobacter aquaeolei* VT8 together with modified *tesA* and *fadD* genes (Liu et al. 2013), whereas high yields of C12-C14 alcohols (0.13 g/g glucose with a titer 1.6 g/l) were produced in a study exploiting an acyl-ACP thioesterase (BTE), FadD, and the same *M. aquaeolei* reductase in an engineered *E. coli* (Youngquist et al. 2013). Improved yields could be obtained by gene expression level balancing and optimized fed-batch cultivation. The photosynthetic fatty alcohol production was enhanced in metabolically engineered cyanobacteria by introducing the fatty acyl-CoA reductase from *M. aquaeolei* VT8 combined with knock-outs of an acyl-ACP reductase and an aldehyde-deformylating oxygenase genes (Yao et al. 2014).

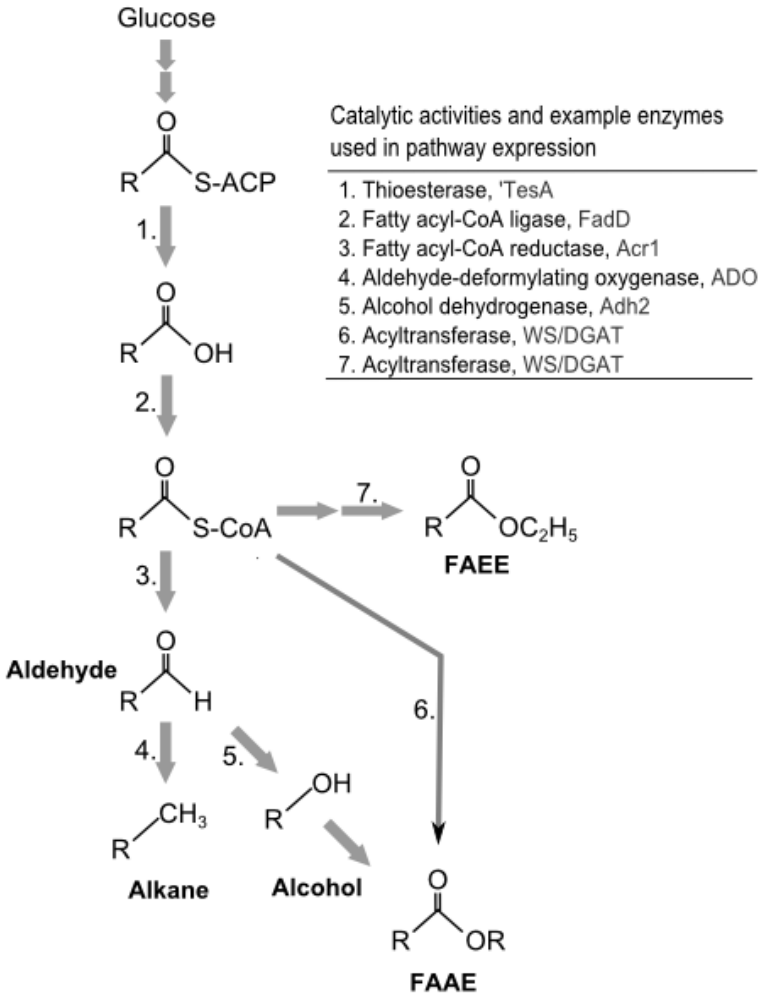

FIGURE 3.4. Biochemical pathways for the production of fatty acid derived compounds. The key enzymatic steps are numbered, and examples of enzymes are provided. FAEE – Fatty acid ethyl ester (biodiesel), FAAE – Fatty acid alkyl ester (wax ester).

Modified esters consisting of diverse fatty acid and alcohol (>C2) moieties can be produced in recombinant hosts. Conventionally, alkyl esters are chemically produced in harsh conditions by Fisher esterification using fossil feedstock, and thus an alternative biological production process is of high relevance. Guo et al. (2014) described an approach of combining a 2-keto acid pathway and an engineered FA synthesis pathway for the optimized production of a variety of branched and aliphatic FA short-chain esters using glycerol as a substrate (Guo et al. 2014). Layton and Trinh (2014) introduced a modular platform for the anaerobic fermentative production of variable butyrate esters in engineered *E. coli,* involving knockouts to block e.g. the competitive fermentative pathways, and insertions of designed individual submodules for the production of acyl-CoAs, alcohols, and an alcohol acyltransferase (Layton and Trinh 2014). In contrast, Rodriguez et al. (2014) constructed several aerobic acetate ester pathways in *E. coli* based on the esterification of acetyl-CoA with branched alcohols produced by the keto acid pathway. A remarkable titer of 17.2 g/l for isobutyl acetate from glucose was achieved, being 80 % of the theoretical yield (Rodriguez et al. 2014). In the same study, a fatty acid reductase complex LuxCDE from *Vibrio harveyi* was exploited for production long-chain tetradecyl-acetate.

Among bacteria, wax esters (WE) are natural products e.g. of *Marinobacter* (Lenneman et al. 2013) and *Acinetobacter* (see Chapter 2.2.3.3) strains. For the recombinant production of WEs, an expression of FAR from *S. chinesis* and WS/DGAT from ADP1, with the supplementation of fatty alcohol substrate, have enabled the production of jojoba-like WEs in *E. coli* (Kalscheuer et al. 2006b). Steen et al. (2010) established the WE synthesis in recombinant *E. coli* without inclusion of external alcohols by a simultaneous expression of exogenous FAR, an endogenous alcohol dehydrogenase, and WS/DGAT. More recently, Kaiser et al. (2013) demonstrated the production of WEs in cyanobacteria by co-expression of the native acyl-ACP reductase, a long-chain alcohol dehydrogenase from *Synechocystis* sp PCC 6803 (slr1192), and WS/DGAT. However, for an unknown reason, the formed neutral lipid inclusions were found to be toxic to the *Synechocystis* cells.

TABLE 3.1. Some key reductases exploited in a recombinant production of fatty aldehydes, fatty alcohols, esters and alka(/e)nes.

Enzyme	Gene	Strain of origin	Preferred substrates	Reference
Fatty acyl-CoA reductase	*acr1*	*Acinetobacter baylyi* ADP1	acyl-CoA; C16-18	(Steen et al. 2010)
Fatty acyl-CoA reductase	Maqu_2220	*Marinobacter aquaeolei* VT8	acyl-CoA; C12-18	(Liu et al. 2013; Yao et al. 2014)
Carboxylic acid reductase		*Mycobacterium marinum*	FA; C6-18	(Akhtar et al. 2013)
Fatty acyl-CoA reductase		*Simmondsia chinensis*	acyl-Coa; C20-	(Zheng et al. 2012b)
Acyl-ACP reductase, AAR	*PCC7942_orf1594*	*Synechococcus elongates*	acyl-ACP; C14-18	(Schirmer et al. 2010; Kaiser et al. 2013)
FAR complex, LuxCDE	*luxCDE*	*Photorhabdus luminescens*	FA (-CoA, -ACP); C10-18	(Howard et al. 2013)
FAR complex, LuxCDE	*luxCDE*	*Vibrio harveyi*	FA (-CoA, -ACP); C10-18	(Rodriguez et al. 2014)
fatty acyl-CoA reductase	*acr*	*Clostridium acetobutylicum*	acyl-CoA; C8-14	(Choi and Lee 2013)
Fatty acyl-CoA reductase, Cer4		*Arabidopsis thaliana*	acyl-CoA	(Zheng et al. 2012b)

Alkanes and alkenes are aliphatic hydrocarbons which are products of a different branch of the above described FA derived pathway typically employing fatty aldehydes as the key precursors. Alkanes can be directly exploited as the constituents of gasoline and jet fuel. Several approaches to microbial alkane production have been described. Schirmer et al. described the microbial production of alkanes by engineered *E. coli* exploiting the alkane synthesis pathway from cyanobacteria. The pathway consists of an acyl-ACP reductase and an aldehyde-deformylating oxygenase (ADO), which convert the intermediates from FA synthesis to alkanes and alkenes, the carbon chain profile ranging from C13 to C17 (Schirmer et al. 2010).

In another study (Choi and Lee 2013) shorter chain ‘gasoline’ alkanes (C9-C14) were produced exploiting a similar pathway involving *E. coli* fatty acyl-CoA synthetase, *Clostridium acetobutylicum* fatty acyl-CoA reductase and *Arabidopsis thaliana* fatty

aldehyde decarbonylase. The titers were further improved employing metabolic engineering approach; β-oxidation pathway was blocked by deleting the *fadE* gene to increase the supply of fatty acyl-CoA substrates to hydrocarbon synthesis. Also, *fadR* was deleted in order to boost up the synthesis of suitable FAs and to hinder the synthesis of unsaturated FAs. To generate FFAs from acyl-ACPs for alkane synthesis, a modified thioesterase was used. A total titer of 580.0 mg/l alkanes could be produced.

Very recently, a platform for the production of renewable short-chain alkane, i.e. propane, using a synthetic metabolic pathway was established in recombinant *E. coli*. The pathway employed a butyryl-ACP specific thioesterase and was complemented with an electron-donating module and elimination of endogenous aldehyde reductases (Kallio et al. 2014).

For long chain alkene production, a three-gene cluster from *Micrococcus luteus* was introduced to FA overproducing *E. coli* strain, resulting in production of C27:3 and C29:3 alkenes (Beller et al. 2010). In a study by Akhtar et al, the expression of a wide substrate range carboxylic acid reductase (CAR) from *Mycobacterium marinum* and an aldehyde reductase, or alternatively an aldehyde decarbonylase resulted in production of C8-18 fatty alcohols and C7-15 alkanes, respectively (Akhtar et al. 2013).

A reconstructed pathway for alkane production exploiting FA reductase complex LuxCDE from *Photorhabdus luminescens* and an aldehyde decarbonylase from *Nostoc punctiforme* was established, resulting in production of alkanes with rationally altered chain lengths (Howard et al. 2013). Further genetic manipulation of the FA substrate pool enabled the production of custom-made branched alkanes.

3.2 Dynamic monitoring tools for enhanced bioproduction

The activity of genes and individual enzymes are affected by several factors inside the cells, resulting in an extremely delicate system in terms of expression levels and productivity. Maximal expression guarantees no maximal productivity; the overproduction of enzymes consumes cellular building blocks and energy, and can be toxic to cells. Thus, optimal distribution of cellular resources and cofactors, and a dynamic response to fluctuations in internal and external stages are required to tune the production systems. Intracellular sensor devices facilitate the screening of optimal genotypes and conditions, and identification of potential pathway bottle-necks. Even more sophisticated integrated sensory-regulatory devices serve as dynamic tools for a

concurrent sensing of the cellular state and responsive regulation of key element expression, resulting in balanced metabolism and consequently improved production yields and titers.

Monitoring tools can function either at transcriptional, translational, or at post-translational levels (Figure 3.5). Sensors functioning at a transcriptional level typically comprise a promoter-transcription factor system responsive to a key metabolite of the pathway, regulating the expression of a reporter, such as green fluorescent protein (GFP). Artificial RNA elements such as riboswitches consisting of ligand-binding aptamer domain and expression platform can specifically regulate transcription or translation (Ceres et al. 2013; Wachsmuth et al. 2013; Berens and Suess 2014; Ma et al. 2014). Qi et al. described a combinatorial approach to engineer ligand-binding RNA fusion molecules that regulate both transcription and translation through allosteric regulation of trans-acting nc-RNAs (Qi et al. 2012).

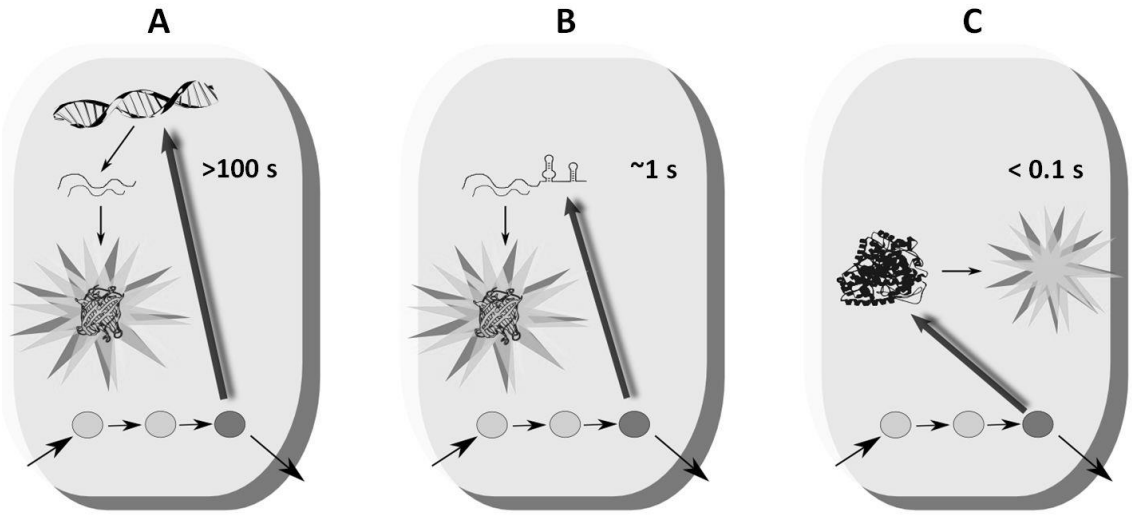

Figure 3.5. Different levels of intracellular monitoring of the product formation exploiting a reporter based sensor tool. The characteristic lag time between the occurrence of the target molecule and the initiation of signal formation is indicated. The approximate response times are adapted from (Olson and Tabor 2012). The time required for signal detection is not considered. A) Transcriptional level: the target molecule induces DNA transcription followed by protein synthesis and signal development. B) Translational level: The target molecule interacts with RNA molecule thus regulating the reporter expression and signal development. C) Post-translational: The target molecule interacts directly with the reporter molecule present in the cell, resulting in instant and dynamic signal production.

A majority of the described sensor-regulatory systems are based on the regulation of key enzyme expression. These approaches suffer from a relatively slow response (up

to minutes) and the loss of temporal and dynamical range with respect to rapidly changing analyte concentrations, since removing a transcription inducer does not instantly remove the reporter signal (Olson and Tabor 2012). Furthermore, maintaining a transcription level sensor requires a significant input of cellular resources and machinery, such as the synthesis of transcription factors. Translational regulation based on RNA elements, on the other hand, is more prone to degradation, and thus less stable. Post-translational regulation and monitoring, which occur through enzyme-enzyme or enzyme-metabolite interactions, can be therefore considered as a more dynamic and perceptive approach to manipulating, balancing, and analyzing the biosynthetic pathway or interest.

An early example of an integrated regulatory circuit controlling gene expression in response to intracellular metabolic states is the improved lycopene production in *E. coli* (Farmer and Liao 2000). In the study, a global Ntr regulatory system was altered in *E. coli*. The engineered system regulated the expression of two key enzymes in lycopene synthesis in response to acetyl phosphate, a precursor to acetate and an indicator molecule for excess glycolytic flux, resulting in 18-fold improved lycopene production through metabolic balancing.

In a more recent study briefly mentioned in Chapter 3.1.3, a dynamic sensor-regulator system for the improved production of FAEEs in *E. coli* was described (Zhang et al. 2012c). The expression of key genes involved in the lipid synthesis was regulated by a specific DNA-binding transcription factor FadR sensing fatty acyl-CoAs combined with synthetic FadR and IPTG regulated promoters. Due to the balanced metabolism and stabilized expression system FAEE titers were significantly improved (1.5 g/l), reaching 28 % of the theoretical maximum.

Recently described *de novo* designed riboregulators called “toehold switches” regulate translation through the detection of endogenous cognate RNAs (Green et al. 2014). The functional sequences of the switches can be modified according to the target RNAs, and thus the switch can serve as an orthogonal and programmable regulator for gene expression. In addition, a switch coupled with a reporter can be potentially exploited as a sensor to monitor the transcriptome levels of the genes of interest. Furthermore, the switches provide wide dynamic range and low cross talk.

Platforms exhibiting both regulation and monitoring function are still in scarce. Indeed, potential mechanisms and tools applicable to such approach have been found in nature, but only recently these complex systems have been employed in novel contexts. In the following subchapter, as an example of a natural robust tool package, a

lux multienzyme complex comprising of unique catalytic and signal producing modules is introduced and considered in the light of synthetic biology.

3.2.1 Lux multienzyme complex

Bioluminescence is a very convenient tool for sensor applications, due to the high specificity and sensitivity and the straight-forward signal determination methods. In nature, a taxonomically diverse group of bacteria can produce visible light, i.e. bioluminescence. The function of bioluminescence is suggested to be related to quorum sensing in dense populations, electron transport, and symbiotic life within the organelles of marine organisms (Meighen 1993). The bacterial luciferase (Lux) system is a multienzyme complex which has been characterized for several bacteria including both terrestrial and marine species, and the most widely studied and the best-characterized Lux systems belong to the bacteria of genus *Vibrio, Photorhabdus* (prev. *Xenorhabdus*), and *Photobacterium* (Engebrecht et al. 1983). Genes behind the multienzyme complex are clustered to form a *lux* operon, with a typical orientations of *luxCDABE* (*Photorhabdus* sp.) or *luxABCDE*, (*Vibrio* sp.), supported by several other related genes including cofactor ($FMNH_2$) generating *luxG* and regulatory genes (Meighen 1994; Gray and Garey 2001; Nijvipakul et al. 2008).

The reductase enzyme complex consists of an acyl transferase (LuxD), an acyl-protein synthetase (LuxE), and a FA reductase (LuxC). In the multistep reaction, LuxD cleaves an activated FA from its carrier and transfers the FA for LuxE. The maximal activity of the transferase is obtained with tetradecanoyl-ACP and tetradecanoyl-CoA substrates. The synthetase LuxE produces an acyl-protein thioester via a fatty acyl-AMP intermediate. Subsequently, the thioester is converted to corresponding fatty aldehyde by the reductase LuxC. The thioester and fatty aldehyde forming reactions require ATP and NADPH, respectively. The bacterial luciferase, consisting of two domains LuxA and LuxB, convert the fatty aldehyde to corresponding fatty acid molecule and visible light (Figure 3.6). In the reaction, a reduced form of flavin mononucleotide ($FMNH_2$) and molecular oxygen form a complex which reacts with the fatty aldehyde, creating a slowly decaying intermediate compound. Light emission occurs along with the oxidation of $FMNH_2$ and the aldehyde substrate. (Meighen 1991)

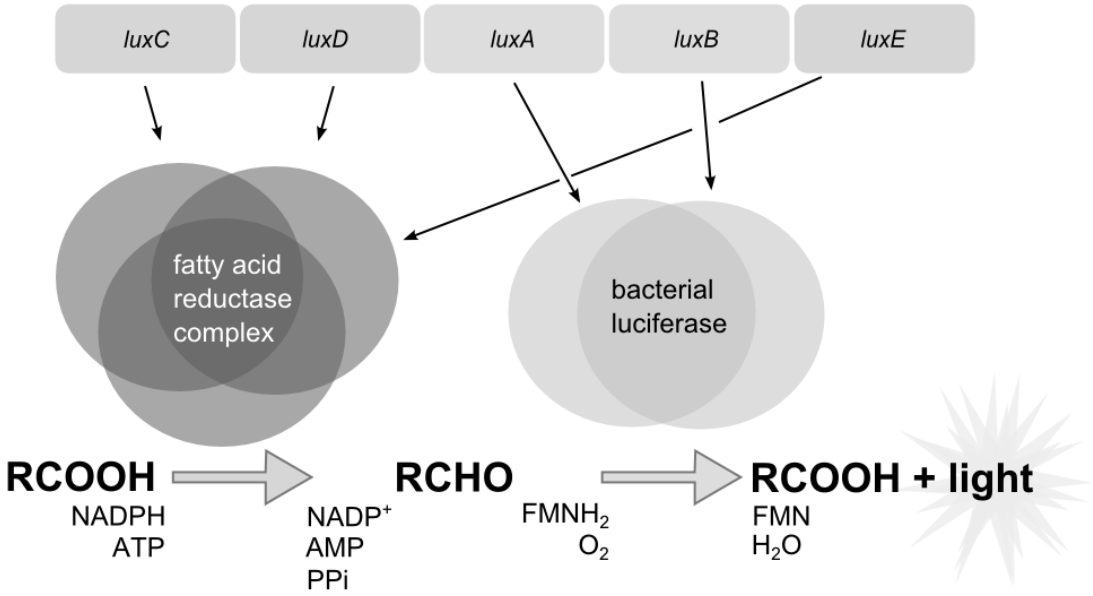

FIGURE 3.6. A schematic representation of the *P. luminescens lux* operon, and the enzyme reactions and cofactors required for bioluminescence production. RCOOH – fatty acid, RCHO – fatty aldehyde.

A majority of bacterial luciferase applications are related to biomedicine (e.g. tumor research) or detection of xenobiotics, heavy metals, or antibiotics in environmental and food samples (D'Souza 2001; Galluzzi and Karp 2006; Close et al. 2012). In a typical whole-cell biosensor, the *lux* operon is expressed under an appropriate inducible promoter (Immonen and Karp 2007; Virolainen et al. 2008), and the bioluminescence production is thus regulated at transcriptional level as a result of defined external stimulus.

As bacterial luciferase produces light specifically through the reaction with an aldehyde molecule, the system holds great potential for new metabolic level sensors; the mechanism enables highly specific and rapid detection which is not dependent on the regulation of transcription or translation. Moreover, the enzymatic reactions serve as indicators for intracellular cofactor metabolism. In a study by Falls et al, the *P. luminescens lux* operon was introduced to a complete library of *E. coli* single gene knock-out mutants. It was shown that the expression of a non-native pathway coupled with a specific gene deletion results in a rearranged distribution of cellular resources and changes in biomass production (Falls et al. 2014). Thus, expressing the Lux system can give implications to studying changes in the cellular performance and physiological state as a result of genetic modifications. The modularity, the instant and linear response to cellular metabolite and cofactor levels, and the long history of

consistent research on the *lux* mechanisms, promote the use of the enzyme complex as a valuable and competent tool for developing elaborate and sophisticated systems for synthetic biology applications (Reeve et al. 2014).

4 Synthetic cocultures

Coculturing of genetically different strains or species is a rapidly evolving new trend in synthetic biology (Brenner et al. 2008; Goers et al. 2014). Cocultures can provide several advantages over monocultures by being catabolically more versatile, balancing biochemical and physical perturbations, increasing the level of modularity, and performing distributed multi-step tasks (Figure 2.12). As a result, more robust production systems with improved bioprocess characteristics and stability can be developed.

Mixed populations with natural cooperation mechanisms and fluctuating population dynamics have been long exploited in applied microbiology, but such natural systems lack the possibility for systematic process control and genetic engineering. In recent years, the focus of studies has shifted from naturally evolved and differentiated strains (Le Gac et al. 2008) towards more controlled and predictable culture platforms. In the synthetic biology approach the principles of rational design and engineering are applied on the coculture systems allowing the establishment of defined artificial connections and cooperation in the consortia.

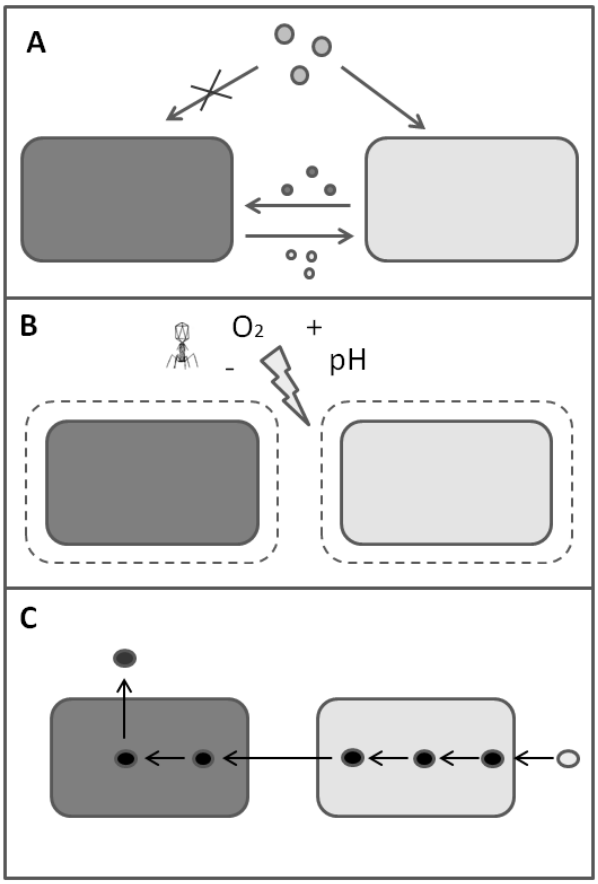

FIGURE 2.12. Cocultures can promote A) an efficient substrate utilization (Eiteman et al. 2008; Eiteman et al. 2009), B) a stability and tolerance against environmental perturbations and rapidly changing conditions (Briones and Raskin 2003; Brenner and Arnold 2011), and C) the performance of complex multi-step tasks (Tsai et al. 2010; Regot et al. 2011).

Coculture approach holds potential for a substantial number of industrial and medical applications (Bermudez-Humaran et al. 2011; Brune and Bayer 2012; Shong et al. 2012; Ortiz-Marquez et al. 2013) and serves as a platform for foundational studies regarding population interactions and cell-cell communication (Xavier 2011; Pawelczyk et al. 2012; Tanouchi et al. 2012). Computational modeling and genome wide analyses of cocultures increase the level of understanding and the predictability of the complex interactions (Salimi et al. 2010; Hanly and Henson 2011; Bernstein and Carlson 2012). In several studies, coculturing has been shown to improve substrate utilization and biomass production, and benefit overall cell performance; in a study by Bernstein et al. (2011), a coculture of ‘primary’ glucose-positive and ‘secondary’ glucose-negative *E. coli* strains resulted in up to 50 % improvement in biomass productivity compared to the monocultures. The improvements were gained through the consumption of end-

metabolites by the secondary strain, thus neglecting the growth inhibiting effects of byproducts, namely acetate (Bernstein et al. 2011). Using similar strategy, cocultures can be engineered for a simultaneous catabolism of different sugars allowing more efficient utilization of heterogeneous feedstocks such as lignocellulose hydrolysates (Eiteman et al. 2008). Interestingly, cocultures can also promote cell survival and resistance to environmental perturbations by coordinated cell self-organization; Brenner and Arnold demonstrated the formation of non-random spatial structures by symbiotic engineered *E. coli* strains resulting in improved biomass accumulation and population persistence (Brenner and Arnold 2011).

As a topical subject, sustainable bioenergy production is a target field in which coculturing can be exploited to tackle challenges related to product tolerance, efficient utilization of industrial heterogeneous substrates, and endured robustness and stability in large-scale bioprocesses. For the improved production of bioethanol, cocultures employing task distribution in engineered populations have been described; in a study by Shin et al. (2010) two *E. coli* populations were differentially engineered to produce and secrete hemicellulases for hemicellulose hydrolysis, and to convert the sugars to ethanol in a single-step process (Shin et al. 2010). In another study by Tsai et al. (2010), a consortia comprising of three different yeast strains was engineered to display a minicellulosome for a direct conversion of cellulose to simple sugars, followed by a conversion of glucose to ethanol (Tsai et al. 2010). For improved hydrogen production, coculturing *Clostridium butyricum* and *E. coli* resulted in increased hydrogen titers and more efficient glucose utilization compared to the monocultures (Seppälä et al. 2011).

Regot et al. (2011) introduced a synthetic biology approach to establish a multicellular network for distributed biological computing, enabling the construction of a complex synthetic device constituting of multiple Boolean logic gates (Regot et al. 2011). In the study, yeast cells were engineered to exhibit different functions according to defined inputs and an output, which could be combined and connected by multiple ways, resulting in the construction of complex synthetic circuits. It was demonstrated that using only 2-5 cell types and three inputs in the system, hundreds of different functions could be executed. Exploiting consortia instead of a single strain for computing allows the construction of more complex, combinatorially powerful, and programmable genetic circuits not realizable in a single cell.

Harnessing quorum sensing mechanisms for coordinated task performance enables the construction of spatially and temporally defined networks. The study of Basu et al. (2005) beautifully demonstrates the power of engineered multicellular communication

systems in the formation of programmed visual patterns and shapes. The pattern formation is based on ‘sender’ cells producing acyl-homoserine lactone (AHL), typical bacterial signaling molecules, and ‘receiver’ cells, producing fluorescent protein in response to defined concentration of AHL. The sophisticated activator-repressor regulator system resulted in protein expression only at a certain distance from the sender cell (Basu et al. 2005). In a study by Tamsir et al. quorum sensing molecules were used as connectors between simple gates in different cells, allowing the construction of all 16 type of logic gates according to the spatial arrangement of sender and receiver cells (Tamsir et al. 2011).

For the lack of universal genetic tools and limited knowledge on the metabolism and genetics of potential hosts, most of the reported rationally engineered coculture systems involve two or more strains or mutants of the same species. Especially in synthetic biology, the work is mostly concentrated on *E. coli* or *S. cerevisiae* based systems, thus partly limiting the coculture characteristics and potential. However, using different species in a coculture is supported by certain aspects; potentially more orthogonal process design can be achieved due to the lack of specific inter-species quorum sensing and regulatory mechanisms. Also, the different carbon metabolism patterns allow more efficient utilization of a wider range of carbon molecules and restrain the accumulation of a single compound in the culture. In scale-up processes, susceptibility to bacteriophage contamination can be decreased by using two species instead of a single strain. In future, the increasing number of well-characterized genetic tools and alternative synthetic biology hosts will probably expand the field of coculturing towards well-defined robust multicellular systems, with distinct properties superior to single-cell based pure cultures.

5 Hypotheses and objectives of the study

The sustainable production of bioenergy molecules and other industrially relevant compounds is one of the central goals of synthetic biology. Long chain hydrocarbons such as fatty aldehydes, fatty alcohols, triacylglycerols and wax esters are exploited by several industries for a broad range of applications. The demand for biological production systems is growing to replace conventional processes using fossil or food-chain related feedstock. In order to achieve sufficient production rates, profound understanding about the biosynthesis pathways and regulatory systems is required. As a major challenge with regard to hydrocarbon production is the limited availability of dynamic, high-throughput monitoring and analysis tools; the conventional methods are laborious, time-consuming, and they reveal no information about the production dynamics.

Efficient tools and expression systems are required in the construction of a robust cell factory. However, an optimal chassis with desired characteristics is of equal importance. Certain aspects such as comprehensive knowledge on the cell genetics and metabolism, and availability of straight-forward engineering tools are essential, but the special features of the cellular machinery should be individually considered according to the application; does the cell provide the required precursors or cofactors at sufficient rate? Is there a need for extensive ‘construction work’ to enable the expression of the pathway? How does the cell cope with the physiological and environmental perturbations caused by the built system? How could the natural properties of the cell be efficiently exploited? In addition to optimizing the host cell, possibilities for multicellular approach promoting task distribution and metabolic balancing should be considered.

To fully realize the production process, that is, to take the process to an industrial scale, issues related to patents and licensing need to be considered. Despite *Escherichia coli* often being the most convenient choice for a process, IPR issues can put obstacles in the way.

To sum up, my study considers the above mentioned limitations, issues, and challenges of existing synthetic biology production systems and approaches with regard to long chain hydrocarbon production and demonstrates the possibilities and benefits of exploiting an alternative bacterial host, *Acinetobacter baylyi* ADP1, as a cellular factory and model system for synthetic biology.

The specific **objectives** and hypotheses of the study are:

1. **To establish a model platform based on *A. baylyi* ADP1 for the improved production of lipid compounds employing metabolic engineering approach (I)**

   According to the hypotheses, the previously constructed metabolic model can be exploited to find gene deletion targets that beneficially affect the lipid, or more specifically, triacylglycerol production. The genome engineering can be conducted by established molecular methods and the predictions are verified experimentally. Single and/or multiple gene deletions increase the lipid titers, productivity or proportion of triacylglycerols in total lipids. The results give important clues of the significance of gene deletions to the lipid metabolism of *A. baylyi* ADP1.

2. **To develop a dynamic *in vivo* tool for studying and monitoring the long chain hydrocarbon metabolism (II)**

   According to the hypotheses, the reporter enzyme bacterial luciferase can be employed as a sensor for intracellular long chain aldehydes. The presence of aldehydes can be detected by measuring bioluminescence produced by the bacterial luciferase. As aldehydes are specific and dynamic intermediates of the wax ester synthesis pathway in *A. baylyi* ADP1, monitoring the bioluminescence gives important information about the wax ester synthesis patterns and production kinetics. The monitoring system can be exploited in the optimization of the production host and conditions. The tool can be also applied to other pathways involving aldehyde intermediate.

3. **To demonstrate the significance of an optimal chassis; developing a monitoring tool for studying alkane metabolism by the integration of a well-known DNA component to a novel context (III)**

   According to the hypotheses, previously described biosensors for alkane detection can be replaced with a simpler design by choosing an optimal cell framework exhibiting the relevant pathway(s) combined with an appropriate sensor element. *A. baylyi* ADP1 provides the required biocomponents for alkane uptake and processing. Degradation of alkanes produces an aldehyde intermediate which can be detected by the bacterial luciferase. The constructed system not only enables the detection of alkanes, but also provides fundamental information about the natural degradation kinetics and patterns.

4. **To reconstruct the wax ester synthesis pathway for modified products in *A. baylyi* ADP1 using synthetic biology tools (IV)**

   According to the hypotheses, the chemical composition of the wax esters of *A. baylyi* ADP1 can be modified by replacing a natural key enzyme of the pathway with an alternative enzyme exhibiting a defined substrate range. A well-characterized biocomponent, fatty acid reductase complex LuxCDE, can be employed for the wax ester synthesis. The reductase complex provides aldehyde precursors mostly consisting of C10-14 chains, resulting in alcohol moieties of respective chain lengths in the final product. Introducing the synthetic pathway allows the regulated production of a custom-made bioproduct.

5. **To design and engineer a synthetic coculture for improved cell performance and product formation (V)**

   According to the hypotheses, using a glucose-negative mutant strain of *A. baylyi* ADP1 supports *E. coli* growth in a coculture by efficiently removing toxic acetate from the culture medium. This improves the *E. coli* biomass production and reduces the need for process control and optimization. Both strains can be harnessed for the production of a relevant compound by well-established engineering tools. The product titers in a simple batch

process coculture are higher compared to the corresponding pure cultures of *E. coli*.

6 Summary of materials and methods

The details for the materials and methods used in the studies are described in papers **I-V.**

6.1 Strains

Acinetobacter baylyi ADP1 (available at German Collection of Microorganisms and Cell Cultures, under accession number DSM 24193) was used in the studies as the wild type strain. Single gene knockout mutant strains (Table 5.1) were kindly provided by Veronique de Berardinis (Genoscope, France). In the single gene knock-out mutants, the gene in question is replaced with a gene cassette containing a kanamycin resistance gene (*kan*^{r}). Double and multiple gene knockout strain constructions are described in 'genetic engineering' section. For coculture studies (**V**), *E. coli* K12 BW25113 (from Yale *E. coli* Genetic Stock Center CGSC, Connecticut, USA) was used.

TABLE 6.1. Single gene knockout mutant strains used in the studies.

gene ID	Gene name	Gene product	EC	Reference
ACIAD2837	*dgkA*	diacylglycerol kinase	EC 2.7.1.107	**I**
ACIAD3383	*acr1*	fatty acyl-CoA reductase	EC 1.2.1.n2	**I, II, III, IV**
ACIAD2844	*glpD*	glycerol-3-phosphate dehydrogenase	EC 1.1.5.3	**I**
ACIAD2425	*cyoA*	cytochrome o ubiquinol oxidase subunit II	EC 1.10.3.-	**I**
ACIAD2426	*cyoB*	cytochrome o ubiquinol oxidase subunit I	EC 1.10.3.-	**I**
ACIAD2291	*cydB*	cytochrome d terminal oxidase polypeptide subunit II	EC 1.10.3.-	**I**
ACIAD3381	*poxB*	pyruvate dehydrogenase (cytochrome)	EC 1.2.2.2	**I**
ACIAD3648	*estA*	carboxylesterase	EC 3.1.1.1	**I**
ACIAD1134	*aesT*	esterase	-	**I**
ACIAD3309	-	lipase	EC 3.1.1.3	**I**
ACIAD1121	*lip1*	triacylglycerol lipase	EC 3.1.1.3	**I**
ACIAD0544	*gntT*	high-affinity gluconate permease (GntP family)	-	**V**

6.2 Genetic engineering

The molecular work was carried out by standard procedures (Sambrook et al. 1990) or according to BioBrick cloning standard (Shetty et al. 2008). Primers were ordered from ThermoFisher Scientific (USA) with appropriate restriction sites.

6.2.1 Plasmid and gene cassette construction

An integrative gene cassette was used as a scaffold for genomic deletions and insertions (**I**, **II**, **III**, **IV**). The gene cassette (Figure 6.1) was constructed *in vitro* and contains the following components: 1) a flanking region upstream of the target gene, 2) a promoter, 3) a multiple cloning site (MCS), 4) a selection marker, 5) a transcription termination loop, and 6) a flanking region downstream of the target gene.

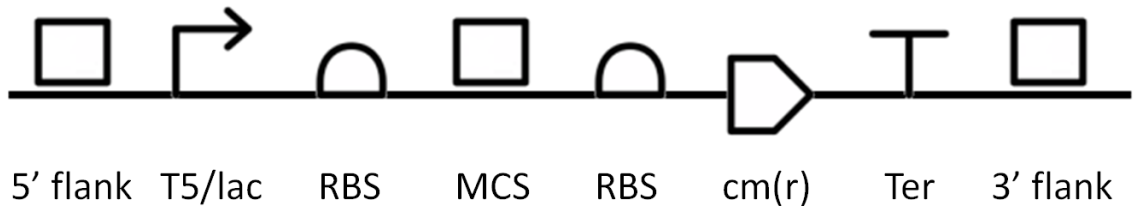

FIGURE 6.1.The synthetic gene cassette employed for targeted gene deletions and chromosomal insertions. 5’ flank – a homologous region upstream the target site, T5/lac – a promoter, RBS – Ribosome binding site, MCS – multiple cloning site, cm(r) – chloramphenicol resistance gene *cat*, Ter – transcription termination loop, 3’ flank – a homologous region downstream of the target site.

For plasmid expression in ADP1 (**IV**, **V**), vectors derived from pBAV1K (Murin et al. 2012) were used (Figure 6.2).

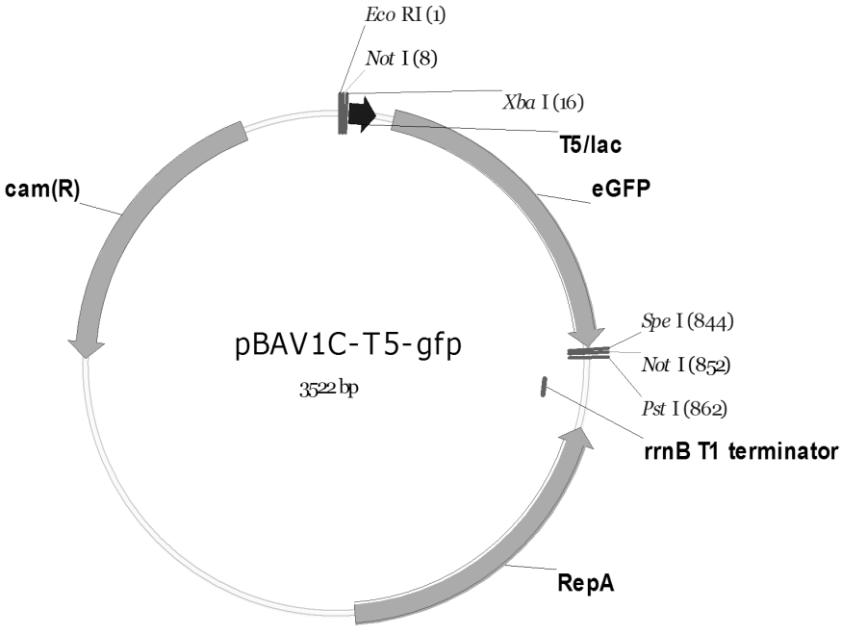

FIGURE 6.2.The expression vector pBAV1C-T5-gfp. The reporter eGFP is expressed under a T5/lac promoter. The vector contains the restriction sites for BioBrick cloning. The described construct was used in In paper **V,** whereas in paper **IV** pBAD and *luxCDE* replaced the T5/lac and *gfp.*

All the genetic constructs used in the studies are listed in Table 6.2.

TABLE 6.2. The list of genetic constructs used in the studies.

Name	Type	locus	Promoter	Insert	Selection marker	Reference
iSM100cz'	Integ. cassette	ACIAD3381-3383	T5/lac		cm	**I**
iluxAB_Cm^r	Integ. cassette	ACIAD3381	T5/lac	*luxAB*	cm	**II**, **III**, **IV**
pVKK81-T-*lux*	plasmid		lac	*luxCDABE*	tet	**V**
pBAV1C-*ara*-*luxCDE*	plasmid		BAD	*luxCDE*	cm	**IV**
pBAV1C-*ara*	plasmid		BAD		cm	**V**
pBAV1C-T5-GFP	plasmid		T5/lac	*gfp*	cm	**V**
sfGFP/pAK400c	plasmid		lac	*gfp**	cm	**V**

*superfolder variant

6.2.2 Transformation

Transformations of *E. coli* were carried out by standard electroporation procedures (Sambrook et al. 1990). For natural transformation of ADP1, a methodology described previously was used (Metzgar et al. 2004); a linear DNA fragment (PCR product) with flanking regions of the target site in genome or plasmid vector was inserted to ADP1 cultivation in an exponential growth phase. The cultivations were conducted at 30 °C and stirring of 300 rpm using LB medium or MA/9 minimal salts medium supplied with glucose. For transformation, 1-2 µg DNA was used per 1 ml of cultivation. After insertion, the cultivations were incubated for 2-3 h and then spread on a selective LA plate supplemented with glucose and appropriate antibiotic. Concentrations for selective antibiotics were 30-50 µg/ml for kanamycin, 25-50 µg/ml for chloramphenicol, and 10 µg/ml for tetracycline, respectively. The plates were incubated at 30 °C until colonies appeared. Negative controls were cultivated in the same method except for insertions sterile water was used instead of DNA. The constructs in the obtained strains were verified with colony PCR and further by sequencing.

6.3 Cultivations

A modified Luria-Bertani medium (**V**; 1 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 3 µM $FeCl_3$) or minimal salts medium MA/9 (**I**-**V**; $Na_2HPO_4 \cdot 2\ H_2O$ 5.518 g/l, KH_2PO_4 3.402 g/l, NH_4Cl 1 g/l, nitrilotriacetic acid 0.008 g/l, NaCl 1.0 g/l, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 3 µM $FeCl_3$, and trace element solution (**I**)) supplemented with a carbon source, casein amino acids, antibiotics, and an inducer, when appropriate, was used in the studies. Gluconate (**I**), glucose (**II**, **III**, **IV**, **V**), alkanes (**III**) or acetate (**V**) were used as carbon sources. For bioreactor cultivations (**V**), a 1-litre vessel (Sartorius Biostat B plus Twin System, Germany) with online pH monitoring

system, stirring, and oxygen supply was used. Cultivations were carried out at 20-37 °C.

6.4 Output analyses

The analysis methods and instrumentation used in the studies are presented in Table 6.3.

TABLE 6.3. Analytical methods and instruments used in the studies.

Analysis	Method/Instrument	Reference
Biomass		
Optical density	Spectrophotometer, 600 nm	I-V
Cell dry weight	Freeze-drying; gravimetric	I-V
Metabolic predictions	*In silico* simulations; FBA	I, V
Lipids		
Total lipids	Solvent extraction; gravimetric	I, II, IV
	+ GC-FID	I
	+ GC-MS	IV
	+ TLC	I, II, IV
TAG	Solvent extraction	I, IV
	+ SPE / FAME derivation, GC-FID	I
	+ preparative TLC; gravimetric	I
	+ HPLC-GPC	IV
WE	Solvent extraction	I, II, IV
	+ TLC	I, II, IV
	+ NMR	
FA, Alcohols	Solvent extraction, GC-FID, GC-MS	IV
Sugars, end-metabolites	HPLC	I, II, V
Luminescence	Microplate Reader (Victor 2)	II, III, IV, V
	Xenogen IVIS Lumina II	II, III
Fluorescence	Microplate Reader	
	(Fluoroskan Ascent FL, ex/em 485/538)	V

7 Summary of results and discussion

This chapter provides an overview of the results obtained in the research, and compiles the thoughts and future prospects arisen during the process. The individual results and observations are more specifically discussed in the original papers **I**-**V**.

7.1 Improving the lipid quantity and quality (I, IV)

For the improved production of TAGs in *A. baylyi* ADP1 (**I**), computational tools and manual comparative analysis were exploited in identifying target genes for the elimination of potential competitive pathways and the redirection of product precursors. An extended constraint-based metabolic model of ADP1 was employed for the computational analysis. After a preliminary screening of eleven potential individual single gene knock-out mutants (Figure 7.1), the four most relevant deletions, fatty acyl-CoA reductase *acr1* (ACIAD3383), pyruvate dehydrogenase *poxB* (ACIAD3381), diacylglycerol kinase *dgkA* (ACIAD2837), and a triacylglycerol lipase (ACIAD3309), were chosen for further studies. Briefly, the hypotheses of the effects of these deletions on TAG metabolisms were the following: A strain lacking a fatty-acyl-CoA reductase is incapable of wax ester synthesis, thus redirecting the carbon flow towards TAG synthesis. Eliminating the TAG lipase potentially prevents the degradation of the product of interest. The diacylglycerol kinase directs 1,2-diaclyglycerol, an important precursor of TAG, to phospholipid synthesis competing with TAG synthesis. The pyruvate dehydrogenase is associated to acetate production, and therefore the deletion can redirect the carbon flux towards storage lipid synthesis.

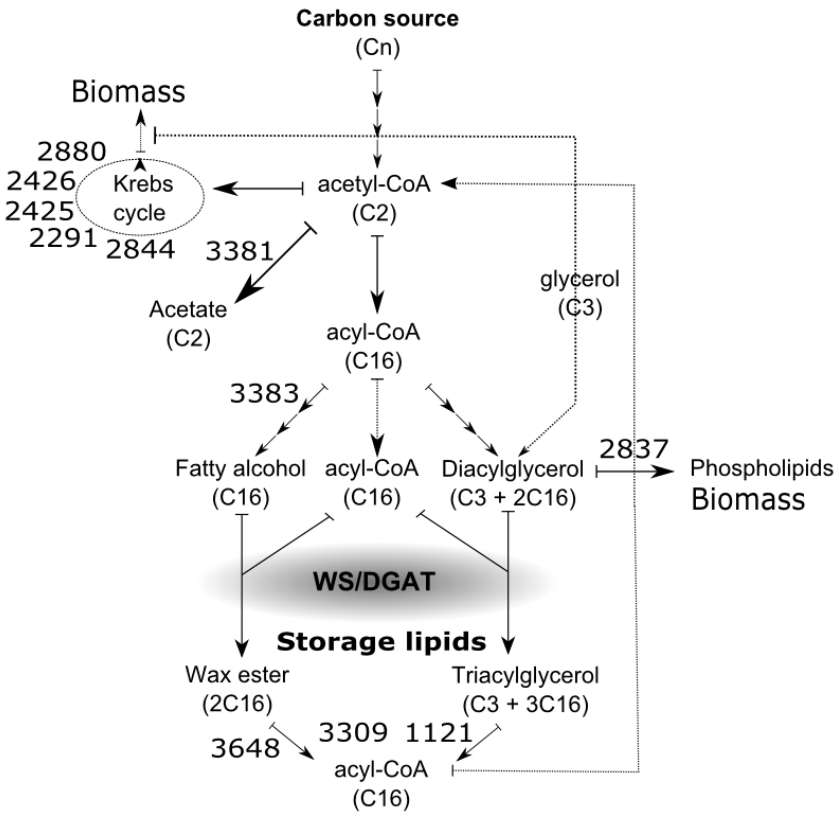

FIGURE 7.1.The metabolic pathways of ADP1 relevant to the storage lipid metabolism. The ACIAD gene numbers of the potentially beneficial gene deletions are shown. The specific functions of the genes and the effect of deletions on the TAG synthesis are described in paper **I** and in (Aho et al. 2012).

Three of the deletions (i.e. *acr1*, *poxB*, and the triacylglycerol lipase) were combined in a single strain (referred as strain MT). As a result, the strain MT produced 5.6 fold more triacylglycerol (mg/g cell dry weight) compared to the wild type strain, and the proportion of triacylglycerols in total lipids was increased by 8-fold. Of individual deletions, the *acr1* knock-out eliminating the WE pathway had the most impact on TAG synthesis; this was further confirmed by the subsequent analyses (**IV**) showing that the diacylglycerol precursors are strongly directed to TAG synthesis in the *acr1* mutant. In addition, it has been suggested that when alcohol substrate is available in a cell, the activity of WS/DGAT is strongly shifted towards WE synthesis (Kalscheuer and Steinbüchel 2003; Kaiser et al. 2013), thus relatively increasing the TAG synthesis in the absence of fatty alcohols. By contrast, deleting the *poxB* or the hypothetical lipase ACIAD3309 results in an increased WE production (Aho et al. 2012).

The study experimentally validated the computationally simulated effects of gene knock-outs on the lipid metabolism of ADP1 and proved the coupled effect of

combining several gene deletions. The study demonstrates the convenience of the strain ADP1 as a host for metabolic engineering purposes and the potential for further investigation as a lipid production platform.

The obtained results give indications of the mechanisms affecting the natural lipid synthesis. While metabolic models can indeed predict the effect of gene deletions on the cell metabolism, the coverage of predictions is restricted to the predetermined reactions. Therefore, all potential bottlenecks and carbon consuming routes which indirectly affect the lipid production are not identified by the model. For example, the extracellular polysaccharide (EPS) production known to consume significant amounts of carbon (Kaplan and Rosenberg 1982) were not considered in the simulations. Furthermore, in the steady-state assuming constraint-based model the gene expression levels or the growth phase are not taken into account, resulting in a qualitative rather than a quantitative output. In addition, the homology-based annotation of genes can lead to inaccurate or false predictions.

For a metabolic model to be truly useful, a continuous evolvement and validation are required. For example, the latest update of the genome-wide metabolic model of *E. coli*, EcoCyc–18.0–GEM, encompasses 1445 genes, 2286 reactions, and 1453 metabolites, afforded by a comprehensive integration of computational and experimental data directly from EcoCyc database (Weaver et al. 2014). According to the developers, the new model offers easy operation, frequent updates, and more accurate predictions enabling the more advantageous use of the computational simulations. For ADP1, the on-going experimental annotation of genes and profound analyses of the transcriptomic and metabolic data can provide means to develop a more accurate and quantitative model for ADP1 (Genoscope 2009; Stuani et al. 2014).

Owing to the laborious and multi-step nature of the preparative TLC analytics conducted, the TAG production of only a very limited number of knock-out strains was thoroughly studied. Furthermore, the screening of several knock-out combinations in a single strain could have resulted in further improved productivity. For example, the deletion of diacylglycerol kinase *dgkA* was considered an interesting knock-out target, but the low biomass yields prevented an accurate determination of the TAG yield of the strain. Recently, it was shown that the deletion of *dgkA* combined with the overexpression of WS/DGAT from ADP1 and *fadD* from *E. coli* resulted in a considerably improved production of TAG in *E. coli* (Janßen and Steinbüchel 2014). Similarly to our study, the negative effects of *dgkA* deletion on growth were observed. Nevertheless, in an optimized fed-batch bioprocess, TAG titers of 530 mg/l (8.5 % of CDW) could be achieved (Janßen and Steinbüchel 2014).

The study of Janßen and Steinbüchel demonstrates the necessity of both gene knock-outs and gene overexpression in achieving significant improvements at the cell level. Moreover, a comprehensive bioprocess optimization is required to obtain improved volumetric yields, since the synthesis of neutral lipids strongly competes with biomass production. Although the main goal of my research was to investigate the effects of gene deletions on the lipid metabolism rather than to maximize the productivity, the overexpression of selected key enzymes could have provided important clues of the rate-limiting steps. For example, in the present *E. coli* based production systems the overexpression of *fadD* and '*tesA*, combined with the deletion of *fadE,* are almost routinely done to improve the supply of FA precursors (see Chapter 3.1.3). Interestingly, the overexpression of the ADP1 thioesterase, which is the corresponding enzyme for the widely applied TesA, has been shown to significantly improve the FA synthesis in *E. coli* (Zheng et al. 2012a). Therefore, in our on-going studies the early steps of the lipid production pathway are investigated, involving both the heterologous expression of non-native genes and regulation of the natural pathway (data not shown).

In natural hosts, the synthesis of storage lipid is regulated by environmental conditions, a nutrition supply, and the growth phase. For example, nitrogen limitation in a stationary growth phase is known to induce lipid accumulation in bacteria (Ishige et al. 2002; Wältermann et al. 2005). Bypassing the natural regulation can thus significantly increase the production rates. However, very little is known about the molecular mechanisms affecting the lipid accumulation. For *R. opacus*, a gene contributing to the regulation of lipid metabolism was previously identified (MacEachran and Sinskey 2013). In nitrogen limiting conditions, the gene (referred as *tadD*) was shown to metabolize glyceraldehyde-3-phosphate to 3-phosphoglycerate yielding NAD(P)H, a crucial cofactor for fatty acid biosynthesis. For ADP1, however, protein homologous to the described TadD is not present.

The first attempt to regulate the lipid synthesis of ADP1 was made in reconstructing the WE synthesis pathway (**IV**). The natural fatty-acyl CoA reductase Acr1 was replaced with a well-characterized FA reductase (FAR) complex LuxCDE from *P. luminescens*. The rewired pathway employing an inducible arabinose promoter pBAD enabled a regulated production of synthetic WEs. The study also revealed a strong temperature dependency of the WE production: within a range of 20-37 °C, most WEs were produced at 20 °C. Due to the different substrate specificity of LuxCDE compared to Acr1, the WE profile of the engineered strain was altered; the alkanol chains of the synthetic WEs were slightly shorter and more saturated in comparison with the WEs of the wild type strain, the major constituent being C16:0 alkanols. Furthermore, C12:0 alkanols, which are very rare in the wild type strain, were detected in the engineered

strain. However, the most preferred substrates of LuxCDE, C14 alkyl groups, were absent. As discussed in paper **IV**, most probably the native aldehyde reductases affect the final product quality. Thus, a feasible approach for neglecting the endogenous aldehyde reductase(s) could be an expression of a multifunctional FAR that converts the FA (-CoA or -ACP) substrates directly to alcohols without the aldehyde intermediate.

Even though the product titers were not improved by the synthetic pathway, the results encourage for further development and enhancement of the WE synthesis platform. Furthermore, demonstrating the utility of the well-characterized LuxCDE complex in the production of hydrocarbons extends the possibilities to choose the most optimal components to build up pathways with specified properties and modularity. Evidently, the increased knowledge on the enzyme functions and pathways facilitates the expansion of the synthetic biology product repertoire (Layton and Trinh 2014; Rodriguez et al. 2014).

With regard to the obtained titers of synthetic WEs, there are several potential reasons why the reconstructed system did not increase the WE production rates. Firstly, considering the role of LuxCDE in its natural context, the activity of the enzyme complex is probably not very high; consuming large amounts of cofactors and valuable carbon molecules for bioluminescence production would not be energetically affordable. In addition, in the constructed expression platform natural regulatory elements upstream of LuxE were disrupted, thus potentially affecting the expression levels. Furthermore, the bacterial luciferase and the FAR complex assumingly recycle the FA substrate in the reactions, thus not significantly interfering with the natural lipid metabolism of the cell. This indicates a relatively low catalytic activity and affinity of LuxD towards the activated cellular FAs, which in turn implies minor contribution of LuxD to the WE synthesis, as discussed in paper **IV**. Furthermore, as implied by Smanski et al. (2014), a simple insertion of a component without further optimization very improbably increases the production rates.

By contrast, the introduction of an alternative LuxCDE complex from *Vibrio harveyi* resulted in production of tetradecanol (C14) in *E. coli* (Rodriguez et al. 2014). A simultaneous expression of an ester forming acyltransferase Atf1 from *S. cerevisiae* led to the production of tetradecyl acetate. It is unclear why the described expression platform produced C14 acyl groups, while they were not detected in the WEs of the engineered ADP1 (**IV**). This discrepancy can be due to the very different cellular environments of the hosts. For example, the ADP1 enzymes involved in the lipid synthesis are typically membrane-bound and may possess unique enzymatic

interactions affecting the substrate flow and preference (Wältermann et al. 2005). In addition, the differences in the specificities between the endogenous alcohol dehydrogenases of *E. coli* and ADP1 probably affect the product quality. It is also possible that the *V. harveyi* and *P. luminescens* FAR complexes, and more specifically the LuxD counterpart, exhibit differential activity (the acyltransferases show 70 % similarity at the amino acid level). For example, the higher affinity of the *V. harveyi* LuxD towards tetradecanoyl-ACPs would theoretically terminate the FA elongation and allow the assimilation of C14 FA for aldehyde synthesis.

The insufficiency of the *P. luminescens* LuxD to provide C14 acyl groups for aldehyde synthesis in a recombinant host was also observed by Howard et al (2013). The *P. luminescens* LuxCDE FAR complex and an aldehyde decarbonylase (*Np*AD) were exploited in the production of variable branched and aliphatic alkanes. The study utilized the FAR complex for a direct conversion of free FAs to aldehydes. This approach was suggested to allow a more tractable specification of the output products via the modifications of the cellular FA pool. It is noteworthy that the acyltransferase LuxD is previously described to utilizing activated FAs as a primary substrate (Meighen 1991). To elucidate this contradiction, the study showed that free FAs can be directly converted to aldehydes by LuxE and LuxC solely. The potential of LuxC to directly accept activated FAs for the reduction reaction has been also discussed in the literature (Wall et al. 1986). In the study of Howard et al., providing exogenous FAs or introducing a specific thioesterase activity, such as FatB1 from *Cinnamomum camphora*, resulted in the production of alkanes with acyl groups of corresponding lengths. However, in the presence of LuxCDE and *Np*AD solely, only minor proportions of the predicted C13 alkanes (yielding from C14 aldehydes) were produced, thus demonstrating the redundancy of LuxD in terms of the transferase activity. However, it was proven that the role of LuxD, albeit not completely clarified, is significant for the catalytic activity of LuxC and LuxE through protein-protein interactions, thus supporting the findings of my study (paper **IV**, suppl.). Altogether, elucidating the role of LuxD, and especially the possibility to engineer the FA(-CoA) pool of ADP1 require further research efforts. Moreover, our future studies will involve the screening of other potential reductases and thioesterases in terms of improved WE production.

Similarly to TAG production, not much is known about the regulation or expression levels of the key enzymes of the WE synthesis pathway. For example, the molecular mechanisms behind the temperature dependency of WE production are not established. It has been shown, however, that only a few copies of the terminal enzyme, WS/DGAT, are present in the cells (Wältermann et al. 2005). We have conducted preliminary studies to investigate whether an overexpression of the native

enzymes improves the WE production. The early results indicate that the overexpression of *acr1* increases the amount of WEs in ADP1, whereas increased WS/DGAT levels do not significantly affect the WE production in the studied conditions (data not shown). Although numerous studies have exploited the ADP1 originated WS/DGAT in the production of esters, other potential acyltransferases/wax ester synthases have been introduced (Barney et al. 2012; Shi et al. 2012). Thus, in order to overcome the potential limitations with regard to the native regulation and product range, the expression of a non-native terminal acyltransferase for lipid production in ADP1 might be profitable.

While only modest WE titers were achieved using glucose as a substrate (**II**, **IV**), it is known that ADP1 naturally produces large amounts of WEs when grown on alkanes. Ishige et al. (2002) obtained 17 % WEs of the cell dry weight in a 10 h cultivation using 3 % n-hexadecane as a substrate. The WEs were almost exclusively (98 %) found to compose of hexadecyl-hexadecanoate, indicating the possibility for very precise determination of the product quality. Even though refined alkanes are not currently considered as a sustainable carbon source, elucidating the mechanisms behind the efficient conversion of long chain hydrocarbons to WEs could promote the processes exploiting more appropriate carbon sources. On the other hand, streams containing impure alkanes, such as waste streams or oil spills, could serve as an economical and sustainable substrate for an efficient recovery of the hydrocarbons in the form of valuable WEs.

Altogether, ADP1 can be concluded to possess potential for the production of long chain hydrocarbons, especially WEs. What is more, a lot of engineering and research efforts have been dedicated to redirect and increase the central carbon flux of *E. coli* towards acyl-CoAs, the key precursors of valuable hydrocarbons (Xu et al. 2014). As a natural producer of acyl-CoA derived hydrocarbons, the central carbon metabolism of ADP1 is able to provide acyl-CoAs at sufficient rates. Furthermore, the existing metabolic network for the long chain hydrocarbon production endorses the straightforward construction of several relevant pathways in ADP1. However, in addition to engineering the individual enzymatic steps to increase the supply of precursors, the redox balance (Singh et al. 2011) and cofactor regeneration (Wang et al. 2013; Akhtar and Jones 2014) need to be considered. As an alternative to produce biomolecules through fermentations from simple sugars, the catalytic diversity of ADP1 could afford selective biotransformations to produce enantiomerically pure biomolecules from provided precursors (Ishige et al. 2005; Molinari 2006).

7.2 Constructing tools for monitoring the hydrocarbon metabolism of ADP1 (II, III)

Conventionally, the lux multienzyme complex has been utilized in an orthogonal manner, i.e. independent of the host metabolism. The complex can be divided into two functional parts, an aldehyde producing unit LuxCDE, as demonstrated in paper **IV**, and a fatty acid and light producing unit LuxAB. In my study, the functional units of the *lux* complex were exploited independently and integrated into the ADP1 metabolism. The aldehyde producing unit provides intermediates for the production of customized long chain hydrocarbons, and vice versa, the integration of the light producing unit enables the detection of endogenous long chain aldehyde formation.

The bacterial luciferase LuxAB from *P. luminescens* was exploited in constructing a real-time monitoring tool for studying the WE production of ADP1 (**II**). The *P. luminescens* luciferase was chosen for its high stability (Szittner and Meighen 1990). The sensor represents a post-translational (metabolite level) detection of the target molecule; the detection of WE formation is based on the light-producing reaction between the bacterial luciferase enzyme and a fatty aldehyde molecule (Figure 7.2). The long-chain aldehyde molecule represents a specific intermediate of the WE synthesis route, enabling a very sensitive and specific detection of the molecule.

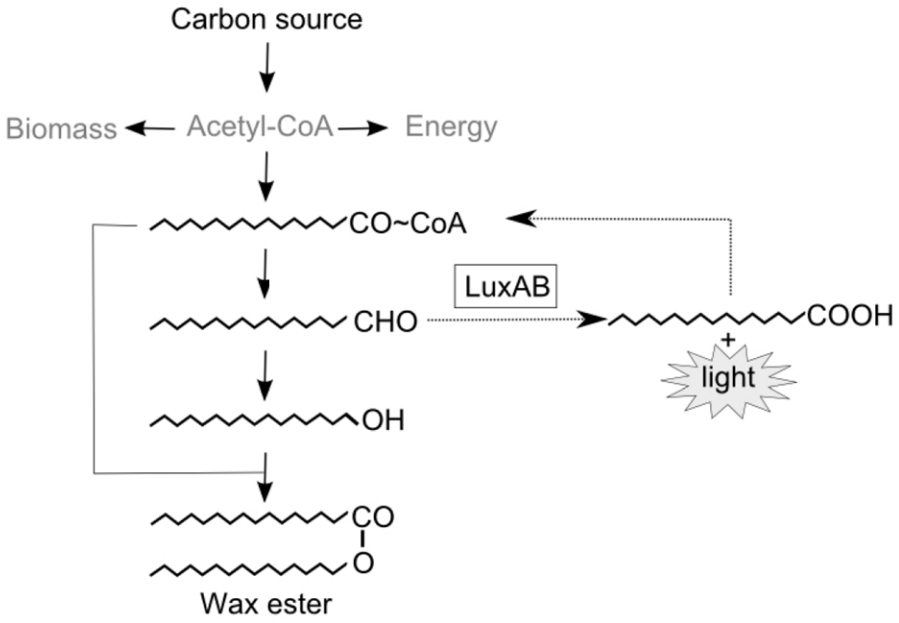

FIGURE 7.2.The proposed WE synthesis pathway of ADP1. The integration of the bacterial luciferase (LuxAB) enables the real-time detection of the fatty aldehyde intermediate. The fatty acid molecule produced in the reaction is returned to the carbon cycle (Modified from paper **II**).

Monitoring the WE production in variable conditions showed correlation between the luminescence signal and the WE synthesis pattern, which was further verified with TLC and NMR analyses. The experiments revealed that WEs are dynamic storage molecules, which are produced and degraded in the cells in different phases of the cultivation. Furthermore, it was confirmed that the WE synthesis is activated in nitrogen limiting conditions. Expressing LuxAB in ADP1 did not significantly affect the WE production rates compared to the wild type strain, indicating that only minor metabolic burden is caused by the tool expression.

The described monitoring system serves as a valuable tool for complementing the laborious lipid analysis methods, especially in high-throughput studies and screening purposes. The sensor does not require inclusion of an external substrate, and thus the monitoring of cultivations can be conducted in an automated manner without sampling. Moreover, the tool provides new information regarding the WE production dynamics, not interpretable by conventional means. In our recent studies, the monitoring tool has been exploited in screening optimal carbon sources and growth temperatures for WE production (data not shown).

The well-characterized and widely applied bacterial luciferase can be readily expressed in a variety of hosts. Apart from studying the native WE metabolism of ADP1, the tool has shown promise in searching novel reductase and dehydrogenase activities, demonstrating its applicability in a broad range of hosts and applications (data not shown). Furthermore, as the long chain aldehyde serves as the key intermediate of the pathways leading to fatty alcohol, fatty alkyl ester, and alkane synthesis, the tool could be potentially generalized for the optimization of advanced biofuel and other valuable hydrocarbon production.

Given that the luminescence emission is a result of direct reaction between the substrate and the enzyme present in the cells, the described tool provides an instant and dynamic response to changing aldehyde levels in the cell. Thus, in contrast to the biosensors functioning through transcription regulation, the described system naturally exhibits very high dynamical range. Furthermore, it is not dependent on the transcription and translation machineries during the detection event, and the lag time between the aldehyde formation and luminescence emission is only dependent on the diffusion of the molecules (<0.1 s). Even though not investigated in paper **II**, the sensitivity and the rate of the tool can be potentially even further tuned by adjusting the luciferase expression levels.

Despite solving some major limitations of lipid analytics, few issues that are not discussed in paper **II** exist. For example, the amplitude of the luminescence signal is dependent on the substrate, i.e. aldehyde chain length and properties, and thus the signal cannot be considered quantitative. Therefore, the signal patterns rather than absolute signals are of higher significance. Moreover, the different affinities towards the aldehyde substrates limit the comparability of results obtained from different culture types, hosts, and target products. As bacterial luciferases are generally applied for acting on aliphatic aldehydes with a typical range of C8-C14 acyl groups, it would be interesting to study the enzyme potential of utilizing other types of aldehydes, such as branched, unsaturated, or cyclic aldehydes as substrates. Thus, in order to take a full advantage of the tool harnessed for metabolic studies, insightful knowledge on the substrate range and engineering potential of bacterial luciferases in the light of synthetic biology is required.

Furthermore, it should be taking into account that different bacterial luciferases (e.g. *Vibrio* vs. *Photorhabdus*) may possess different substrate preference and catalytic activity depending on the conditions. On one hand, the distinct properties of the luciferases, such as the heat lability of *Vibrio* spp. luciferases (Escher et al. 1991), can

be potentially exploited in selective and dynamic redirecting of metabolic fluxes, evolving the idea of Falls et al (2014) introduced in Chapter 3.2.1.

Aldehydes themselves are important molecules. In addition to being key intermediates, i.e. one catalytic step away from alcohols, alkanes, FAs, and WEs (Kaiser et al. 2013), aldehydes have intrinsically industrial use, for example in the food industry, and in the synthesis of plastics and rubbers. Moreover, compared to alcohols, the high volatility of aldehydes simplifies the product recovery and alleviates the product toxicity to cells. As a natural producer, the strain ADP1 serves as a potential host for the production of aldehydes. However, the endogenous aldehyde reductases which further convert the aldehydes to alcohols complicate the sufficient production of aldehydes as the final product. Rodriguez and Atsumi have comprehensively investigated the *E. coli* reductases to allow an efficient production of aldehydes and alkanes; the deletion of five potential reductases coupled with a relevant pathway engineering of *E. coli* resulted in production of 35 g/l isobutyraldehyde and 10 g/l isobutanol (Rodriguez and Atsumi 2012). The work was previously continued with the elimination of all 13 known aldehyde reductases, resulting in 90-99 % loss of aldehyde reducing activity (Rodriguez and Atsumi 2014). It can be speculated, that ADP1 probably contains several reductases exhibiting such activity. The key aldehyde reductase(s) involved in the WE synthesis pathways has yet to be characterized although some suggestions have been presented (Kaiser et al. 2013).

It was proven that the degradation of WEs in carbon limiting conditions did not generate background luminescence emission and thus not interfered the specific monitoring of WE synthesis. It was shown, however, that the sensor system could be modified for the monitoring of alkane degradation (paper **III**). Deleting the fatty acyl-CoA reductase *acr1* eliminates the aldehyde production from endogenous sources, thus allowing the unambiguous detection of alkane degradation by the bacterial luciferase (Figure 7.3).

The monitoring system was demonstrated to be applicable to the studied range of aliphatic alkanes C12-18 and for diesel fuel. The tool was functional both for cells actively growing on alkanes and for static cell cultures exposed to alkane samples. Furthermore, the tool possessed high stability in longer cultivations. Thus, the sensor can be proposed for investigating the kinetics of alkane degradation as well as for the detection of alkanes or fuel components in environmental samples. Furthermore, the tool holds potential for the screening of alkane degrading or alkane producing strains or new enzymatic activities.

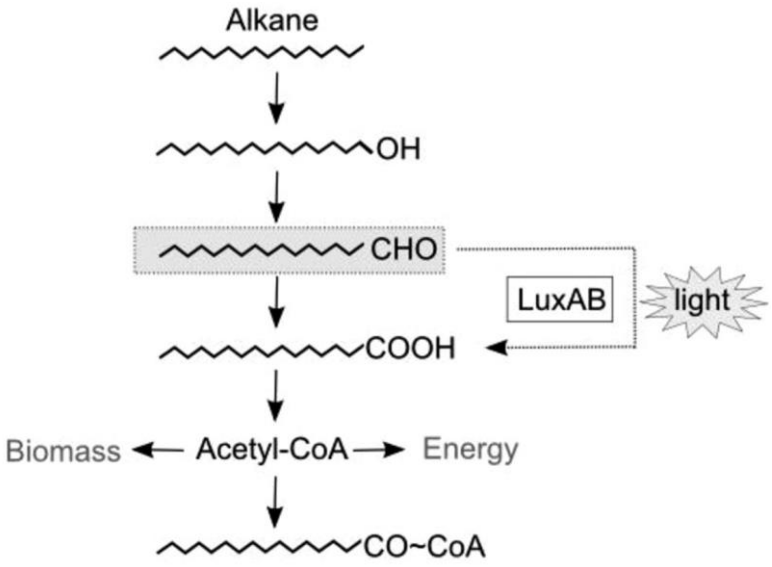

FIGURE 7.3. The proposed alkane degradation pathway of ADP1. The integration of the bacterial luciferase (LuxAB) enables the real-time detection of the fatty aldehyde intermediate in the catabolic pathway. Elimination of the strain's natural aldehyde producing pathway results in an unambiguous and simple monitoring system (modified from paper **III**).

The study demonstrates the differences in kinetic responses and signal amplitudes with regard to various alkyl chain lengths. While the longer alkanes C16 and C18 turned out to be more convenient substrates for biomass production, the shorter C12 alkanes resulted in relatively higher luminescence signals. The variables affecting the signal development include the transportation efficiencies of different alkanes, and the substrate preference of both the bacterial luciferase and the enzymes catalyzing alkane oxidization. Furthermore, the tool also detects the extracellular long chain alcohols and aldehydes, which could reduce the tool specificity. Therefore, as for the WE monitoring, the tools should be further developed by specifying and validating the system for different substrates.

In general, biological robustness can be improved by designing modular and hierarchical systems exhibiting dynamic control (Zhu et al. 2012). In addition, constructing an optimal genetic platform imposing minor stress to the cells promotes the persistence and performance of the host cell. However, it is very difficult to evaluate or predict the effects of heterologous gene expression on cells in response to environmental perturbations and changing metabolite levels, and introducing multiple non-native activities increase the metabolic and physiological burden. Thus, the ideology to choose an optimal host cell intrinsically promoting the application has been increasingly brought up in the context of synthetic biology (Nandagopal and Elowitz

2011; Fisher et al. 2014; Nikel et al. 2014). The described alkane monitoring tool represents an example of a systematic and rational approach to a step-wise construction of a stripped-down device exploiting an optimal host cell. The sequential steps include:

1) the specification of the target pathway or the product,

2) the choice and validation of an optimal cell framework to afford the system with minor modifications and convenient characteristics with regard to the device purpose,

3) the determination of a specific intermediate or other key compound which is

    a) linked to the synthesis pathway,

    b) compatible with an amenable biosensing component

4) the design, construction, and validation of the device with appropriate molecular components.

The described monitoring tools demonstrate the straight-forward applicability of ADP1 framework for integrating standard DNA components to create streamlined and operative devices with only few genetic modifications. Theoretically, the metabolic characteristics of ADP1 enable the utilization of the network for establishing several pathways for the studying and production of relevant biomolecules.

7.3 Engineering a synthetic coculture (V)

A rationally designed and engineered coculture of *E. coli* and *A. baylyi* ADP1 was constructed to improve the cell performance and product formation (**V**). The coculture was engineered to possess a carbon channeling system, which enables an efficient removal of a common inhibitory molecule, acetate, from the cultivation, and redirects the carbon flow to the biomass and the product. It was shown that using a glucose-negative mutant strain of *A. baylyi* ADP1 supported the *E. coli* growth and recombinant protein (GFP) production in variable conditions; as a result of the cocultivation, the *E. coli* biomass and protein production were improved both in minimal and rich medium batch cultures without pH control or optimization. Furthermore, it was demonstrated that both the strains could be readily engineered to produce GFP with a single genetic construct, resulting in improved volumetric titers.

As discussed in paper **V**, the coculture most probably cannot exceed the maximal productivity of an optimized fed-batch monoculture. However, the results suggest faster growth for the coculture compared to the *E. coli* monoculture in all studied conditions. In batch processes, the cultivation time directly affects the process feasibility and is thus an important parameter to consider. The compromise between the maximal productivity and process timespan of the two culture types is illustrated in Figure 7.4. Furthermore, considering a process development for a new product starting from scratch, carefully designed cocultures with less need for optimization provide a shortcut to reach the critical level of productivity.

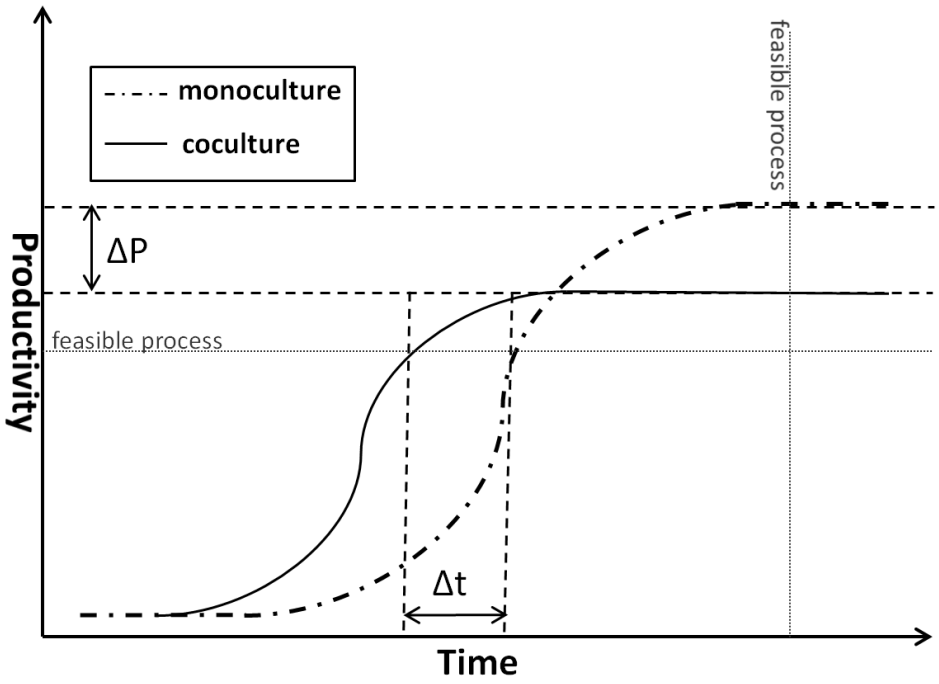

FIGURE 7.4. A hypothetical illustration of a coculture and a monoculture batch processes. Productivity P is given as a function of time t. A threshold for an economically feasible process in terms of cultivation time and product yield is indicated in light grey. An optimized monoculture results in higher maximal productivity whereas coculture possesses faster growth.

Evidently, several factors affect the choice between a monoculture and a coculture. Another benefit of the coculture can be realized in processes exploiting sustainable, but challenging feedstocks possessing varying sugar concentrations and limited possibility for process control and optimization. In such conditions, *E. coli* can readily shift to overflow metabolism, and the environmental perturbations cannot necessarily be balanced by a single strain population. Even though strategies for alleviating the negative effects of acetate in monocultures are proposed (Wang et al. 2014a), they are

often unable to address the other potential issues related to for example substrate and metabolite imbalance and carbon loss.

Substrate costs constitute the major expense in bioprocesses, and therefore industrial secondary flows and lignocellulose hydrolysates are preferred substrates. However, the toxic compounds present in the liquors restrict their use (Palmqvist and Hahn-Hägerdal 2000). *Acinetobacter* spp. are known to tolerate and/or degrade a variety of toxic compounds such as phenols, furfurals, and organic acids, including the 5-hydroxymethylfurfural and ferulic acid present in lignocellulose hydrolysates (Beshay et al. 2002; Lopez et al. 2004). Furthermore, our preliminary studies indicate that some typical toxic compounds are not only metabolized into a harmless form, but can be directed to the synthesis of valuable compounds (data not shown). Thus, exploiting a well-designed coculture may broaden the possibilities to exploit challenging heterogeneous liquors as substrates.

For the convenience of the product determination, the functionality of the coculture was demonstrated with GFP production. However, the coculture could be potentially harnessed for the production of industrially more relevant compounds, such as long chain hydrocarbons. Especially for products involving several enzymatic steps and intermediates, a task distribution could be a feasible option. Given that *E. coli* and ADP1 exhibit very different metabolic environments, employing the hosts for different tasks would allow the optimal use of the benefits and constraints of each host. For example, *E. coli* has been successfully engineered for the overproduction of free fatty acids, whereas engineered ADP1 could efficiently convert them e.g. to alkanes or wax esters (Lennen et al. 2010). Distribution of functions can solve issues related to metabolic burden, carbon flow, metabolite accumulation, and product tolerance. The possibilities of cocultures are beyond imagination; the “supercell” environment provides unique possibilities to build up dynamic sensory-regulatory interactions, precisely timed functions, and complex genetic circuits (Bacchus and Fussenegger 2013).

Maintaining the robustness of expression systems in industrially relevant conditions is a major challenge. Upscaling often leads to the instability of genetic constructs and loss of functionality (Moser et al. 2012). In single strain processes, redundant synthesis pathways could provide stability, but they impose an unwanted burden to cells (Zhu et al. 2012). In contrast, the cocultures involving one common product target naturally possess “a genetic backup” facilitating the production stability and endurance. In paper **V**, *E. coli* and ADP1 were engineered for GFP production with the same genetic construct. However, depending on the product, using genetically different expression systems or biochemically alternative pathways for the production could improve the

process reliability and robustness. For example, employing alternative enzymes that consume different cofactors for a specific catalytic step could stabilize the redox balance and improve the energy distribution of a culture. Furthermore, the naturally different growth phases and metabolisms equalize the physicochemical characteristics and fluctuations of the culture.

A major challenge in building up an ADP1 based process, albeit a coculture, is the lack of experience and knowledge on ADP1 bioprocessing. The unique sugar metabolism of ADP1 can set constraints for high-cell density cultivations, although efforts to improve the growth kinetics by metabolic engineering have been made (Kannisto et al. 2014). On the other hand, neither substrate inhibition nor overflow metabolite production has been recognized for ADP1, thus simplifying a bioprocess. Furthermore, the wide substrate range provides flexibility in terms of the available feedstocks. However, extensive investigations regarding the ADP1 bioprocess optimization need to be carried out before steps toward a further process development can be taken.

Acinetobacter strains are conventionally exploited as model hosts in fundamental research regarding microbial genetics, metabolism, or mechanisms related to pathogenesis (*A. baumannii*). My research has introduced new aspects of utilizing the strain ADP1 in biotechnology and synthetic biology, and I want to challenge the tradition of using *E. coli* as the only worthy host in modern biosciences. In particular, the study reveals the opportunities for the production of fatty acid derived biocompounds in ADP1. Although modest improvements were gained in the study, the results encourage for further development of the strain for industrial purposes. Thus, I hope that my research will serve as a trigger for broader interest toward ADP1 based applications. In addition to ADP1 bioprocess development and optimization, I propose other acute research targets, such as improving the genomic stability, genome streamlining, increased transformation efficiency, engineering of the sugar metabolism, and harnessing the broad enzymatic repertoire of ADP1 for developing novel tools for synthetic biology.

While developing an ultimately optimal chassis, the concepts of a minimal cell, a completely synthetic cell (Gibson et al. 2010), and an *in vitro* synthetic biology (Shin and Noireaux 2012; Chappell et al. 2013) are emerging. These systems provide a high level of programmability, orthogonality, and predictability, and are thus well suited for synthetic biology purposes. Is there a possibility that these synthetic platforms displace the natural cells in future applications, diminishing the need for further understanding about the complexity and behavior of natural cells? Regardless of the host or the platform origin, the elegant groundwork of the nature cannot be easily overridden.

8 Concluding remarks

The metabolic diversity and genomic characteristics of *A. baylyi* ADP1 facilitate the strain utilization in synthetic biology applications. The unusual genomic environment featured with unique engineering opportunities creates an appealing playground for modifying and rewiring the existing pathways and designing new approaches to exploit the formable cellular machinery. The natural competence of ADP1 enables straight-forward genomic insertions, deletions, and modifications in an automated manner, and the unique orientation of genes promotes the possibilities for further genomic streamlining. The strain ADP1 has been previously shown to be an ideal model host for genetic investigations and metabolic studies, and this research further supports the findings.

The metabolic features of ADP1 were exploited in the development of a model platform for studying and engineering the long-chain hydrocarbon metabolism. The neutral lipid quantity and quality were improved by metabolic engineering and synthetic biology means, and ADP1 proved to be a superior platform for developing straight-forward tools for studying bacterial hydrocarbon metabolism. The developed monitoring tools were shown to fulfill the urgent needs for robust metabolic sensor devices.

Furthermore, it was shown that ADP1 can serve as a potential counterpart for rationally engineered coculture systems, especially in processes involving inhibitory concentrations of substrates, metabolites, or toxic compounds. The findings support the views of cocultures providing metabolic balance and robustness for bioprocesses, and the introduced concept will be further developed in future.

Some *Acinetobacter* spp. have been proposed for industrial production of bioemulsifiers and lipases, as well as for bioremediation, biodetoxification, and biosensoring purposes in the field of environmental biotechnology. In addition, further development and engineering of the strain for the production of neutral lipids, especially wax esters, or other high-value hydrocarbons could be profitable, as demonstrated here. Nevertheless, to build up truly viable applications, further research efforts are required to uncover the mechanisms of the wide-ranging metabolism of *A. baylyi* ADP1.

In synthetic biology, two trends in creating an optimal chassis hold the field: the other approach utilizes the rapidly evolving techniques to construct logic synthetic cells with streamlined genomes and programmable features, while the other aims at exploiting the natural machineries in the creation of “cyborg cells” with less need for engineering and more possibilities for complex designs. In the light of the findings provided by this research, *A.baylyi* ADP1 well represents the latter approach. ADP1 serves a platform for the creation of novel and unique metabolic tools and systems by an apt fusion of natural and synthetic traits.

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ORIGINAL PAPERS

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V

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