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Chapter 7

Summary and Discussion

Prelude

This thesis focusses on adaptations of cyanobacteria to nutrient starvation stress. The research was done with the model organism *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis*). In particular, nitrogen starvation stress was studied by means of transcriptomic, proteomic and physiological experiments. To identify patterns of regulation, advanced experimental techniques were established and applied.

Full genome sequences for expression analysis

These days, genome sequencing has become a widely applied method. To answer questions in the domain of biodiversity, generally only a limited number of genes well suited for phylogenetic inference are studied (Woese, 1987; Pace, 2009; Rajendhran and Gunasekaran, 2011). However, for expression studies the real puzzle starts only after a full genome sequence has been established (e.g., Lashkari *et al.*, 1997; Swarbreck *et al.*, 2008; Gnerre *et al.*, 2011). With the genome sequence at hand, careful comparison of homologies with other genome sequences may enable recognition of functional units in the sequence, called open reading frames (ORF). Those basic minimal units of DNA ultimately provide for cell proteins after transcription and translation. Even for highly studied organisms many of the identified open reading frames are not yet annotated, and thus represent functionally unknown proteins. Even worse, for many ORF transcripts the translation may still be disputed; these ORFs are annotated as encoding hypothetical proteins. Despite the relatively small genome size of *Synechocystis* (3.6 Mbp) and the early date of its full genome sequencing, also for this model organism a large proportion of ORFs have no defined function yet. Extending beyond the common metabolic schemes and regulatory patterns earlier observed by more traditional biochemical methods, gene transcription studies under a range of conditions may reveal patterns of regulation indicative of functional gene products. This may help to annotate unknown and hypothetical proteins. Some interesting discoveries have been made in the work reported in this thesis (Chapter 2) that allowed ORFs of unknown or hypothetical proteins to become truly known genes that encode functional enzymes.

Early DNA-microarrays

At the time the work for this thesis was started, many of the instruments widely in use at present were not yet available. Our investigations started with an early transcriptomic platform, a so-called “genomic microarray”, which was kindly provided by the Japanese team that succeeded already in 1996 to landmark *Synechocystis* as the first fully sequenced photosynthetic prokaryote (Kaneko *et al.*, 1996). This event inspired many teams around the world to start working with the cyanobacterium *Synechocystis*. An early setup for transcriptomics was acquired from the first cyanobacterial sequencing project, which had the single-stranded constructs made for sequencing directly applied to a blot material. This platform included thousands of M13 clones spotted on nylon filters, which contained the sheared *Synechocystis* chromosome. Hence, in each spot many different ORFs were present.

Quite often those ORFs had different roles in cell physiology, making precise investigation of the functional expression of each individual ORF rather limited. Despite this handicap the survey on differential expression was started. To this end, a procedure for enrichment of radioactively labeled cDNA belonging to the small pool of mRNA transcripts was designed. The concomitant removal of abundant cDNA related to ribosomal transcripts (over 90% of all RNA) significantly increased the signal to noise ratio. However, the fact that each spot on the genomic macroarray represented 1-10 different genes made interpretation of the transcription data quite challenging.

In order to discriminate between specific and general stress responses of *Synechocystis* we conducted experiments with three types of environmental stress: two different nutrient stresses and high salt stress (Chapter 2). Despite our inability to test for the statistical significance of the observed transcription changes, we were able to highlight sets of differentially expressed genes for each stress condition, based on careful comparison of individual blots. This stimulated in-depth interrogation of these differences in gene expression using a context of common and specifically regulated metabolic categories. Several ORFs upregulated under high-salt stress were members of the hypothetical and unknown protein categories, and of these two ORFs were targeted for further study using knock-out mutagenesis. Proof was thus found for participation of two adjacent ORFs (*slr1208* and *ssr2016*) in cyclic electron flow around Photosystem I in *Synechocystis* (Yeremenko *et al.*, 2005). Also, unique changes in transcription were disclosed for phosphate limitation stress. These studies revealed that different environmental stresses give rise to different response intensities as exemplified by the number of regulated spots. The completed macroarray experiments are covered in further detail in the second chapter of this thesis. Yet, despite the new insights that we obtained from the macroarray data, the overall conclusion was that better experimental techniques were required for the aims of our research project.

The age of DNA-microarrays

In 2001, colleagues in Japan had arrived at a first true microarray platform for *Synechocystis* which was based on spotted cDNA probes of individual ORFs (Hihara *et al.*, 2001). The technical and statistical challenges of the early macroarray approach together with the appearance of the new Japanese setup (with restricted access for third parties) inspired our group to create our own *Synechocystis* microarray for custom order printing. Our goal was to dig deeper into transcription profiling and to decipher stress adaptation. Requirements for the design of this novel microarray platform included, amongst others, a high specificity of the probes and a statistically sound number of probes for each ORF. After consideration, we based our platform choice on spotted 60-mer oligonucleotide microarrays produced by Agilent. Eventually, the design was arranged locally in our laboratory and was largely realised by Dr. Eneas Aguirre, at that time PhD student in our laboratory. The creation of 'our' DNA microarray was based on the known 3264 ORFs of *Synechocystis*. Each of the ORF/genes was represented on the microarray by one to four specific oligonucleotides

and printed in 11K format on a standard glass slide. More recently, Cyanobase updated the *Synechocystis* annotation and now reports the presence of 3317 genes on the *Synechocystis* chromosome and 408 additional genes located on plasmids (<http://genome.kazusa.or.jp/cyanobase>). Detailed information on our whole genome *Synechocystis* DNA microarray is covered in the thesis of Aguirre von Wobeser (2010).

Working with DNA microarrays

Any microarray experiment requires sophisticated data treatment in order to obtain a statistically significant pattern of differential gene expression. Chapter 3 of this thesis presents the design of a data analysis pipe-line, which was applied to decipher differential gene expression in response to nitrogen starvation. Data analysis was based on a widely used open source programming language for statistical analysis called “R” (www.r-project.org). In particular, I made use of the Bioconductor project, which provides tools for scientists and programmers working on problems in bioinformatics and computational biology (Gentleman, 2008; www.bioconductor.org). The Bioconductor software is specifically tailored for the analysis and comprehension of high-throughput genomic data. My data analysis pipeline was based on Limma, a software package for microarray data using linear models for the analysis of designed experiments and the assessment of differential gene expression (Smyth, 2005). Data handling included raw microarray data import, correction for background variation, and data normalization (Smyth and Speed, 2003). Differential expression of genes was analyzed by fitting a linear model to the normalized data of treatments and controls, and subsequent tests of the statistical significance using empirical Bayesian statistics with control of the false discovery rate (Smyth, 2004). At each step of data analysis, we also inspected the data using intensity plots for individual microarrays, density and box-plots, and hierarchical clustering of all replicates. Finally, the results of the data analysis were visualized with MA-plots, Venn-diagrams and heat-map representation of the clustering of specific groups of genes (Hahne *et al.*, 2008). Pioneering the application of these new techniques was quite demanding, and nowadays these labour intensive operations would be outsourced to specialists in bioinformatics. In retrospect, our development of the data treatment procedures has required a substantial amount of time and effort, but with that done, the real investigations could start.

Testing of DNA microarrays

In order to test the newly designed microarray platform, we initiated a series of pilot experiments. These consisted of dye-swapped technical hybridizations of three biological replicates of 12 hours nitrogen-starved cultures that were compared to non-starved control cultures of *Synechocystis*. The time point of 12 hours was chosen on purpose as at this stage cellular adaptation to nitrogen starvation has already started. This was clearly visible by changes in a marked cell property, as the colour of the cells shifted from bright green to yellow. However, it was anticipated that at this time point during nitrogen starvation, the physiological changes are not yet too dramatic. Typically, cells can store nutrients, thus

permitting continuation of cellular metabolism during quite some time after the onset of nutrient starvation (Görl *et al.*, 1998; Sauer *et al.*, 2001). In this respect, gradual nutrient starvation differs somewhat from other stresses, such as pH, temperature, salt, osmotic or high light stress, with a more immediate impact on cellular function.

Several nutrients can potentially become limiting for cyanobacterial growth in natural waters, including C, N, P and Fe. Of these, nitrogen limitation was chosen as the topic for my thesis work, because nitrogen is well represented in the macromolecules that make up living cells while only limited information exists on the transcriptome response of cyanobacteria to nitrogen starvation. One exception is the work of Osanai *et al.* (2006), who studied the transcriptome response of *Synechocystis* after 4 hours of nitrogen starvation. Although a 4-hour treatment seems relatively long for many standard transcriptome applications, it is still relatively short given that cyanobacteria can continue to be active on their internal nutrient storage for quite some time. Osanai *et al.* (2006) highlighted the interesting induction of sugar catabolic and nitrogen assimilation genes, and also noticed the repression of photosynthetic and ribosomal genes. However, longer-term nitrogen starvation responses were not documented in their work.

From our 12 h pilot experiment, we concluded that our microarray platform performed technically very well and generated highly reproducible and biologically interesting data. In response to nitrogen starvation, we observed repression of genes for the photosynthetic apparatus, including many genes of Photosystems I and II, phycobilisome-related genes, all subunits of the ATP synthase, and a number of genes related to CO₂ fixation. Conversely, as expected, nitrogen assimilation genes were induced. We came across several examples of ORFs encoding hypothetical or unknown proteins that suggested upgrading to true genes *sensu stricto*. Furthermore, we spotted consistent induction of genes encoding respiratory terminal oxidases and hydrogenases. In total, this experiment gave some first insights on how cells can redistribute their internal nitrogen stocks and reorganize their physiology to enable continued cell growth in an economically feasible manner when nitrogen falls short. The results of this pilot experiment are described in Chapter 4 and provided a good starting point for further experimentation.

Using microarrays to pursue transient changes in continuous culture

Investigations continued with a study of the whole-genome response of *Synechocystis* in continuous cultures subjected to a shift from nitrogen- to light-limited conditions, and then back to nitrogen-limited conditions (Chapter 5). Continuous culture provides experimental control over the specific growth rate of the organisms by diluting the culture at a fixed rate. At the onset of the experiment, cells were acclimated to N-limitation. These cells likely put all of their abilities to collect as much N as possible from the relative low supply of N to the culture. We monitored changes in cellular physiology and the transcriptome before and after addition of non-limiting aliquots of N to the medium supply, to determine how the cells would respond.

In a macroscopic sense, the culture got a much greener complexion after N addition, a sign that the growth conditions changed from N limitation to light limitation. The microarray experiments revealed numerous changes in the transcriptome, permitting insight into the relative transcription of all ORF/genes in context. Analysis of the microarray data highlighted eight clusters of regulated genes, each cluster showing different temporal patterns of gene expression during the transition from nitrogen to light limitation. We investigated possible correlations between these genetic changes and the physiological parameters that were measured simultaneously. For instance, a number of photosynthesis-related genes clustered together and showed up-regulation during light-limited growth and down-regulation during nitrogen-limited growth. The behavior of this gene cluster proved well correlated with measured physiological parameters such as specific growth rate, light attenuation coefficient, photosynthetic potential and maximum electron transfer rate. Another cluster including genes related to nitrogen assimilation showed the opposite pattern: down-regulation during light-limited growth but up-regulation during nitrogen-limited growth.

Special attention was given to a comparison of our findings with existing data from other laboratories. In particular, our transition from light-limited to nitrogen-limited growth permits comparison with nitrogen starvation for a relatively short period of four hours performed in the batch cultures of Osanai *et al.* (2006). While many genes responded similarly in both experiments, several interesting discrepancies were also observed. This may be attributed to differences in growth conditions, with continuous culture permitting inspection of gradual changes in nutrient limitation rather than a trajectory towards complete nitrogen starvation as in batch cultures. The results of these continuous culture experiments are reported in Chapter 5 (Aguirre von Wobeser *et al.*, 2011) and provide a detailed insight into the numerous genes that change transcription during the transition from N- to light limitation. Notably, even under N limitation, the continuous culture is provided with low amounts of nitrogen all the time, as fresh medium is dripped in 24 h a day at a given rate. Here, there is always at least some N available for growth. This condition optimally exploits the window for in-depth investigation of changes in gene transcription while cells sustain growth.

Study of differential expression during N-starvation in batch cultures

Nitrogen starvation in batch culture is likely to impose other growth conditions than nitrogen limitation in continuous culture. In particular, although nitrogen-starved cells may temporarily sustain their growth on internally stored nitrogen, they will ultimately cease growth when all available nitrogen in batch culture has been depleted. To investigate whether this would lead to differences in whole-genome expression, we monitored changes in the transcriptome during nitrogen starvation in batch cultures (Chapter 6; Krasikov *et al.*, 2012). Nitrogen starvation was experimentally imposed by an abrupt change of growth medium from nitrate-containing to nitrate-free medium at time zero. The cellular physiology and transcriptome were sampled at several time points, from 6 hours up to 4 days of starvation. In addition, we investigated the *Synechocystis* transcriptome in recovery mode, after adding

back nitrate to the mineral medium.

The results showed a gradual response of the cells to nitrogen starvation, consisting of three major phases: (1) an immediate response, (2) short-term acclimation, and (3) long-term survival. As immediate response, at the early stage of nitrogen starvation, cells continued growth but with enhanced gene expression of uptake and transport systems for nitrogen assimilation and even transient increase of expression of Photosystem I and phycobilisome-related genes. The second phase was characterized by gradual decreases of the growth rate, the nitrogen to carbon ratio of the cells, and the phycocyanin content. Interestingly, photosynthetic activities of photosystems I and II were well retained during this phase, as supported by measurements of photosynthetic and respiratory capacities by means of polarographic oxygen assays and PAM fluorescence. Hence, after phycobilisome degradation, the cells still retained their capacity for chlorophyll-excited photosynthetic electron transfer. The transcriptome of this phase showed suppression of genes encoding phycobilisomes, and also of genes related to carbon fixation. In contrast, genes encoding terminal oxidases and hydrogenases were induced, probably playing a role in quenching of excess electrons, thus compensating for a decreased demand for carbon fixation and nitrogen assimilation. In addition, protein levels after 12 hours of nitrogen starvation showed a two-fold decrease of the cellular contents of key enzymes of the dark reactions of photosynthesis, consistent with the similarly lowered transcript levels of the corresponding genes. In the latest phase, *Synechocystis* ceased growth and almost all other activity, as reflected by measurements of the physiological parameters. However, transcription levels of genes encoding PSI were sustained at some level, in contrast to the decreased expression of PSII, phycobilisomes and carbon fixation genes.

We believe that the sustained transcription of PSI genes together with the continued induction of nitrogen assimilation genes prepares cells for rapid recovery in case of increased availability of nitrogen. This rapid recovery was illustrated by rapid induction of many genes upon N addition to the starved cultures, although we retrieved quite some interesting genes that did not respond immediately to N enrichment as well.

In total, these results show that different genes respond at different time scales, thus enabling a gradual and smooth response of the organisms to rather abrupt changes in environmental nutrient availability.

Prospects

Our study of the physiology and transcriptomics of *Synechocystis* gives insight into the interplay between different cellular adaptation processes in response to nitrogen limitation, and also suggests new lines of investigation for future research. Regulatory networks involved in stress adaptation are quite complex. Therefore, attempts to resolve these networks and their pathways for regulation should include an integration of transcriptome analysis, high-throughput proteomics, comparative genomics and physiological experiments. In particular, comparative genomics in which the results of various stress conditions are overlaid to arrive at unique or nearly unique signals that could serve as witness of environmental conditions are

of evident interest for continued research and its applications.

Furthermore, technical possibilities grow rapidly and further integration of new techniques is likely. For example, since changes in gene expression do not necessarily match changes in protein expression, time-resolved high-throughput experiments investigating proteomes of stressed cultures would be desirable. This may be accomplished by an approach recently applied to *Synechocystis* acclimated to low CO₂, using iTRAQ based proteomics (Battchikova *et al.*, 2010). Another recent technical approach is full metabolomics, investigating whether changes in the cellular metabolite pools actually reflect the restraints and opportunities that regulated expression and translation confer.

Another major source of curiosity, with a lot of potential for new breakthroughs, is provided by the large number of open reading frames on the *Synechocystis* chromosome with a not yet assigned function. In our continuous cultures and time-resolved nitrogen starvation experiments, we found orchestrated changes in expression of many genes encoding hypothetical and unknown proteins, thus pointing to their actual involvement in stress adaptation or central metabolic processes of the cell. Careful time-resolved cluster analysis of these genes, using our transcriptome data as well as transcriptome results obtained under other stress conditions may suggest potential targets for site-directed mutagenesis with subsequent analysis of physiology and transcriptome analysis of generated knock-out and/or overexpression mutants. Such approaches may bring not only discoveries of the function of unknown genes, but will also extend our understanding of the crosstalk between carbon and nitrogen assimilation, photosynthesis and energy metabolism regulatory networks in cyanobacteria.

In recent years a new topic arose in studies addressing regulatory networks in cyanobacteria. Several studies have identified non-coding RNAs (ncRNA) and antisense RNA (asRNA) participating in cyanobacterial gene regulation in response to environmental stress (Georg and Hess, 2011; Gierga *et al.*, 2012). The classic example of asRNA in *Synechocystis* is IsrR – 177 nt asRNA complementary to the central region of the *isiA* mRNA, controlling the amount of transcript for the iron-stress inducible protein (IsiA) (Dühring *et al.*, 2006). Georg *et al.* (2009) identified 60 ncRNAs and 73 asRNAs in *Synechocystis* and proposed that asRNA might affect expression of up to 10% of all *Synechocystis* genes. Most recently, it was reported that around a quarter of the protein-coding genes have antisense transcripts in *Synechocystis*, and the *Synechocystis* chromosome encodes around 300 ncRNAs (Mitschke *et al.*, 2011). So far, functional relevance has been shown for asRNAs participating in cold stress, high light stress, iron and carbon limitation. It seems feasible to include targets for ncRNA and asRNA into microarray design, and to extend investigation of their regulatory functions in, for example, nitrogen starvation.

Finally, I would like to add that the early initiative to develop our own microarray platform with access for others was well accepted by the international cyanobacterial research community, as reflected in several joint publications (Eisenhut *et al.*, 2007; Tuominen *et al.*, 2008; Schriek *et al.*, 2008; Hackenberg *et al.*, 2009 and 2012). In the meantime new

techniques have evolved. For instance, the concept of tiling arrays makes array design much faster, because tiling arrays do not require the design of specific probes that target known genes but simply use a large set of probes to span the entire genome. Tiling microarrays allow a higher resolution map for the cyanobacterial transcriptome as recently demonstrated for *S. elongatus* PCC 7942 (Vijayan *et al.*, 2011), permit resolving operons more convincingly than with the current ORF based arrays, and also detect transcription of ncRNAs and asRNA (Georg *et al.*, 2009). Because tiling arrays are not biased by prior gene annotations, they can facilitate the discovery of hypothetical and unknown ORF and their metabolic control. DNA-microarrays have now become cheap enough to permit direct use in environmental studies. For large-scale analysis, microarray techniques may be complemented by direct RNA sequencing (Mitschke *et al.*, 2011).

It is foreseeable that environmental studies will profit from knowledge of the relative expression of “reporter genes” that can serve well as indicators of specific environmental conditions. An example of that work was shown for P-limitation in freshwater phytoplankton (Dignum *et al.*, 2004). The present work has revealed a rich list of interesting candidate reporter genes for nitrogen limitation stress. Genes reporting N limitation together with genes reporting other key environmental parameters like P, C and Fe limitation may be printed on small array platforms for rapid evaluation by end users in the world of water management. The idea is to combine these expression arrays with probes for biodiversity analysis, such that interrogation of e.g. environmental nutrient levels can be combined with insight into microbial species diversity and seasonal shifts in strain composition. Such data will likely contribute to a better early-warning system that can anticipate upcoming changes in surface water quality, for instance by revealing environmental conditions that shed advantage to unwanted species such as the harmful cyanobacterium *Microcystis*.

In conclusion, in this thesis we have exploited the fast moving field of microarray analysis, from its early rise till a first valuable product. A newly developed microarray platform for *Synechocystis* permitted whole-genome transcriptomics of cyanobacteria exposed to environmental stress. This brought new insights into the molecular and physiological adaptations of cyanobacteria to nitrogen limitation and nitrogen starvation. In a broader context, this work may contribute to an improved understanding of the response of aquatic microorganisms to changing environmental conditions, thus providing a deeper appreciation of the complexities of microbial life in aquatic ecosystems.

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