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Systems and synthetic biology for the biotechnological application of cyanobacteria

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Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis. Their evolutionary relation to plastids in eukaryotic phototrophs and their increasing utilization as green cell factories initiated the use of systems biology approaches early on. For select model strains, extensive 'omics' data sets have been generated, and genome-wide models have been elucidated. Moreover, the results obtained may be used for the optimization of cyanobacterial metabolism, which can direct the biotechnological production of biofuels or chemical feedstock. Synthetic biology approaches permit the rational construction of novel metabolic pathways that are based on the combination of multiple enzymatic activities of different origins. In addition, the manipulation of whole metabolic networks by CRISPR-based and sRNA-based technologies with multiple parallel targets will further stimulate the use of cyanobacteria for diverse applications in basic research and biotechnology.

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Introduction

Systems biology uses a holistic approach to describe properties of organisms functioning as a set of biological items or objects that combine as parts into a mechanism, complex network or organism [1]. Fundamental to this approach has been the development of various 'omics' technologies, which provide comprehensive non-biased data sets of high quantity and quality. Metabolic and regulatory models established on the basis of such data sets lead to advanced insight into complex biological systems.

Cyanobacteria attracted systems biology approaches early on. These organisms evolved oxygenic photosynthesis approximately 2.5 billion years ago, and it was later conveyed via endosymbiosis into eukaryotes, giving rise to the development of eukaryotic algae and plants [2]. The resulting close evolutionary relation between cyanobacteria and plant plastids motivated early genomic analyses, leading to the first complete and publicly available genome sequence of *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) more than 20 years ago [3]. Based on the established genetic tools [4], *Synechocystis* became one of the most widely used models among cyanobacteria. Due to their environmental and biotechnological importance, more than 200 complete genome sequences of cyanobacteria belonging to different clades are now publicly available [5].

The available genome sequences facilitated the use of transcriptomic and proteomic approaches. Transcriptomics, initially performed with DNA microarrays, permits for example the systematic analysis of gene expression patterns under different environmental perturbations [6]. More recently, RNAseq in its different flavours has become more popular. These approaches not only permit genome-wide quantification of gene expression but also may be utilized for annotation of transcriptional starting points, promoter regions and the identification of many new genes, largely for non-protein-coding RNAs [7] and previously overseen microproteins with less than 100 amino acid residues [8•]. Hence, this technique has been instrumental to our understanding of complex and mixed regulatory networks, which include traditional protein regulators and RNA regulators in a hierarchical manner [9,10•]. Many of the transcriptomics data sets available for *Synechocystis* are accessible in a single database [11].

Cyanobacterial proteomics started initially with two-dimensional gel electrophoresis combined with mass-spectrometry-driven peptide identification. These approaches displayed between 10% and 20% of the entire proteome of *Synechocystis*. Gel-free approaches using fractionated protein preparations can now identify more than 50% of the entire *Synechocystis* proteome. However, such a high coverage requires much effort and is difficult to apply to large sample sets [12]. Recently, selected reaction monitoring (SRM) has been established for a subset of the *Synechocystis* proteome [13]. SRM requires substantial effort to set up, but once established, it allows the precise quantification of targeted proteins. SRM has been used to quantify proteins of regulons, which are

controlled by the transcriptional factor SufR [13] or by the small RNA IsaR1 [10•]. In addition to the quantification of proteins, proteome methods can be used to identify protein modifications, such as phosphorylation [14,15] or glutathionylation [16], that are potentially involved in regulating protein activities.

Metabolomics aims to quantify the complete set of cellular metabolites and may directly display the dynamics of cellular metabolism. The combination of gas chromatography or liquid chromatography with mass spectrometry permits quantitative analysis of approximately 100 metabolites in cyanobacterial cells, yet the spectrum of identified substances varies between the applied methods [17]. Flux measurements (fluxomics) using stable isotopes [18,19] have also been applied to obtain deeper insight into carbon partitioning occurring in cyanobacterial cells under different growth conditions.

For the evaluation of flux and systems biology approaches using cyanobacteria, that is, the integrated analysis of 'omics' experiments, appropriate models are necessary. For *Synechocystis*, Knoop *et al.* presented [20] the first metabolic network reconstruction of a cyanobacterium in 2010. Interestingly, the model identified some missing or not yet annotated reactions in the primary metabolism of *Synechocystis*. For example, serine biosynthesis in *Synechocystis* was initially thought to be performed via photorespiratory metabolism, but it actually also proceeds through the phosphoserine pathway [21]. In recent years, cyanobacterial models became more comprehensive, for example, by including photosynthetic light reactions [22,23]. Most models used the flux balance analysis approach, which describes the reaction network of an organism and optimizes flux to optimal growth output. However, this approach does not allow simulation of different growth conditions or the analysis of specific roles of isoenzymes. Recently, a comprehensive genome-scale model was published for *Synechococcus* sp. PCC 7002 [24•] including variable biomass objective functions, which reflected changes in cellular composition under different light regimes. This approach allows for the prediction of carbon flux changes in cells under different growth conditions, which was validated by ^{13}C-flux experiments [24•]. Kinetic modelling approaches are more demanding and difficult to apply to the entire metabolic network of a cyanobacterium. However, kinetic modelling permits the quantitative analysis of metabolic subsets under different growth conditions, revealing the crucial role of isoenzymes for the rerouting of organic carbon in *Synechocystis* cells grown under different inorganic carbon regimes [25,26].

Systems biology of cyanobacteria and biotechnology

Recent climate change concerns and the increasing demand for renewable energy sources has raised much interest in photoautotrophic cyanobacteria for use in the production of biofuels and chemical feedstock [27,28]. Hence, cyanobacterial strains were engineered to produce diverse products, such as ethanol [29,30], isobutyraldehyde and isobutanol [31], fatty acids [32], sucrose [33], 3-hydroxypropionate [34], and isoprene [35,36]. However, in most cases, the production rate was low, making economic feasibility for production questionable.

Systems biology approaches that apply different 'omics' technologies were used to identify strategies to increase the biofuel yield. Despite high ethanol productivity, surprisingly few transcriptomic changes were identified in the *Synechocystis* producer strains when compared to wild type [37], whereas proteomics showed many alterations in ethanol as well as lactic acid producer cells [38]. *Synechococcus* sp. PCC 7002 ethanol producer cells exhibited a low-carbon phenotype despite their cultivation under CO_2-supplemented aeration, and their metabolome analysis revealed a limiting carbon flux into the precursor pyruvate [39]. Optimization of carbon allocation into precursor synthesis and of the subsequent biofuel pathways will probably be one of the major challenges to improving the productivity of cyanobacteria. For example, isoprene productivity was nearly threefold higher when the non-naturally occurring mevalonic acid pathway, which promotes the synthesis of the isoprene synthase precursor, was expressed in *Synechocystis* [40]. Isotopically nonstationary ^{13}C flux analysis was used to optimize isobutyraldehyde production in *Synechococcus elongatus* PCC 7942. Moreover, the method revealed potential bottlenecks in precursor pyruvate synthesis, which were at least partly overcome via overexpression of selected enzymes, leading to enhanced pyruvate and product synthesis [41••].

Another strategy to increase product yield aims to reduce carbon flow into competing pathways. For example, glycogen represents the main storage unit of organic carbon in cyanobacterial cells and many researchers aim to minimize this pool to increase carbon flux into the product. Accordingly, deletion of the *glgC* gene for ADP-glucose pyrophosphorylase, which synthesizes the glycogen precursor ADP-glucose, resulted in enhanced *Synechocystis* ethanol production [42]. However, reduced fitness and stress tolerance were reported for glycogen-deficient mutants of *Synechocystis*. Under nitrogen-starvation, they showed a metabolic carbon-overflow, leading to the excretion of pyruvate and 2-oxoglutarate [43]. Since 2-oxoglutarate is a precursor for ethylene biosynthesis, it was surprising that decreased ethylene yield was measured when the ethylene synthesis pathway was expressed in the *Synechocystis glgC* mutant background [44]. Therefore, instead of abolishing glycogen synthesis, it might be better to increase glycogen breakdown for the utilization of organic carbon. Moreover, glycogen breakdown was recently observed as a result of the