Synthetic Biology Assisting Metabolic Pathway Engineering

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Abstract

Synthetic biology is mainly focused on the design and construction of new biological parts. Assembly of these parts aids with the creation of synthetic biological pathways. This ultimately leads to an overlap of disciplines in which the synthetic parts are applied to a microbial production host and metabolic engineering for production of value-added compounds. This chapter intends to highlight the supportive input of synthetic biology parts, devices and modules to metabolic pathway engineering for the production of chemicals and fuels. Synthetic bricks find applications on the genome, transcriptome and proteome levels. By exemplifying several applications on the different levels, this chapter intends to give a brief overview of recent developments in the field; furthermore, upcoming developments in protein scaffolding and spatial organisation of pathways in bacterial microcompartments are discussed.

7.1 Introduction

7.1.1 Metabolic Engineering

Especially in the domain of pathway engineering for the production of metabolites, chemicals or biofuels, the two disciplines of metabolic engineering and synthetic biology interlock. The creation and engineering of metabolic pathways for the economically feasible production of desired compounds from renewable resources is a major aim of the discipline of metabolic engineering. In this context, the microbial cell is often compared to a chemical factory, which converts a given substrate via several consecutive steps into one or more products. The advantage of microbial cell factories is the incorporation of these steps into one "vessel" or chassis. Lee et al. (2012c) state the expectation that materials currently produced by the petrochemical industry will be replaced by increasingly diverse chemicals and materials produced in microbial cell factories.

One aim of metabolic engineering is to take an existing metabolic pathway, which leads to the desired product and to maximise the flux towards the product. In order to do so, bottlenecks in the pathway have to be identified, for example, by metabolic flux analysis and opened up by genetic or protein engineering to enhance enzyme activity and turnover of intermediate substrates towards the product. Competing pathways that drain substrates from the pathway of interest have to be deleted when possible, or at least the flux through the competing pathways has to be reduced to the minimum necessary in order to maintain the cell's viability.

The genes and enzymes directly involved in the cells metabolism only account for a small part of the open reading frames of a genome (roughly 12–20 %).

Much more account for the regulatory network of the cell (Nielsen 2003). However, looking at a metabolic map of an organism, it seems that certain branches of the network are rather far apart from each other, but one should not forget that even distant reactions can be interconnected by common cofactors such as ATP, or NADH and NADPH, which link the carbon metabolism to energy and redox metabolism. Furthermore, certain metabolites (like glucose, pyrophosphate, coenzyme A, NH_3) participate in more than ten different reactions, which depicts the tight connection even more. The overall cell metabolism therefore has to be seen as the entity of reactions converting carbon sources to cell building blocks as well as reactions supplying the cell with energy (ATP) and redox equivalents (NADH) (Nielsen 2003). Even more important for engineering the production of chemical compounds is the fact that all of the interesting metabolites and macromolecules produced by a microbial cell are derived from 12 precursor metabolites (Nielsen and Jewett 2008; Förster et al. 2003). These precursor metabolites represent sugar phosphates, intermediates of glycolysis and the TCA cycle, thereby depicting the field in which the pathway engineer has to intervene: the core of the tightly regulated central carbon metabolism.

7.1.2 Pathway Engineering

To gain a substantial product output, Yadav et al. (2012) have outlined the different levels to act on in order to enhance the flux through a metabolic pathway. One step is to increase the substrate uptake followed by eliminating competing pathways leading to the formation of undesired by-products. An enhanced flow of precursor metabolites as well as the sufficient supply of cofactors has to be provided. The activity of enzymes in the pathway has to be optimised to ensure a high flow rate from substrate via several intermediates to the product, and finally, the export of the product to the cultivation medium has to be ensured. Furthermore, when designing a metabolic pathway, one has to keep in mind that thermodynamics pose constraints on the direction in which the enzymatic reactions will lead (Noor et al. 2014), as well as the prerequisite that the oxidation state of the substrates will be balanced with the oxidation state of the products.

The transition of a classically metabolically engineered pathway to a pure synthetic pathway is fluent. Nielsen and Keasling (2011) illustrated this by describing on one hand a pathway in a naturally occurring production organism which has to be engineered by "classical methods" to eliminate by-product formation and the other aforementioned perturbations, on the other hand, a purely synthetic organism that is designed and constructed for the special production task only. Clearly, most of the approaches to date lie in between these two opposing examples (Lee et al. 2012b).

At the time being, successful examples for metabolic engineering are very much dependent on the host background used and therefore not directly applicable to other potential production organisms (Yadav et al. 2012). One of the major advantages that synthetic biology can add to metabolic engineering is the generalisation of parts and devices that perform independently of the host/chassis

background or at least the parts and devices are already characterised in the background of the desired chassis. This chapter intends to highlight the supportive input of synthetic biology parts, devices or systems to metabolic pathway engineering for the production of chemicals and fuels.

7.1.3 Getting Synthetic

Although synthetic biology is more focused on the design and construction of new biological parts, the assembly of these parts could or will result in the creation of a synthetic biological pathway. This ultimately leads to an overlap of disciplines in which the synthetic parts are applied to retrofit a microbial production host to the metabolic engineering goal of producing value-added compounds (Nielsen et al. 2014).

Synthetic biological parts are assembled into synthetic devices, which can be further assembled to modules. By the lack of a completely synthetic minimal cell, these modules have to be integrated in a host chassis, which has the advantage of providing the necessary building block metabolites, cofactors and energy. For further information on the construction of chassis cells, see the respective chapter in this book. In doing so, a completely synthetic pathway can be tested and will show the production of a new compound, most probably in a low amount at the beginning (König et al. 2013). The disadvantage is the not completely predictable interaction of the module with the chassis metabolic network. For this reason, synthetic biologists have to apply the same omics technologies as the metabolic engineer to predict or verify the effect of the module in the hosts' background and in a further step to increase the flux towards product formation. In this respect, systems biology will guide the design and optimisation of modules for synthetic pathway construction, and vice versa, the application of better devices and modules will allow systems biology to gain deeper insight into chassis metabolic networks.

7.2 Levels of Action

7.2.1 Genome Level

The production of lactic acid with *Saccharomyces cerevisiae* is an excellent example for an unwanted by-product pathway competing with the production pathway, tightly connected to the central carbon metabolism. By introducing a heterologous L-lactate dehydrogenase (L-LDH) in *S. cerevisiae*, pyruvate is directly converted to L-lactic acid. The competitor for pyruvate is the native pathway to ethanol starting with pyruvate decarboxylase (PDC) converting pyruvate into acetaldehyde which is further converted to ethanol. Engineering strategies which applied knockouts of the three PDC genes (PDC1, PDC5 and PDC6) present in *S. cerevisiae* observed a very reduced growth rate and fitness of the production strain (Sauer et al. 2010). Yamanishi and Matsuyama (2012) proposed a Cre-lox-

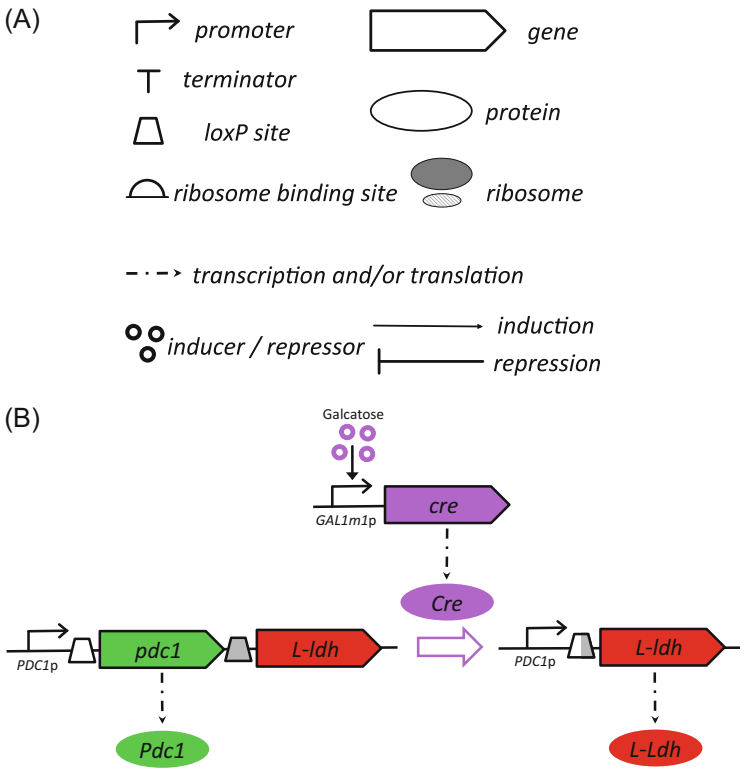

Fig. 7.1 (**a**) Symbols for parts and reactions used throughout this chapter. (**b**) Upon induction with galactose, the Cre recombinase is expressed, and the PDC gene flanked by loxP sites is excised from the genome. The excision of PDC results in the expression of L-LDH under the control of the native PDC promoter

based switch for dividing the process into a growth and a production phase. For this purpose, PDC5 was deleted from the host genome, PDC6 was left unchanged due to lower activity and PDC1 coding sequence was replaced by a cassette consisting of PDC1 coding sequence flanked by two loxP sites followed by the coding sequence of a L-LDH (Fig. 7.1). The Cre recombinase was also inserted on the genome under the control of an inducible promoter. Upon induction, Cre recombinase is expressed and cuts out the PDC1 coding sequence from the genome simultaneously putting the L-LDH coding sequence under the control of the PDC1 promoter, thereby inhibiting ethanol production and activating L-lactic acid production. Although lactate yields could be increased from 8.3 % to 85.4 %, the authors state that the control and induction strategy would need further improvement, due to leakiness of the system. This result depicts the problematic application of genomic intervention during the production process. The following examples will depict more convenient

ways of redirecting carbon flux towards the desired product by synthetic parts and devices.

7.2.2 Transcription Level

7.2.2.1 Promoter Strength

Flux imbalances in a synthetic pathway can be overcome by tuning the promoter strength for each of the pathways catalysing enzymes. Varying the promoter strength for each enzyme one at a time is a tedious and time-consuming effort. To enhance the speed in such an approach, Du et al. (2012) developed the customised optimisation of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) strategy. As a case study, COMPACTER was employed to engineer *S. cerevisiae* for xylose utilisation. As xylose is the most abundant C-5 sugar from lignocellulosic feedstocks, it is important to make it accessible for microorganisms devoted to a biorefinery application. The fungal xylose utilising pathway based on reductase-hydrogenase reactions was favoured in comparison to the bacterial isomerase reaction-based pathway. The fungal xylose pathway applied consists of three consecutive reactions. The xylose reductase (XR in this study from *Candida shehatae*) reduces xylose to xylitol, further on xylitol is converted to xylulose by xylitol dehydrogenase (XDH from *Candida tropicalis*), and finally, xylulokinase (XKS from *Pichia pastoris*) phosphorylates xylitol to channel it into the pentose phosphate pathway (PPP). So there are three enzymes whose transcript levels have to be orchestrated to balance the flux to the PPP and further on into glycolysis. For each enzyme, another constitutive *S. cerevisiae*-derived promoter was applied. From each promoter (PDC1 for XR, TEF1 for XDH and ENO2 for XKS expression), a mutagenised library was constructed by nucleotide analogue mutagenesis (Alper et al. 2005). These promoter mutants were assembled with their respective enzyme coding sequences and terminator sequences onto a single-copy plasmid by the DNA assembler method (Shao et al. 2009). Each plasmid created harboured the three expression cassettes for XR, XDH and XKS representing a mutant pathway for xylose utilisation. The reason for assembling the mutant pathway on a single-copy plasmid, instead of inserting it to the genome of *S. cerevisiae*, was the possibility to conveniently transfer an identified favourable mutant pathway to another strain background. The identification of a favourable mutant pathway was conducted by plating transformants of DNA assembler plasmids on xylose and selecting favourable phenotypes by colony size. The phenotypes created were characterised in liquid culture testing their performance on xylose utilisation and ethanol production under aerobic conditions. Expression levels of XR, XDH and XKS were determined by quantitative PCR. Du and coworkers tested the COMPACTER in two different strain backgrounds: a laboratory strain and the industrial “Classic Turbo Yeast”. For the laboratory strain, the selected phenotype had an improved ethanol yield of 60 % and a 70 % faster xylose consumption rate coupled to a 1.5 times faster ethanol production rate compared to the same strain background with the

non-mutated promoter equipped xylose utilisation pathway. The findings for the industrial strain were even more promising exhibiting a xylose consumption rate of 0.92 g/L/h and an ethanol yield of 0.26 g/g xylose. The benefit of COMPACTER is underlined by the finding that the industrial strain with the non mutated promoter pathway performed very poor by a non detectable ethanol production and total consumption of only 9 % of xylose provided. However, the most important outcome of the study is the comparison of expression levels in the laboratory strain and the industrial strain, which has shown that in the industrial strain, a higher XDH level than XR level is necessary for xylose consumption. In contrary, in the industrial strain, a higher XR than XDH level is favourable. Furthermore, by exchanging the best performing mutant pathways to the other strain, a decrease in performance was detected highlighting the host background dependency of synthetic pathways, which can be attributed to the overall availability of cofactors and variable stress responses of diverse host strains (Du et al. 2012). The synthetic character of this example lies first of all in the brick wise assembly of expression cassettes and further on a whole synthetic pathway for expanding the substrate range of a desired production host as well as the transferability of a selected combination to another chassis strain. The universal applicability of the COMPACTER has been proven by its application to a cellobiose utilising pathway additionally.

An even more advanced approach of improving xylose utilisation has been published recently by Latimer et al. (2014). To point out the differences to the aforementioned example:

1. Instead of using mutated promoter libraries, the authors used five characterised promoters (Lee et al. 2013) spanning evenly distributed the strength of three orders of magnitude including the strongest constitutive promoter known in *S. cerevisiae*. Plasmids harbouring the pathways with varying promoters for every single gene were constructed using the Golden Gate assembly technique (Engler et al. 2009).
2. All the necessary pathway genes were isolated from *Scheffersomyces stipitis*.
3. Additionally to the genes for XR, XDH and XKS, four genes of the nonoxidative PPP and the gene for pyruvate kinase were tested for overexpression. Expression cassettes were cloned onto two different plasmids, one containing the xylose utilisation pathway (resulting in 5^3 125 possible combinations) and one containing the PPP (5^5 3125 possible combinations) to allow the screening of 5^8 possible combinations. For identification of preferable combinations, the previously established TRAC method (Lee et al. 2013) was applied.
4. For enrichment of favourable promoter gene combinations, both aerobic and anaerobic conditions were used. Especially the anaerobic enrichment is of industrial interest owing to fermentations which are rather economically feasible when performed in large scale under anaerobiosis.

The results from this study show a clear difference in promoter strength for the three-gene pathway (xylose utilisation only) to the eight-gene pathway (xylose

utilisation plus PPP) selected under aerobic conditions. While the eight-gene pathway was enriched towards strong promoters driving XR expression, the three-gene pathway was enriched for medium strength promoter activity. Additionally, the eight-gene pathway strains showed a much faster growth on xylose. Authors hypothesised that the strain background used might be limited in the PPP especially in the expression of transaldolase (TAL), as the enrichment showed a preferable combination of the strongest promoter with TAL. Furthermore, the authors also suggest that the limiting PPP activity might explain the differences between the laboratory strain and the industrial strain from Du et al. (2012). Consequently, using a strain limited in PPP activity will only show a local optimum of expression tuning when the three-gene pathway is applied. Finally, the authors tested aerobically enriched strains under anaerobic industrially relevant conditions resulting in inferior performance of these strains, underlining the importance of adjusting selection criteria to the industrially relevant framework. This example highlights that it is often not enough to equip a production chassis with an additional synthetic pathway to broaden the substrate spectrum. One might not forget to tune the chassis native metabolism in order to benefit from the new substrate.

A more advanced concept in this context is multivariate modular metabolic engineering (MMME). This approach is based on grouping a given biosynthetic pathway into modules. For each module, the expression of every enzyme is adjusted to ascertain an optimal carbon flux through the module. Then the expression levels of the entire modules, which complete the pathway, can be varied against each other in order to find the optimal level for each module to maximise the pathway's productivity. This approach helps to find the right compromise between the necessity of knowledge for a complete rational engineering approach and the necessity for extensive screening for a (simple) combinatorial approach. MMME aims for a rationally based approach with moderate knowledge of the system. Therefore, MMME is applicable independent of host and pathway but still limiting the design space in which analyses have to be done to abandon the need for a high-throughput method. Biggs et al. (2014) reviewed a collection of examples where MMME was applied for the enhanced biosynthetic production of taxadiene, resveratrol, β-carotene, fatty acids and more, highlighting the universal applicability independent of host or product.

7.2.2.2 Toggle Switch

Precursor metabolites like acetyl-CoA are tightly connected to the central carbon metabolism. In a variety of chemical and fuel production processes (e.g. mevalonate pathway to artemisinic acid, 1-butanol, cadaverine, isobutanol), acetyl-CoA serves as a starting point of biosynthesis. Ultimately, there is a keen competition for building blocks of the desired product with the cells' needs for growth and energy. A knockout of competing genes from the cellular metabolism in this case is not feasible, because most processes depend on a moderate to high cell density during the production to maintain a high titer and yield of the production process. Several microbial chemical production processes show a distinct partition of biomass and product formation, for instance, the microbial production of itaconic

acid with *Aspergillus terreus* and citric acid production with *Aspergillus niger* or *Yarrowia lipolytica*. This partition is mostly owed to the limitation of compounds in the production medium other than the carbon source. To create a similar situation of disconnecting the growth phase from the production phase, Soma et al. (2014) applied a synthetic toggle switch for the production of isopropanol. To turn off the drain of acetyl-CoA into the TCA cycle at a certain time point, the *gltA* gene coding for citrate synthase was deleted from the *E. coli* chassis genome and supplied on a so-called switching plasmid. The switching plasmid carries a $P_L lacO_1$ promoter which controls the expression of a TetR repressor which inhibits transcription of *gltA* from a $P_L tetO_1$ promoter. Furthermore, the isopropanol pathway itself is encoded on an operon which is also controlled by $P_L lacO_1$ (Fig. 7.2a). This design enables the simultaneous activation of the isopropanol pathway as well as the repression of citrate synthase expression upon the addition of IPTG to the culture broth. To have a tight control over the two $P_L lacO_1$ promoters, an additional repressor source was added by a plasmid expressing the *lacI* repressor. Before testing the performance of the isopropanol pathway, the authors characterised the effect of the toggle switch controlled *gltA* expression in a strain without the isopropanol pathway plasmid. When the switch was not induced by IPTG, the respective strain performed comparable to a control strain considering growth, glucose consumption and acetate production. When IPTG was added at different time points of cultivation, the functionality of the switch was observed via an increased formation of acetate and an inhibitory effect on growth, especially when the addition was done at the early time points of cultivation (0 h, 6 h and 9 h). The effect of the toggle switch was also monitored by measurement of intracellular metabolite levels which revealed a decrease in citrate level down to 3.6 % compared to a control strain, and the same drop was observed for α-ketoglutarate to 5.2 %. Acetyl-CoA levels were increased by a factor of 3.2, which is in line with the aforementioned acetate accumulation, since acetyl-CoA serves as a precursor of acetate. Furthermore, also the acetyl-CoA precursor pyruvate increased 15.9-fold compared to the control strain. As the proof of concept was done, the effect of acetyl-CoA provision was tested for the production of isobutanol. Results show that especially switching off *gltA* at early time points of cultivation (6 h and 9 h) has a significant effect on isopropanol production. IPTG addition at 6 h and 9 h resulted in titers of 48.3 mM and 50.9 mM isopropanol, respectively. The yields were 47.3 and 48.5 mol/mol%, 2.8- to 3.1-fold higher compared to the respective control strain, whereat the theoretical maximum yield for isopropanol production from glucose is 1 mol isopropanol per 1 mol glucose.

By this attempt, Soma et al. (2014) demonstrated the applicability of a synthetic toggle switch for rerouting metabolites, closely connected to the central carbon metabolism, towards a synthetic pathway. The isopropanol pathway was selected as an example, but the applicability to other synthetic pathways using acetyl-CoA as a precursor metabolite is obvious. Moreover, the insertion of a toggle switch like this is feasible in further branch points of the central carbon metabolism, where synthetic pathways draw their precursor metabolites from.

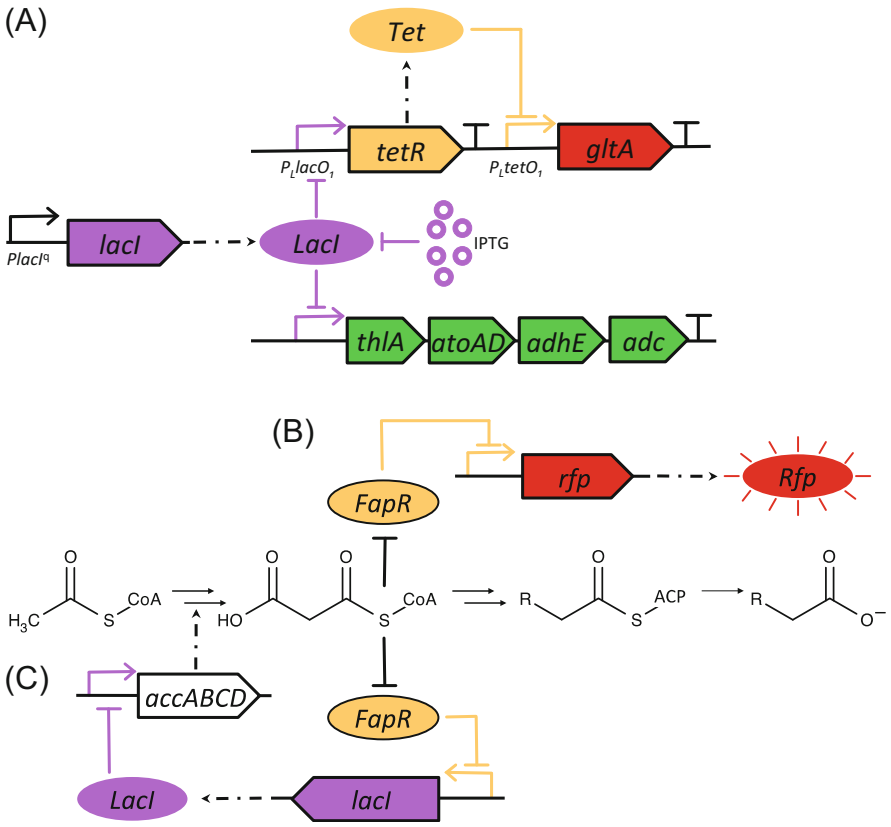

Fig. 7.2 (**a**) LacI represses the expression of TetR and the biosynthesis pathway of isopropanol. Upon induction with IPTG, TetR represses the expression of gltA (citrate synthase), while the expression of the isopropanol pathway is induced. (**b**) The malonyl-CoA-responsive transcription factor FapR represses the expression of Rfp. When malonyl-CoA is present in the system, it binds to the FapR transcription factor, thereby releasing FapR from the promoter and turning on Rfp expression. Fluorescence intensity of Rfp is directly correlated to the level of malonyl-CoA present in the cell. (**c**) Depending on the intracellular malonyl-CoA pool, the expression of acetyl-CoA carboxylase (accABCD) is regulated. High levels of malonyl-CoA induce the expression of LacI, which represses the expression of acetyl-CoA carboxylase, thereby reducing the formation of malonyl-CoA

7.2.2.3 Malonyl-CoA Sensor-Actuator System

When engineering chassis for accumulation of precursor metabolites, the engineer has to be cautious. The accumulation of certain precursor metabolites can also be detrimental to cell growth and further on to productivity due to toxicity effects of the precursor/intermediate. The biosynthesis of malonyl-CoA is such an example. Acetyl-CoA carboxylase catalyses the formation of malonyl-CoA from acetyl-CoA. Malonyl-CoA is the precursor of fatty acid synthesis, in which the activity of acetyl-CoA carboxylase represents the rate-limiting step. By overexpression of

acetyl-CoA carboxylase, the production of fatty acids can be enhanced, but the accumulation of the precursor metabolite malonyl-CoA hampers cell growth. To balance the expression of acetyl-Co carboxylase with the pace of the fatty acid biosynthesis to circumvent malonyl-CoA accumulation, Liu et al. (2015) developed a malonyl-CoA sensor-actuator system. They constructed a negative feedback circuit which is able to up- or downregulate expression of acetyl-CoA carboxylase, depending on the intracellular malonyl-CoA pool. The design of the sensor-actuator circuit is based on the malonyl-CoA responsive transcription factor FapR from *Bacillus subtilis*. In its native host, FapR represses fatty acid and phospholipid biosynthesis by binding to a 17 bp DNA sequence. At the onset, Liu and coworkers inserted the FapR-binding site in the −10 region of a synthetic promoter controlling the expression of red fluorescent protein (RFP). When malonyl-CoA is present in the system, it binds to the FapR transcription factor, thereby releasing FapR from the promoter and turning on RFP expression. All the expression cassettes necessary for the verification of the operability of the sensor-actuator system as well as fatty acid production in *E. coli* were constructed on separate plasmids with compatible origins of replication. The expression of FapR was controlled by a P_{BAD} promoter, whereas the expression of acetyl-CoA carboxylase was controlled by an IPTG-inducible T7 promoter (Fig. 7.2b). Upon induction with different concentrations of IPTG, acetyl-CoA carboxylase is expressed and malonyl-CoA is formed. The surplus of malonyl-CoA present in the cell releases the FapR repressor from the synthetic promoter giving rise to an RFP signal which can be quantified. By measuring the intracellular malonyl-CoA levels and correlating them to RFP signal intensity, the researchers have established a quick method for quantifying intracellular malonyl-CoA levels. By mutating the FapR-binding site, it was even possible to create promoters with varying strength and dynamic ranges, which is essential for fitting the triggered response to the actual malonyl-CoA level in the system. The final aim was the construction of a negative feedback regulatory circuit. For this reason, the expression of the acetyl-CoA carboxylase was controlled by a LacI-repressed T7 promoter. The expression of LacI was controlled by the FapR-regulated promoter, thereby closing the loop. A further coexpression of a cytosolic thioesterase *tesA* enables the release of the free fatty acids from the acyl carrier protein of the fatty acid biosynthesis pathway (Fig. 7.2c). The resulting strain produced 2.03 g/L of free fatty acids which was 34 % higher in titer than the respective control strain. In line with this result also, the fatty acid productivity was increased by 33 %. Also in respect to growth rate, the sensor-actuator equipped strain entered the exponential growth phase earlier than the control strain, reflecting the relief of the growth inhibitory effect of malonyl-CoA accumulation. It will be interesting to see in the future whether this malonyl-CoA sensor-actuator device will be applied to other production systems that need malonyl-CoA precursors like flavonoids, polyketides and 3-hydroxypropionic acid production strains.

7.2.3 Translation Level

7.2.3.1 Synthetic Ribosome Binding Sites

Besides tuning the promoter strength of a given synthetic pathway (acting on transcription), the expression level of catalysing enzymes can be tuned by the insertion of synthetic ribosome binding sites (RBS, acting on translation). Lin et al. (2014) demonstrated this for the production of riboflavin in *E. coli*. The riboflavin biosynthetic pathway is a branched pathway starting from GTP and ribulose-5-phosphate as precursors. Several strategies have been applied to enhance riboflavin production. Among them, synthetic operons of the riboflavin pathway from *E. coli* and *Bacillus subtilis* have been tested on plasmids with different copy numbers. However, most importantly, riboflavin itself is a precursor for FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), and FMN acts as a potent feedback regulator on the riboflavin biosynthesis pathway. To overcome this feedback inhibition, Lin et al. (2014) attempted to reduce the expression level of *ribF*, the bifunctional riboflavin kinase/FMN adenylyltansferase, which catalyses the formation of FMN and FAD from riboflavin. To this end, they constructed a library of RBS sequences weaker than the native RBS using the RBS calculator from Salis (2011). Introducing this RBS library to the production strain, nearly 2/3 of the presented strains exhibited a higher riboflavin production compared to the parental strain. The best clone accumulated a 77 % higher titer of the vitamin. Further improvements of the cell factory could involve an upregulation of the precursor pathways to GTP and ribulose-5-phosphate. In this case, stronger RBS sequences could be used to tune expression. This is also one of the tools which Ng et al. (2015) used to improve NADPH provision in *E. coli*, underlining the potential of the modification of translational efficiency by RBS engineering.

7.2.3.2 Dynamic Metabolic Valve

To redirect the flux of glucose away from glycolysis directly to a heterologous pathway for gluconate production by the activity of glucose dehydrogenase (Gdh), Solomon et al. (2012) engineered a dynamic metabolic valve tuning the expression of glucose kinase (Glk). To highlight the synthetic character of this approach, it is noteworthy that all the building blocks used in this study were obtained from the Registry of Standard Biological Parts (http://partsregistry.org). The authors tested the inducible transcription of antisense RNA (asRNA) to reduce Glk activity, thereby reducing the specific growth rate of the production host *E. coli*. asRNA constructs were designed by inverting the *glk* including 20 bp of the upstream region of glk encompassing the RBS. Transcription of asRNA was controlled by a Tet promoter (P_{tet}) (Fig. 7.3a). Four constructs of asRNA of different length were tested, whereby constructs of 100 bp and 1000 bp showed markedly reduction in specific growth rates upon induction with anhydrotetracycline (aTc). The 100 bp asRNA construct indicated a decreased Glk activity of 12 %, whereas the 1000 bp asRNA construct indicated a decrease of 25 %. The mode of action of these constructs seemed to be different. The 100 bp construct did not have any influence on mRNA levels of Glk, indicating a RBS occlusion mode of action. For the

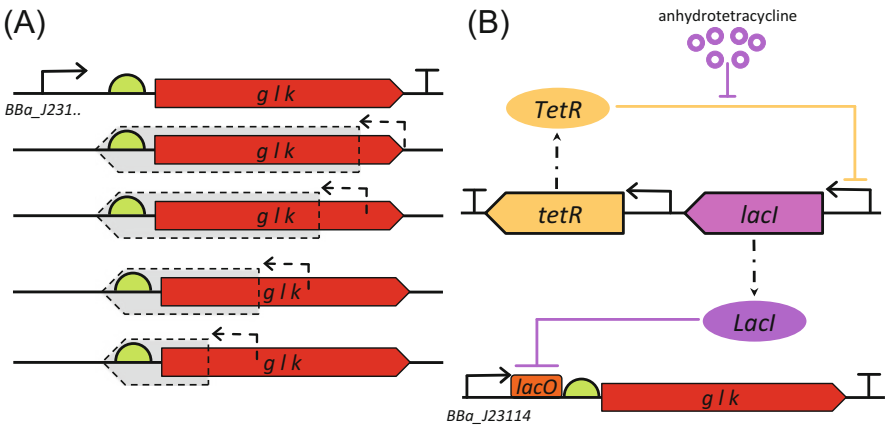

Fig. 7.3 (**a**) Illustrates the expression of antisense RNA of different lengths from an inducible promoter (*dashed arrows*), by inverting the coding sequence of glucose kinase Glk including 20 bp of the upstream region of *glk* encompassing the ribosome binding site. (**b**) The constitutively expressed tetR represses the expression of LacI which binds to the lac operator of the Glk expression cassette turning Glk expression off. Upon induction with anhydrotetracycline, tetR repression is released from LacI, thereby turning off the expression of Glk

1000 bp construct in contrary, a reduction of mRNA levels more than 85 % was observed, suggesting that binding of the asRNA increased mRNA degradation. Testing these constructs on glucose medium, the authors witnessed what they termed a "growth-mediated buffering" effect. The effect of inhibition of Glk reduces growth rate, thereby concentrating the remaining Glk in the cells. Glk is a fairly stable protein, and it could only be diluted in the culture by cell growth, which was detained upon asRNA transcription. In another attempt, Solomon et al. (2012) constructed an inverting gene circuit as a dynamic glucose valve activator consisting of a constitutively expressed tetR which represses expression of lacI. Upon aTc addition, TetR is repressed, thereby driving the expression of LacI which binds to the lac operator of the Glk expression cassette turning Glk expression off (Fig. 7.3b). Applying this circuit with an appropriate amount of 100 ng/mL aTc, an 80 % repression of mRNA levels was observed. Furthermore, the inverter was able to reduce growth by 50 %. Testing the inverter for the production pathway of gluconate, a 30 % increase in gluconate titers and additionally a 40 % reduction in acetate formation were observed, which accounts for carbon loss via by-product formation.

7.2.3.3 Riboswitches

A riboswitch is the regulatory part of an mRNA, located in the upstream region of a coding sequence. It consists of two elements, an aptamer and an expression platform. When a specific metabolite (e.g. lysine) binds to the aptamer region, a structural change of the expression platform is induced, which can block the access to, e.g. the ribosome binding site (Fig. 7.4a). Zhou and Zeng (2015) tested two

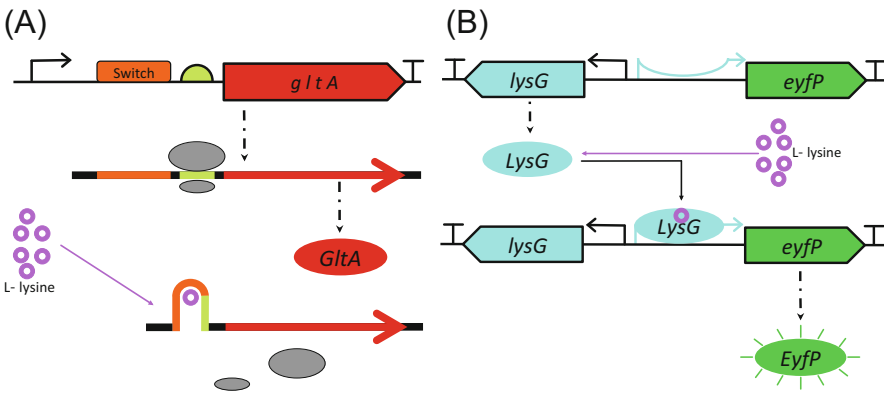

Fig. 7.4 (**a**) Intracellular accumulation of L-lysine induces a conformational change of the mRNA by binding to the aptamer region of the riboswitch controlling the translation of citrate synthase *gltA*. (**b**) The transcription factor LysG is activated by L-lysine and binds to the promoter region of lysE which controls the expression of eYFP. The fluorescence signal of eYFP enables the cell sorting for single cells with elevated levels of L-lysine

lysine riboswitches, one from *E. coli* and the other from *B. subtilis*, first in *E. coli* and further on in the lysine producer *Corynebacterium glutamicum*. Comparable to Soma et al. (2014) mentioned above, Zhou and Zeng (2015) wanted to gain control over expression of citrate synthase *gltA* but without the necessity of external addition of an inducer molecule. By replacing the regulatory sequence of the native *gltA* 5′UTR with the lysine riboswitches, they confirmed that the lysine pool present in *E. coli* was sufficient to downregulate *gltA* translation resulting in a striking reduction of cell growth and glucose consumption in nearly one order of magnitude. To further characterise the strength of the two riboswitches, a β-galactosidase reporter plasmid was constructed, finding that the *E. coli* riboswitch confers a much stronger lysine repression than the one from *B. subtilis*, tested in an *E. coli* background at least. After transferring both these riboswitch-regulated *gltA* expression cassettes to the chromosome of a *C. glutamicum* lysine overproducer strain, results confirmed the stronger repression of the *E. coli*-derived riboswitch. While the growth of lysine riboswitch carrying mutants was retarded, the glucose consumption rate was nearly as high as for the control strain. The redirection of the carbon flux towards product formation was confirmed by a 63 % and 38 % higher product yield for the *E. coli* and the *B. subtilis* riboswitch, respectively, compared to the control strain. Measurements of enzyme activities of citrate synthase indicated that compared to the control strain, the riboswitch mutants had only 30 % and 43 % of activity for the *E. coli* and *B. subtilis*, respectively.

Future perspectives for the development and application of riboswitches and a status quo have been recently reviewed by Berens et al. (2015). One of the striking massages is that an aptamer domain can be selected in vitro for virtually any given small molecule. This comes in very handy for sensing a metabolite of choice in a producing cell to regulate the activity of growth or by-product pathways.

Furthermore, metabolite levels could be made visible in the cell comparable to the RFP signal quantification described by Liu et al. (2015) above, whether for a quick quantification for intracellular metabolite levels or the online monitoring of the overall cell status during a production process. A further example of metabolite sensing via an optical signal is given in the following section.

7.2.4 Protein Level

Enzymes catalysing a biosynthetic overproduction pathway are often feedback inhibited by the respective product. To overcome such a feedback inhibitory effect, it is worthwhile to engineer the catalysing protein itself by eliminating or reducing the affinity of the enzyme to the inhibitory product. For this purpose, Schendzielorz et al. (2014) applied the metabolite sensor pSenLys-Spc. This sensor is based on the metabolite binding transcription factor LysG which binds to the promoter region of its target gene *lysE* upon activation by elevated levels of basic amino acids (L-lysine, L-arginine and L-histidine) in *C. glutamicum* (Fig. 7.4b). Fusing an *eyfp* (enhanced yellow fluorescent protein) coding sequences to the *lysE* promoter enabled a high-throughput sorting for cells with elevated intracellular product formation by FACS (fluorescence-activated cell sorting). To enable the accumulation of elevated intracellular product levels, a *C. glutamicum* strain with a chromosomal deletion of a basic amino acid exporter, carrying the pSenLys-Spc reporter, was employed. This strain was transformed with mutant libraries of the product-inhibited pacemaker enzymes for L-arginine (*argB*), L-lysine (lysC) and L-histidine (HisG). Sorting cells via FACS enabled the identification of mutants of each enzyme which showed reduced feedback inhibition, resulting in elevated product formation of 34 mM of L-arginine, 45 mM of L-lysine and 17 mM of L-histidine, compared to titers of 0.4 mM, 1.2 mM and 0.0 mM of the respective control strains.

7.2.5 Spatial Organisation

The picture of a bacterial cell as a bag of enzymes is being overhauled. Today, a wide variety of organisational features in bacteria and archaea are known, which have recently been reviewed by Saier (2013). In this section, only organisational forms related to metabolism like substrate channelling via enzyme aggregation, bacterial microcompartments and natural scaffolds will be discussed. The benefits and properties of these natural phenomena will be discussed in the context of current efforts aiming at their exploitation as synthetic biology tools to artificially create organisation in heterologously introduced metabolic pathways.

7.2.5.1 Substrate Channelling in Central Carbon Metabolism of Bacteria

The idea of the central carbon metabolism in bacteria as a result of random molecular collisions involving substrates, cofactors and enzymes is becoming increasingly unrealistic (Saier 2013). Recent studies found several protein-protein

interactions in glycolysis and TCA cycle of *Bacillus subtilis* (Commichau et al. 2009; Meyer et al. 2011). In glycolysis, an interaction between phosphofructokinase, phosphoglyceromutase and enolase has been identified, for the TCA cycle citrate synthase, isocitrate dehydrogenase and malate dehydrogenase on the one hand, and fumarase, aconitase and malate dehydrogenase on the other hand have been detected as interaction partners for catalysing sequential reactions (Commichau et al. 2009; Meyer et al. 2011). These interactions are thought to establish substrate channelling of intermediates of the central carbon metabolism to enhance metabolic flux through these pathways.

One emerging strategy to overcome problems in metabolic engineering is the colocalisation of enzymes similar to the multienzyme complexes found in nature. By bringing enzymes in close proximity, substrate channelling to prevent carbon loss due to diffusion or secretion of intermediates and side reactions in host cell networks is achieved (Lee et al. 2012a). Strategies aiming at colocalisation of enzymes usually involve the construction of a synthetic scaffold to which enzymes are attached. This creates a space with high enzyme "density" where intermediates are passed from one enzyme to the next, with reduced probability of leaking to the cytosol. Attaching enzymes to scaffolds furthermore allows for control of stoichiometry between different enzymes to balance flux in a pathway.

7.2.5.2 Protein Scaffolds

A first example of improving a heterologously expressed pathway with the aid of a scaffold was shown by Dueber et al. (2009). There, protein-protein interaction domains were used as a scaffold, and the enzymes of the mevalonate pathway, acetoacetyl-CoA thiolase from *E. coli*, together with hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA reductase from *S. cerevisiae*, were attached to this scaffold by tagging the enzymes with peptide ligands specific for the interaction domains. The modularity of the interaction domains allowed for changing the stoichiometry of the enzymes within the scaffold. Controlling the stoichiometry of the overexpressed enzymes was used to optimise the flux through the mevalonate pathway, as the expression of the enzymes without the scaffold showed an imbalanced flux resulting in intermediate accumulation. In order to lower metabolic burden, pathway enzyme and scaffold expression were separated from each other and individually titrated for optimal production. Low induction of the pathway enzymes and high induction of the protein scaffold turned out to be optimal for mevalonate production. The pathway with optimised stoichiometry showed a 77-fold increase in mevalonate production compared to expression of the pathway without a scaffold. Other studies showed improved production of glucaric acid (fivefold) and butyrate (threefold) by the use of the same protein scaffold (Moon et al. 2010; Baek et al. 2013).

Another example for the application of protein scaffolds is the hydrolysis of cellulose. This reaction is carried out by anaerobic bacteria in a complex, multienzyme structure called cellulosome. The cellulosome usually consists of several cellulose-binding modules and a series of different cohesin-dockerin attached cellulases. A highly ordered structure and close proximity are key features of

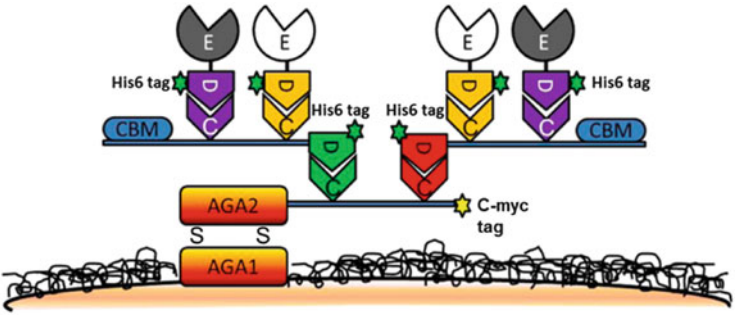

Fig. 7.5 Schematic diagram of the cellulosome complex assembled on the yeast surface using the adaptive assembly. The two adaptor scaffoldins (*red* and *blue*) serve as templates for enzyme recruitment to the yeast surface via specific interaction with the surface-displayed anchoring scaffoldin. *Green* (scaffoldin/dockerin from *Acetivibrio cellulolyticus*), *red* (scaffoldin/dockerin from *Bacteroides cellulosolvens*), *purple* (scaffoldin/dockerin from *Clostridium thermocellum*), *yellow* (scaffoldin/dockerin from *Ruminococcus flavefaciens*). *CBM* cellulose-binding module, *E* enzyme. Reprinted with permission from Tsai et al. 2013. ACS Synth Biol. 2013. Copyright 2013 American Chemical Society

natural cellulosomes. Tsai et al. (2013) recently showed the construction of a synthetic cellulosome displayed on yeast surface to increase cellulose hydrolysis 4.2-fold compared to the use of free enzymes. In their study, they combined cohesin-dockerin modules from different bacteria and created an artificial, tetravalent cellulosome. Briefly, an anchor scaffoldin was attached to the yeast cell surface, containing two cohesin domains. Connected to each of these cohesion domains was a dockerin tagged with two enzymatic subunits, endoglucanase and β-glucosidase, and a cellulose-binding module (Fig. 7.5). It was further shown that ethanol production increased twofold with the tetravalent cellulosome compared to a divalent cellulosome with similar enzyme loading. The authors hypothesised that enzyme proximity has a higher impact on cellulose hydrolysis than overall number of cellulolytic enzymes.

7.2.5.3 Nucleic Acids as Scaffolds

Plasmid-Based Scaffolds

Another approach proven to be feasible is the use of DNA as scaffold. Conrado et al. (2012) used plasmid DNA with synthetic binding sites as the scaffold and fused zinc fingers specific for these binding sites to the enzymes of a metabolic pathway. Production improvement using *E. coli* as host was largely dependent on the scaffold architecture, namely, the ratio of binding sites for the individual enzymes and the length of the spacer on the plasmid between the different binding sites. By varying the length of the spacer, the arrangement of the enzymes on the plasmid DNA is changed, with the optimal arrangement leading to close proximity of the different enzymes. Furthermore, the number of scaffold units on the plasmid was also found to be important, as in general, the plasmid copy number even with a

high-copy-number plasmid is significantly lower compared to the number of enzyme molecules produced in an overexpression strain. Therefore, multiple scaffold units were included on the plasmid to accommodate all enzyme molecules on the scaffold. Using optimal scaffold architecture for different pathways, 5-fold, 4.5-fold and 2.5-fold titer improvement for resveratrol, 1,2-propanediol and mevalonate, respectively, was observed compared to expression of the enzymes without scaffolds. Another study employed DNA scaffolding for improved production of N-acetylglucosamine and obtained a 2.5-fold titer improvement when glucosamine synthase and N-acetylglucosamine/N-acetyltransferase were colocalised on a DNA scaffold (Liu et al. 2014). The review by Lee et al. (2012a) named different interaction motifs as well as variable length linkers between binding sites without solubility issues as potential advantages of DNA scaffolds, whereas heavy enzyme modification by lengthy zinc finger domains and limitation of the number of scaffolds per cell by the plasmid copy number (~500) were mentioned as possible drawbacks.

RNA Molecule-Based Scaffolds

Single RNA molecules with two aptamers as well as RNA molecules that can be polymerised in one or two dimensions have been employed in a recent study (Sachdeva et al. 2014). For that purpose, a library of eight aptamers and corresponding RNA-binding domains was constructed. By tagging split green fluorescent protein fragments with RNA-binding domains, fixation to an RNA scaffold colocalised the fragments resulting in measurable fluorescence. The study further showed application of RNA scaffolds to a two-enzyme pentadecane pathway and a three- and four-enzyme pathway for succinate production, which for both metabolites resulted in a 1.8-fold increase in production.

Bacterial Outer Membrane Vesicles as Scaffolds

Another interesting approach for a scaffold has been shown by Park et al. (2014). Bacterial outer membrane vesicles (OMVs) were used as the scaffold for extracellular display of cellulolytic enzymes. In detail, an ice nucleation anchor was connected to the surface of the OMV surface. Attached to the anchor was a trivalent scaffold containing cohesin domains and a cellulose-binding domain. By interaction with the cohesin domains, enzymes tagged with the respective dockerins could be assembled in an ordered fashion on the surface of OMVs. Glucose production was shown to be improved 23-fold compared to expression of free enzymes.

7.2.5.4 Bacterial Microcompartments

Bacterial microcompartments (BMCs), macromolecular structures entirely made up from proteins, are simple organelles with a large number of different structures and functions (Chowdhury et al. 2014; Axen et al. 2014). A first example of a BMC was discovered in the 1950s, when carboxysomes were observed in the electron microscope (Chowdhury et al. 2014; Drews and Niklowitz 1956). Despite the knowledge about bacterial microcompartments in some bacteria, it has only recently become fully appreciated how widespread bacterial microcompartments

are as an organisational form in bacteria (Lee et al. 2012a). Assisted by bioinformatics and an increasing number of bacterial genome sequences available, today, BMCs have been discovered in hundreds of bacteria with a variety of metabolic functions assigned to them (Axen et al. 2014). The genetic information encoding these macromolecular complexes is either organised in operons (e.g. pdu, eut and α-carboxysomes) or spread throughout the bacterial genome (e.g. β-carboxysomes) (Frank et al. 2013). Operons have most likely been spread among different bacteria via horizontal gene transfer. BMCs are polyhedral in shape and have a diameter of approximately 100–150 nm. The protein layer forming the shell of BMCs is thought to be 3–4 nm thick and consists of up to 20,000 polypeptides of 10–20 different types, and so far, no evidence for the presence of lipids has been found (Cheng et al. 2008). Encapsulated by the shell, 10,000–15,000 protein molecules are found in the lumen of the BMC required for the metabolic function (Cheng et al. 2008; Yeates et al. 2008). The interior of the microcompartments is connected to the rest of the cell by small selective pores in the protein shell (Frank et al. 2013). These pores are thought to allow substrates and products to enter and leave BMCs, while toxic and volatile intermediates are retained within the lumen. This provides protection to the rest of the cell. Additionally, intermediate loss due to diffusion is thought to be reduced, and a higher metabolic efficiency is achieved by creation of an improved microenvironment in the lumen of the BMC.

Therefore, the main function of BMCs is thought to be the optimisation of metabolic pathways (Chowdhury et al. 2014). The models of the two best studied BMCs support this hypothesis. The anabolic carboxysome is involved in the carbon fixation in cyanobacteria and functions as part of a carbon dioxide-concentrating mechanism (CCM) (Cheng et al. 2008). CO_2 is concentrated within the carboxysome, which improves the catalytic efficiency of RuBisCO, the rate-limiting enzyme of the Calvin cycle. In addition, the shell of the carboxysome prevents entry of molecular oxygen and, thus, lowers photorespiration activity, a process otherwise draining up to 50 % of carbon fixed in the Calvin cycle (Cheng et al. 2008; Cannon et al. 2001). The second example, the catabolic pdu metabolosome, plays a key role in bacterial degradation of 1,2-propanediol and glycerol. Both substrates are converted into an alcohol and a carboxylic acid. The intermediate of this conversion is in both cases a toxic aldehyde. The pores of the pdu metabolosome are thought to be selective in retaining the intermediate within the lumen of the BMC while allowing substrates and products to enter and exit (Chowdhury et al. 2014). Therefore, the main function of the pdu metabolosome is thought to protect the cell from cytotoxicity and DNA damage by sequestering the toxic aldehyde intermediate (Sampson and Bobik 2008; Chowdhury et al. 2014).

Because of their properties, BMCs have been proposed as a potential synthetic biology tool for metabolic engineering strategies. Targeting of a complete, non-natural pathway composed of a number of different enzymes to a BMC might provide the same advantages as for the pathway naturally found in the BMC.

A prerequisite for exploitation of these structures as nanobioreactors would require targeting of foreign enzymes to the interior of a BMC. Encapsulation of

metabolic enzymes naturally located within a BMC is thought to be mediated by interaction of the enzymes with shell proteins during BMC assembly (Fan et al. 2010; Chowdhury et al. 2014). Fan and coworkers first observed short N-terminal extensions on pduP, a propionaldehyde dehydrogenase, when they performed sequence alignment of different pduP homologues. It could be shown that the deletion of the first 14 or 18 amino acids from pduP of *Salmonella enterica* had little effect on activity, but the enzyme largely lost its association with the BMC. When GFP was N-terminally tagged with the first 18 amino acids of pduP, fluorescence localised to the BMC was observed. This clearly proved the role of this peptide in encapsulation of enzymes in the BMC of *S. enterica*. Other luminal enzymes such as pduD and pduV were also shown to have similar N-terminal targeting sequences. However, for other encapsulated enzymes, no such N- or C-terminal extensions could be identified, and the mechanism of encapsulation of these enzymes is not yet understood. It is thought that the mechanism underlying encapsulation is an interaction between α-helices at the N-terminus of the enzyme and the C-terminus of one of the shell proteins and that manipulation of these interactions could allow for the encapsulation of multiple enzymes with a defined stoichiometry (Fan et al. 2012; Chowdhury et al. 2014). This would mark an important feature of a bespoke nanobioreactor. Another study showed a potential tool for rapid flow cytometric quantitation of protein encapsulation (Kim and Tullman-Ercek 2014). This fluorescence reporter-based system could be of great use for engineering targeting sequences as it allows fast evaluation of the targeting efficiency.

A first milestone in making bespoke BMC nanobioreactors was the study of Parsons et al. (2010), which showed recombinant production of empty BMCs in *E. coli* with a minimal set of six shell proteins, namely, pduA, pduB, pduJ, pduK, pduN and pduU, from *Citrobacter freundii*. Targeting of GFP to recombinant BMCs containing the fusion construct mCherry-pduA resulted in a colocalised red and green fluorescence signal, strongly indicating targeting of GFP to intact mCherry-labelled protein shells. Other studies later showed recombinant production of functional carboxysomes with a minimal set of ten genes, and empty recombinant microcompartments form ethanolamine (eut) metabolosome shell proteins in *E. coli* (Bonacci et al. 2012; Choudhary et al. 2012). The study of Lawrence et al. (2014) marked the first example of a simple BMC nanobioreactor in *E. coli*. The pyruvate decarboxylase and alcohol dehydrogenase of *Z. mobilis* were targeted with P18 and D60 targeting sequences, respectively, and expressed along with six shell proteins pduA, pduB, pduJ, pduK, pduN and pduU from *C. freundii*. This resulted in a 30 % higher production of ethanol compared to expression without the shell proteins.

For efficient exploitation of these fascinating structures as reaction chambers, an understanding of the pore selectivity is required. Knowledge about how pores function would allow adaption of pore properties for molecules associated with a foreign pathway. However, pore selectivity is still poorly understood. The models of shell proteins available show distinct differences regarding pore residues. Since one of the main hypotheses about BMCs is that these structures confer protection of

the cell by sequestering toxic intermediates, all pores from the different shell proteins must show similar selectivity in allowing substrates and products to enter and leave the BMC and sequestering toxic intermediates. Crystallographic studies of pduB of *L. reuteri* in the presence of glycerol showed blocking of the pore by three molecules of glycerol, which would allow only glycerol to enter the BMC and preventing the toxic aldehyde intermediates from relocating to the cytosol (Pang et al. 2012). How the products propionaldehyde-CoA and propanol are exported from the BMC is not yet understood.

The ability to control pore selectivity and therefore transport to and from the BMC would be a key requirement to make bespoke BMCs as nanobioreactors in metabolic engineering strategies. To date, available studies dealing with changing pore selectivity are very limited. Cai et al. (2014) showed the possibility of random mutagenesis of pore residues as well as combining shell proteins from different types of BMCs to create chimeric shells. The review of Lee et al. (2012a) has suggested random pore mutations coupled with an effective screening to alter pore selectivity. Transcriptional regulators as biosensors for desired metabolites coupled to the expression of an antibiotic resistance gene or a cytotoxic protein would allow for positive and negative selection (Lee et al. 2012a). Additionally, engineering of FRET sensors for pathway metabolites and sequestering of these sensors in BMCs have been proposed to directly measure metabolite permeability (Lee et al. 2012a).

7.2.6 Combined Approaches

Metabolic regulation of natural cells is generally extremely tight and robust due to the combination of regulatory events on all levels. As outlined before, excellent tools and devices have been constructed acting on different levels of the cellular networks. Finally, the metabolic engineer has to do what nature does—to combine different approaches on different levels to obtain optimal results. Clearly, here we are looking into the future as the development of synthetic tools, and a systems wide understanding of cellular networks is just emerging. One example for a combination of approaches has recently been shown by Ng et al. (2015) who combined the construction of synthetic bacterial operons, RBS engineering and MAGE to obtain a library of more than 600 *E. coli* strains, which they screened for improved NADPH provision. Combining this further with an optimised terpenoid pathway increased the terpenoid titer by 97 %. This points nicely into the direction how synthetic biology will aid metabolic engineering very efficiently in the future.

7.3 Commercial Application

A remarkable story of commercialisation of a synthetic pathway-derived chemical is Genomatica's process for the production of 1,4-butanediol (BDO). The synthetic pathway to the highly reduced BDO (relative to the potential carbohydrate

substrates available for bioproduction) has been designed from scratch. By using a genome-scale metabolic model of *E. coli* and their in-house biopathway prediction algorithm, 10,000 possible ways were predicted to form BDO from *E. coli*'s central carbon metabolism. The reason for this vast number of predicted ways might be that the applied SimPheny Biopathway Predictor software uses transformation of functional groups of known chemistry, instead of known enzyme reactions. By visualising the predicted pathways, Genomatica's scientists had to decide which way to go. The decision was made by ranking the possible ways by predicted thermodynamic feasibility, maximum yield, length of pathway, steps without currently known enzymes and the number of steps from the central metabolism to the product (Yim et al. 2011). A decision was made to test two pathways starting from the TCA cycle intermediates succinate and α-ketoglutarate from where pathways were predicted to lead to the intermediate metabolite 4-hydroxybutyrate (4HB) and further on to BDO. Similar to the modulation described above, the predicted pathway was divided into an upstream part leading to 4HB and a downstream part leading from 4HB to BDO. For both upstream and downstream pathways, several heterologous enzymes from different organisms were introduced in *E. coli*, testing several plasmids with varying copy number. Combining up- and downstream parts, it was found to be favourable to have the upstream part on a medium copy level and the downstream part on a high copy plasmid. As soon as the complete pathway was established, scientists at Genomatica started to engineer the production host by eliminating competing by-product pathways predicted by the OptKnock algorithm. It was shown that a strain engineered in this way changed the product pattern in a fermentation run away from by-product formation towards the desired product. Titers of 18 g/L BDO were reported in comparison to the non engineered strain yielding 7.5 g/L. Finally, the applicability of this BDO-producing strain in a biorefinery context was proven by testing the strain on sucrose, xylose and crude mixed sugars from biomass hydrolysis.

7.4 Conclusions

The future is bright for synthetic biology applications in metabolic pathway engineering. Several examples depicted in this book chapter will find or already have found their way towards a commercialised application. The standardisation and characterisation of biological parts and devices and the concept of transferability between different laboratory strains and industrially relevant production organisms add a big plus to the recent examples of successful metabolic engineering, which are rather isolated showcases. As outlined in the text, synthetic devices can assist metabolic engineering efforts on all the possible levels of action. It is noteworthy that also recent metabolic engineering efforts tend to systematically standardise their tools like using tunable promoters, manipulating ribosome binding sites, diversifying gene order in an operon and scaffolding to colocalise enzymes participating in a pathway. Moreover, to circumvent imbalances in a heterologous pathway, enzymes with a comparable turnover are grouped into modules, and

further on modules with different turnovers are orchestrated by adjusting their concentration by applying promoters of different strength or varying the copy number of a module (Yadav et al. 2012).

As already mentioned in the beginning, there are no "stand-alone" definitions for metabolic engineering and synthetic biology, rather than an overlap of disciplines that will cross-fertilise in the future. With the ongoing improvement in both disciplines, the field is moving forward, and future will show if synthetic biology can keep up with the prediction to heal us, feed us and fuel us (Editorial 2014).

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