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From systems biology to metabolically engineered cells — an omics perspective on the development of industrial microbes

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Green routes are indispensable for a sustainable production of energy, chemicals and materials, and health and nutrition products from renewable resources. Naturally, microbes are capable to conduct many of the desired biochemical conversions involved, however, only at rather low efficiency. It is therefore essential to metabolically engineer them towards efficient cell factories, which enable a high product titer, yield and productivity, exhibit a good process robustness and a broad substrate spectrum, and are safe to be used, to name a few prominent points from the wish list for industrial bioproduction. Such optimization of a microbial cell typically involves the implementation of several up to multiple traits into its genome, which then mediate the desired phenotype. While the genetic modification step itself is straightforward due to the much advanced genome editing methods, the selection of what exactly has to be optimized out of the manifold possibilities is still a challenge. Here we will discuss, how systems biology can offer guidance to orchestrate the hundreds to thousands of biochemical conversions of a microbe into a concert of desired performance. To this end, we will highlight recent success stories, where systems biology approaches have enabled next-level cell factories and bioprocesses.

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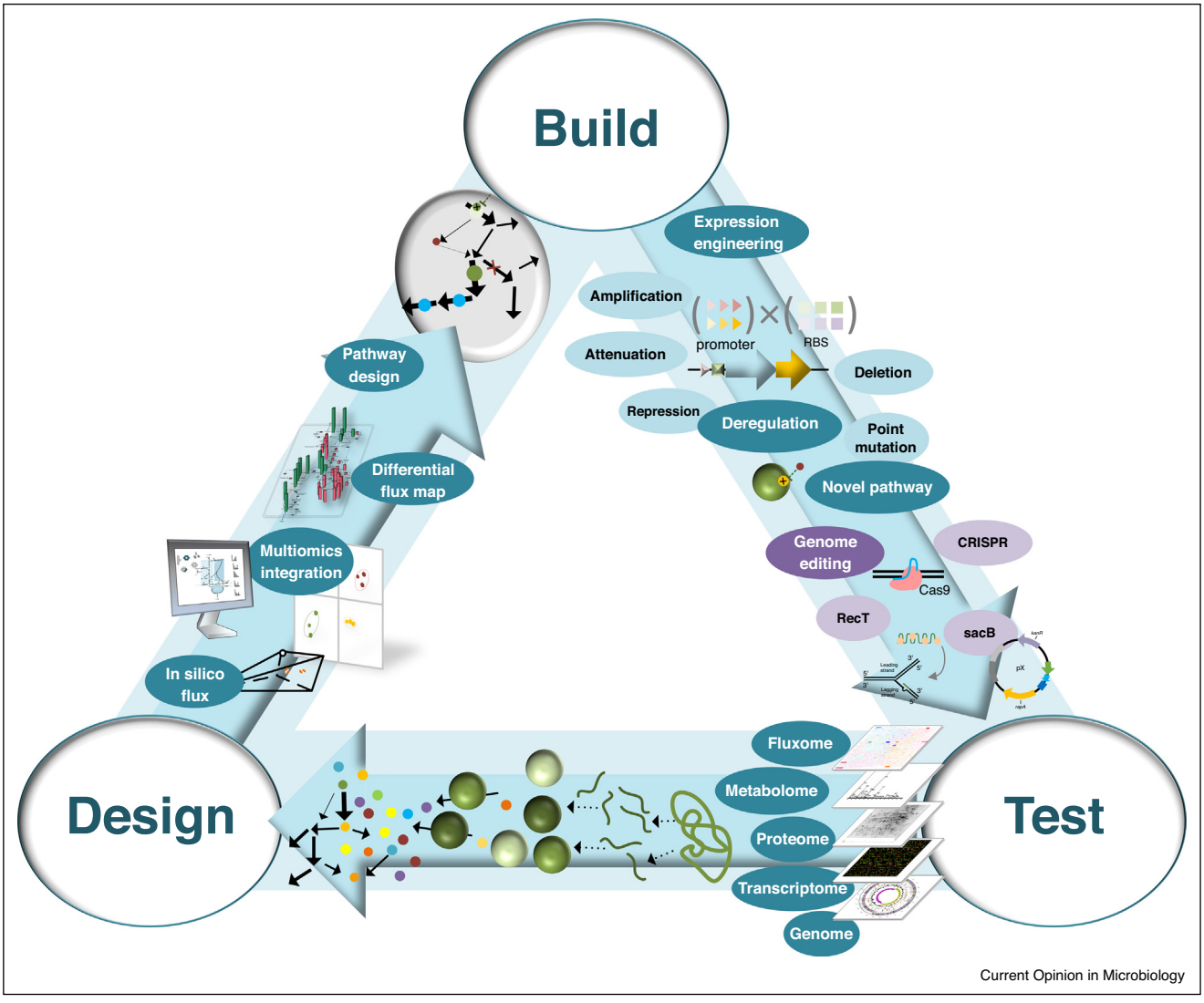
Introduction

The technological breakthrough in omics-based analysis has enabled quantitative insights into cellular systems at a new level of detail and survey. Systems biology continuously and substantially deepens our understanding of biology and additionally provides a powerful toolbox to re-design existing cellular functions and create new ones. The sequencing of entire genomes and the quantification of cellular components and their interaction at a global level (transcripts, proteins, metabolites, and fluxes) are major drivers of design-based systems metabolic engineering [1,2]. Whole-genome sequencing of microbes and metagenome analysis [3,4] unravels the genetic repertoire of an organism and has laid the foundation for genome breeding [5], genome mining [4–7], and genome-scale metabolic modelling [8,9]. In the post genomic research, systems-wide profiling of transcriptome, proteome, metabolome, and fluxome has proven valuable to better understand network operation and regulation on a global scale [1,2]. Embedded into an iterative design-built-test cycle (Figure 1), omics technologies meanwhile open the door to create tailored cell factories for numerous products provided by Natures treasure chest and derive novel attractive industrial processes [3]. Their particular value lies in the guidance of researchers to select the most promising combinations of genetic traits for a desired phenotype out of the manifold possibilities, or to conduct fine-tuned changes to the industrial process environment. Guided by knowledge, strains with impressive performance were developed within a comparably short time period, providing traditional bio-based products such as the amino acids L-lysine [10], L-threonine [11], L-arginine [12] and L-valine [13], but also completely new ones, including diaminopentane [14,15], aminovalerate [16,17], artemisinin [18–20] and other high-value molecules [21–24]. In this review, we will highlight prominent examples of very recent studies that used the analysis of transcriptome, proteome, metabolome, fluxome, and combinations thereof for industrial strain and process optimization.

Transcriptomics-based metabolic engineering

A breakthrough in DNA sequencing technology promoted the development of transcriptome analysis already about 20 years ago. Pioneering methods such as microarrays and RT-PCR are meanwhile complemented by RNA sequencing, substantially increasing throughput and additionally targeting RNA species, not captured by previous methods [25]. In the past years, transcriptome profiling has proven valuable to support rational strain design.

Figure 1

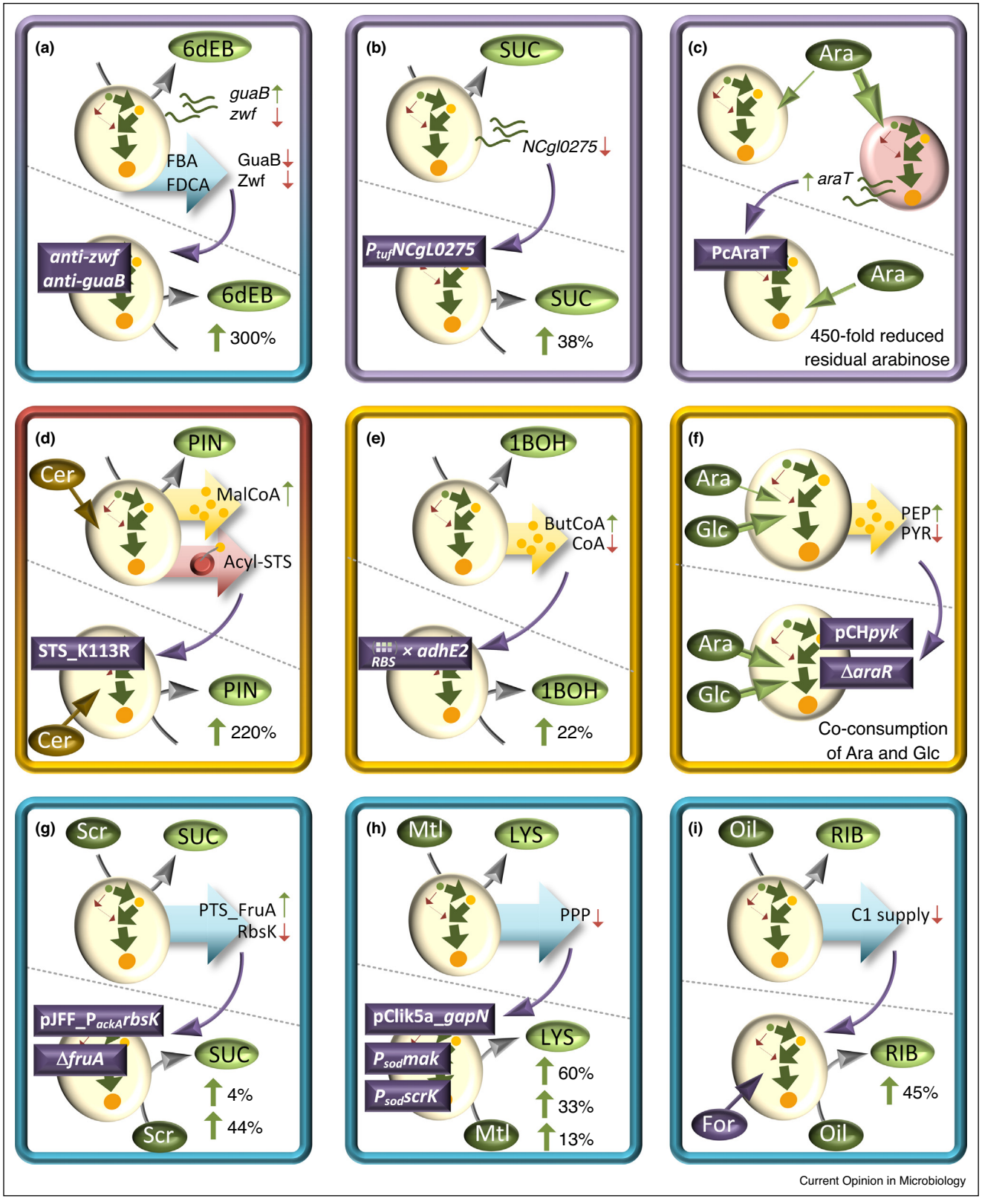


Iterative Design-Build-Test cycle for the creation of optimized producer strains for industrial application through systems metabolic engineering. Systems biology technology platforms (omics) are used to decipher genome, transcriptome, proteome, metabolome, and fluxome. Through comprehensive data integration, computational modelling and simulation, a metabolic blueprint is designed and then translated into a 'genetic engineering language' for genome editing of the desired phenotypes.

In a recent example, microarray-based transcriptomics, combined with *in silico* flux modelling, was used to improve heterologous production of the polyketide 6-deoxyerythronolide (6dEB) in *Escherichia coli* [26]. Polyketides are natural products with a tremendous value for the pharmaceutical industry, but their heterologous production in workhorses such as *E. coli* remains challenging, related to the complex biosynthetic enzyme cluster, often incompatible with the host's physiology [21]. In particular, the comparative analysis of 6dEB-producing and non-producing cells identified pentose phosphate pathway and nucleotide metabolism as relevant pathway modules to be engineered. For these, *in silico* prediction and transcriptome data were most divergent, for example, genes were upregulated, whereas the flux model suggested to reduce pathway activity. The down-regulation of all 25 identified targets was then addressed using synthetic antisense RNAs [26]. Combinatorial repression of beneficial targets increased 6dEB production by almost 300% (Figure 2a) up to a remarkable titer of 210 mg/L [26].

Transcriptome studies were also successfully applied to optimize the fermentative production of bulk chemicals. During succinate production in *Corynebacterium glutamicum*, elevated product levels had detrimental effects on glucose consumption and succinate production rate, as well as cellular fitness [27•]. Expression profiling, again

Figure 2



Success stories of omics-based systems metabolic engineering, using the analysis of the transcriptome (purple), the proteome (red), the metabolome (yellow), the fluxome (turquoise), and combinations thereof **(a,d)**, to identify bottlenecks and guide strain and process engineering for

using microarrays, revealed that metabolic pathways, transport processes, transcriptional regulation and DNA repair were upregulated or downregulated at high succinate concentration. Among the downregulated genes, several were tested to restore glucose consumption rate. While the overexpression of genes involved in central metabolism was not successful, overexpression of the transcriptional regulator *NCgl0275* restored cellular fitness and increased succinate production by 38% (Figure 2b). The combined amplification of the anaplerotic carboxylation, fueling the succinate pathway, generated a strain, which accumulated 152 g L^{-1} succinate at a yield of 1.1 mol mol^{-1} [27•].

In addition, transcriptome analysis guided metabolic engineering for enhanced substrate utilization. The industrial workhorse *Saccharomyces cerevisiae* is no natural degrader of arabinose, an ingredient of hemicellulose streams [28]. Consequently, heterologous pathway engineering is required to make arabinose accessible for the fermentation process [29]. In the yeast, the uptake of the sugar is mediated by the homologous GAL2 transporter, which, however, suffers from poor substrate affinity and inhibitory effects. In contrast, the filamentous fungus *Penicillium chrysogenum* is well known for its capacity to degrade arabinose, implying the presence of at least one arabinose uptake system [29]. *P. chrysogenum* was hence considered as promising donor strain for such a transporter to be expressed in *S. cerevisiae*. For transporter identification, comparative microarray-based transcriptome studies were carried out, either using glucose, arabinose, or ethanol as carbon source. Out of five identified genes, encoding a putative arabinose transporter, PcAraT restored arabinose-based growth of engineered *S. cerevisiae*, lacking the native GAL2, and even improved uptake and utilization (Figure 2c) [29]. Because of a high affinity and specificity of this novel transporter and its low sensitivity to inhibitory effects by competing substrates, it is attractive for bio-refinery applications on sugar mixtures.

Taken together, transcriptome analysis provides routine access to cellular regulation on a global level. Supported by the greatly advanced technologies available today, it appears most useful to understand (and then optimize) the response of cells to their environment or to genetic perturbations and discover unknown proteins that mediate a desired or undesired function. Gene expression, however, often does not correlate to metabolic activity, but can show even opposite trends, due to the various layers of post-transcriptional control. When used for metabolic engineering, transcriptome data therefore need careful evaluation and interpretation.

Proteome-based metabolic engineering

Proteomics is done by a combination of chromatographic separation techniques and sophisticated mass spectrometry [25] and has a track record to investigate the effect of environmental changes [30,31] and genetic perturbation [32,33] in microorganisms and to see, how cells cope with stress conditions [34,35••,36,37].

An elegant study on production of the polyphenol pinosylvin in *E. coli* recently demonstrated, how proteomics can guide metabolic engineering [38••]. Pinosylvin exhibits antimicrobial and antifungal activity and is of interest for the pharmaceutical industry. To obtain improved production, the metabolic engineers considered the interaction of the introduced synthetic pathway with the physiology of the host. An elevated availability of malonyl-CoA, obtained through addition of cerulencin, improved production, but higher levels had a negative effect. Proteome analysis then revealed, that the negative effect was related to global protein malonylation, triggered by the elevated malonyl-CoA level. This was similarly true for the native proteome as well as for the implemented pinosylvin pathway [38••] and repressed the latter at the level of 4-coumarate-CoA ligase (4CL) and stilbene synthase (STS). Upon this finding, the malonylation sites Lys113 and Lys161 of STS were replaced by arginine to avoid protein acylation. The STS_K113R enzyme variant tolerated higher intracellular levels of malonyl-CoA and allowed 220% increased pinosylvin production, when only 18 μM cerulencin was added (Figure 2d) [38••]. More generally, this work highlights the importance of considering post-translational modifications for metabolic engineering.

In another study, combined proteome and metabolome analysis was carried out to survey the metabolism of quiescent (Q-cell) *E. coli* [37]. Q-cells, created by addition of the proton ionophore indole, maintain a high metabolic activity decoupled from cell growth. Comparative

(Figure 2 Legend Continued) a tailored optimization [26,27•,29,38••,42•,43,51,55•,58••]. 1BOH: 1-butanol; 6dEB: 6-deoxyerythronolide; *adhE2*: gene, encoding butanoyl-CoA reductase; Ara: arabinose; *araR*: arabinose repressor gene; PcAraT/*araT*: arabinose transporter from *Penicillium chrysogenum* and encoding gene; ButCoA: butanoyl-CoA; Cer: cerulencin; CoA: coenzyme A; FBA: flux balance analysis; FDCA: flux distribution comparison analysis; For: formiate; *gapN*: NADP-dependent glyceraldehyde dehydrogenase gene of *Streptococcus mutans*; Glc: glucose; GuaB/*guaB*: IMP dehydrogenase and encoding gene; LYS: lysine; NCgl0275: protein/gene involved in transcriptional regulation in *C. glutamicum*; MalCoA: malonyl-CoA; *mak*: fructokinase gene of *E. coli*; Mtl: mannitol; PEP: phospoenolpyruvate; P_{ackA}: promoter of acetate kinase; P_{fuf}: promoter of elongation factor tu; PIN: pinosylvin; PPP: pentose phosphate pathway; P_{sod}: promoter of superoxide dismutase; PTS_FruA/*fruA*: fructose specific subunit of phosphotransferase system and encoding gene; PYR: pyruvate; RBS: ribosomal binding site; *rbsK*: fructokinase gene of *B. succiniciproducens*; RIB: riboflavin; Scr: sucrose; *scrK*: fructokinase gene of *Clostridium acetobutylicum*; STS/Acyl-STS: stilbene synthase/acylated STS; SUC: succinate; Zwf/*zwf*: glucose 6-phosphate dehydrogenase and encoding gene.

proteome analysis of growing and Q-cell cultures revealed an increased expression of stress response proteins upon the addition of indole. The stress proteins likely primed the Q-cells to tolerate the metabolic imbalances. The imposed imbalance resulted in an accumulation of acetyl-CoA and phosphoenolpyruvate, which was then exploited to drive the production of 3-hydroxypropionate (3HP) via an artificial operon. Q-cells were found to produce twice as much 3HP (39 g L^{-1}) as compared to the control (19 g L^{-1}), despite a strongly reduced biomass formation [37]. This, however, was not designed from proteome data, but these were considered more for explanatory reasons.

In summary, there are surprisingly not as many application examples of proteomics in the metabolic engineering context. So far, this field has remained more at a descriptive level and thus contributed mainly to our general knowledge gain than to specifically improved strains. However, proteomics technologies underwent a tremendous development and meanwhile offer well-designed, largely automated workflows [25]. The beauty of the examples presented here, recommend to do more proteomics-based metabolic engineering in the future. Given the importance of, for example, posttranslational protein modification, isoenzymes, protein complexes, and metabolons for cellular function, it seems absolutely worth.

Metabolome-based metabolic engineering

Metabolomics provides access to the metabolites of primary metabolism that are pathway intermediates, energy nucleotides and redox cofactors, and furthermore to anabolic building blocks and the spectrum of secondary metabolites, totally up to 10 000 different species per cell [39,40]. Typically, it couples elaborated techniques for sampling and sample work-up with different analytical instruments to address the low molecular weight compounds of a cell, substantially differing in abundance, chemical properties, and turnover [41].

When applied to 1-butanol producing *E. coli*, metabolome studies revealed an imbalanced CoA metabolism upon disruption of the *ack-pta* pathway to the undesired by-product acetate [42•]. The resulting decrease of free CoA inside the cell triggered the formation of other by-products: pyruvate and butanoate. The activity of butanoyl-CoA reductase within the butanol pathway, catalyzing butanal formation under release of free CoA, was identified as the bottleneck causing this phenotype [42•]. Fine-tuned expression of butanoyl-CoA reductase through modification of the RBS could then increase the 1-butanol titer by 22% up to 18.3 g L^{-1} (Figure 2e) [42•].

Metabolome analysis was also successfully applied to optimize arabinose metabolization in *C. glutamicum* [43]. *C. glutamicum* ATCC 31831 is a natural arabinose degrader and can equally grow on glucose and arabinose. However, when both substrates are present, glucose is preferentially consumed, while arabinose assimilation is repressed. Metabolome analysis identified the phosphoenolpyruvate-pyruvate switch point as key target for optimization. Overexpression of pyruvate kinase was found crucial for simultaneous utilization of glucose and arabinose, particularly when the arabinose metabolism was stimulated by deletion of the repressor gene *araR*. This provided the first *C. glutamicum* strain, which simultaneously degrades hexoses and pentoses without inactivation of the phosphotransferase system (Figure 2f) [43].

Compared to, for example, transcriptome and proteome, the analysis of the metabolome is technically far more challenging with regard to timescale and analytical instrumentation [41,44]. To account for the fast turnover of metabolites within the cell, immediate quenching of all metabolic activity is required at the time point of sampling. Resulting effects thereof on cell integrity, often entailing metabolite leakage, can severely bias metabolome data [45,46]. Subsequent analytics need to cover a chemically diverse, partly instable set of molecules over a concentration range of several orders of magnitude in a complex sample matrix. Moreover, it is still difficult to obtain compartment-specific metabolite levels, which complicates interpretations for higher cells, such as yeast and fungi. These challenges might explain, why we have not seen so many success stories here. However, if properly performed, metabolomics can be of high value to guide strain engineering, as demonstrated by the given examples.

Fluxome-based metabolic engineering

Through a comprehensive approach of ^{13}C isotope experiments, MS and NMR based analysis and computation of metabolic and isotopomer models [8,47,48], ^{13}C metabolic flux analysis provides quantitative estimates on the *in vivo* reaction rates of enzymes (the fluxome) as the functional output of all cellular components, interlinked by transcriptional, translational, post-translational and metabolic control [49]. Routinely, this gives access to about 100–150 reactions of central carbon metabolism. This core set of reactions establishes a ubiquitous and interconnected network that catalyzes the major material flows and provides building blocks, energy, and redox power [50].

In a straightforward study, ^{13}C-based metabolic flux analysis unraveled the complex architecture of sucrose metabolism in *Basfia succiniciproducens* [51], a potent producer of succinate [52]. The dicarboxylic acid is a platform chemical of recognized value for different industrial branches [53]. Sucrose, on the other hand, is one of the most preferred raw materials for biotechnology, so that an efficient production of succinate from this raw material

is regarded highly attractive. Microbial metabolism of sucrose forms a network of specific complexity, involving uptake of the disaccharide, intracellular cleavage and further metabolization of the two monomers glucose and fructose, including secretion and re-uptake of the latter [54]. To quantify the metabolic flux distribution of succinate producing *B. succiniciproducens* on sucrose, a comprehensive set-up of four parallel ^{13}C tracer experiments with GC/MS analysis of 460 different mass isotopomers allowed a precise quantification of all pathway fluxes in the central metabolism. This led to the discovery of fructokinase, so far unknown in *B. succiniciproducens* [51], which contributed to a low extent to phosphorylation of the sucrose-subunit fructose in addition to the known fructose PTS. For production efficiency, fructokinase plays a key role, because it can reduce the fructose PTS-related loss of the valuable succinate precursor phosphoenolpyruvate into pyruvate [51]. On the basis of these findings, metabolic engineering eliminated the PTS (*fruA*) (not a promising target without knowing about the novel fructokinase) and enhanced expression of fructokinase (*rbsK*) (not obvious at all before). Both targets resulted in improved succinate production at concomitantly reduced by-product formation (Figure 2g). The best strain reached a yield of 2.5 mol mol^{-1}, thus almost touching the theoretical optimum (3 mol mol^{-1}) [51].

Metabolic flux analysis was also the key to success for the recent design of an efficient lysine-producing *C. glutamicum* cell factory, which could use mannitol as a substrate, a major constituent of seaweed, which in turn is an attractive third-generation renewable [55•]. The study revealed that, due to a low flux via the pentose phosphate (PP) pathway, the NADPH supply in mannitol-grown *C. glutamicum* was strongly impaired. In fact, the substrate was almost exclusively channeled through the Emden–Meyerhof–Parnas (EMP) pathway. The PP pathway is the major route to supply NADPH in this microbe. This flux phenotype limited production, which has a high demand for redox power: four molecules of NADPH have to be supplied per molecule of lysine. Guided by the flux information, the production performance could be improved stepwise. In a first attempt, a heterologous fructokinase was implemented to re-direct the mannitol influx towards the PP pathway. The novel mutant, however, exhibited only a minor improvement. Its evaluation on the flux level then revealed a rigid flux partitioning around the PP pathway [55•], suggesting to design a PP pathway-independent strain to by-pass the observed bottleneck. Efficient production was then achieved by coupling the NADPH supply to the highly active EMP route and expressing the NADP-dependent glyceraldehyde dehydrogenase as additional NADPH source (Figure 2h). The resulting SEA-3 strain accumulated lysine from mannitol in shake flask at a yield of 0.24 mol mol^{-1} and a productivity of 1.3 mmol g^{-1} h^{-1}, as high as the performance of glucose-based lysine cell factories [55•].

Commonly, ^{13}C metabolic flux is applied to study microbes under well-defined conditions that are single carbon sources and defined media. In recent years, however, the approach has been taken further to resolve fluxes also under industrial process conditions, involving undefined fermentation broth, microbial consortia and complex pathway layouts [56,57]. In an elegant approach, ^{13}C tracer substrates were now used to elucidate metabolic fluxes in the riboflavin producing fungus *Ashbya gossypii* under industrial production conditions [58••]. Riboflavin (vitamin B2) is essential for animal and human health and nutrition and its commercial production has almost doubled in the past decade [59]. The production process with *A. gossypii* is based on a complex medium (vegetable oil, yeast extract, and different precursors) and exhibits separate phases for growth and production. Using a sophisticated set-up of parallel ^{13}C experiments and labelling analysis with GC/MS, LC/MS, 1D NMR, and 2D NMR in combination with computational simulations, this process was elucidated in detail. The flux approach revealed that the supply of carbon one building blocks formed a severe bottleneck at one specific time point of the culture: the initial phase of riboflavin production starting once growth ceased. Time-resolved feeding of formate as a carbon-one donor then successfully replenished the transiently limiting carbon-one supply, resulting in an impressive 45% increase of the final riboflavin titer [58••]. This nicely demonstrates, that systems biology approaches are not only valuable for developing strains, but also for targeted optimization of bioprocess control.

Among all omics levels, the fluxome is most close to the phenotype of a cell. In the past decade, the analysis of metabolic fluxes has massively deepened our understanding of biology [8,9] and emerged as a key technology to guide metabolic engineering [60]. Admittedly, its analysis is technically not as simple as DNA or RNA sequencing, but provides a most direct view on how to optimize a cell. In metabolic engineering, this is where it is at. As shown above, fluxome analysis particularly provides light in the darkness of more complex scenarios, such as an industrial fermentation set-up, which appear difficult or even infeasible to be understood from other data.

Conclusion

The past years have seen a tremendous development of technological platforms for microbial systems biology [1,2,61,62]. The highlighted examples of this review (and other studies that could not be covered here) show that systems biology offers a huge power to guide strain and process engineers in academia and industry. In this sense, microbial systems biology and systems metabolic

engineering have become a design science. A few things are finally worth mentioning.

Firstly, the most important omics in the biotechnology area is probably economics. Omics techniques are embedded into industrial strain and process development in a problem-oriented and target-driven manner. Their (often expensive) use should always manifest in value, that is, a measurable improvement, which emerges more from specific knowledge about a microbe of interest, than from general knowledge. This implies to first identify the exact problem and then extract the essential information as fast as possible: only as much as needed, but preferably not more. The examples in this work serve as inspiring cases that systems biology offers a box full of tools, which each can provide unique insights into a cell or process, not covered by the others. Therefore, each approach available today appears important to drive the field. It very much depends on the purpose and the aim of a planned study, which omics tool(s) promise(s) to address the problem best and therefore should be selected. In line, we base the choice of a specific omics approach based on the problem to be solved: the search for an unknown export protein is well supported by transcriptomics [63], a novel pathway is discovered most straightforward by metabolomics [64], whereas systems-wide pathway redirection is best guided by fluxomics [10].

Secondly, the whole (of a microbe) is (still) more than the sum of its parts. In contrast to a first intuitive guess a beginner might have, there often is no linear correlation between the expression of a gene, the level of the encoded protein and its *in vivo* catalytic activity, due to the various layers of cellular control [35••]. In fact the different levels can substantially differ or even show opposed trends. This inherent complexity can only be understood (and subsequently optimized in a desired manner) by integrating complementary data, which resolve the underlying interplay [49,65–67]. As a consequence, ^{13}C metabolic flux analysis becomes more and more important to obtain complete pictures on cellular systems [1,47,60], because it has the power to link cellular components into functional relations and exclusively provides a real measure of network operation. Moreover, we will see more multi-omics studies beyond the examples realized so far, which directly address this problem [1,35••,68].

Thirdly, the impressive improvement in microbial systems biology indicates that we will soon see studies of similar resolution and design power in biological systems of much higher complexity. Even whole plants are meanwhile studied on a systems level, providing important insight into their nitrogen and carbon metabolism [69].

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