Dynamic changes in gene expression of the cyanobacterium Synechocystis sp. PCC 6803 in response to nitrogen starvation

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Chapter 1

General introduction

Contents

1. Cyanobacteria

1.1. Prelude

Cyanobacteria represent a large phylum of Bacteria able to perform photosynthesis. Some of the oldest fossils on Earth, from Australian Archaean rocks dated around 3.5 billion years ago, belong to ancient cyanobacteria (Schopf, 1993 and 2006). Nowadays, cyanobacteria may be found in almost every imaginable habitat on Earth: in oceans and fresh water, on rocks and in soil, and some of them have even been isolated from hot springs and Antarctic ice sheets. They may be single-celled, may form multicellular filaments or aggregate to colonies of various shapes (Fig. 1).

Probably the most striking feature of cyanobacteria is their ability to fix carbon dioxide and produce oxygen in the process of oxygenic photosynthesis. In fact, it is believed that photosynthesis by ancient cyanobacteria has been responsible for the large amounts of oxygen in the atmosphere of our planet. Oxygenic photosynthesis is performed on thylakoid

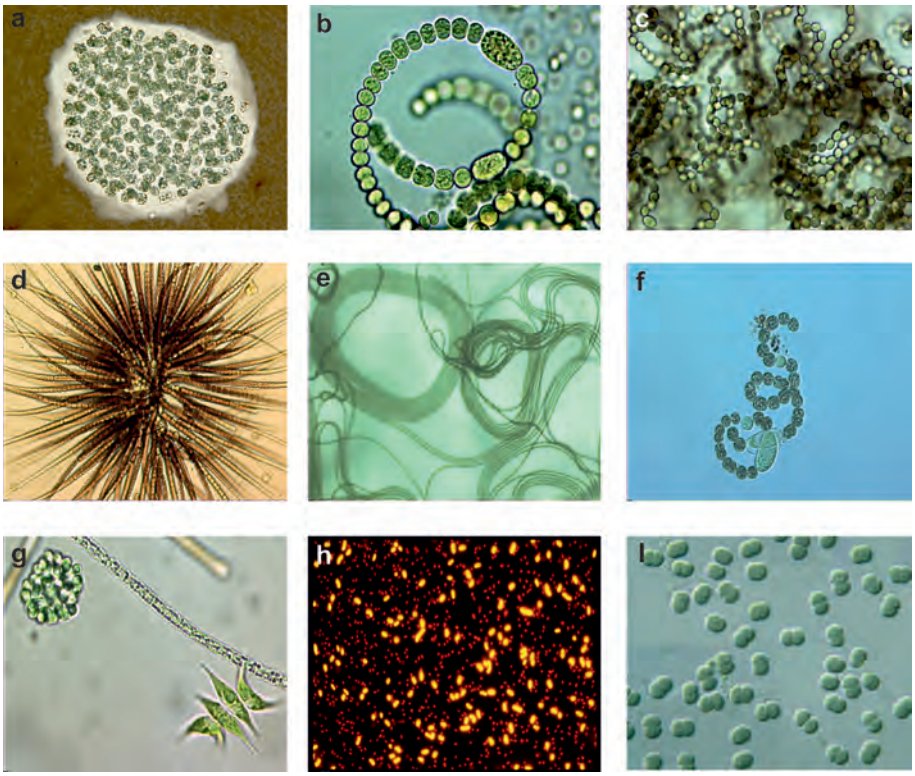

Figure 1. Cyanobacterial biodiversity. A. *Microcystis*, **B**. *Anabaena*, **C**. *Nostoc*, **D**. *Gloeotrichia*, **E**. *Planktothrix*, **F**. *Anabaena*, **G**. *Planktothrix*, *Scenedesmus* and *Gomphosphaeria*, **H**. *Synechococcus* spp. from the Baltic Sea, **I**. *Synechocystis* sp. PCC 6803. Photos are courtesy of the research group Aquatic Microbiology, University of Amsterdam.

membranes. These internal membranes embed sophisticated protein complexes involved in the light reactions and photosynthetic electron transfer by tandem action of Photosystem II and Photosystem I. These protein complexes of cyanobacteria are regarded as the evolutionary origins of those used for oxygenic photosynthesis in chloroplasts of eukaryotic algae and higher plants. Water is used as electron donor for Photosystem II, releasing oxygen as by-product. Some cyanobacteria may also use hydrogen sulfide as electron donor (Cohen *et al*., 1986). This rare relict may indicate how cyanobacteria themselves gained their photosynthetic ability from purple and green sulfur bacteria (Blankenship, 1992), although other analyses suggest that photosynthesis originated in the cyanobacterial lineage and the first phototrophs were anaerobic "procyanobacteria" (Mulkidjanian *et al*., 2006). A full respiratory chain, including a cytochrome aa3 type of terminal oxidase, can provide cyanobacteria with energy in darkness (Matthijs *et al.*, 1984; Hart *et al*., 2005). In some strains fermentation during anaerobic growth conditions has been shown as well (Stal and Moezelaar, 1997). These multiple physiological attributes have provided extreme versatility to cyanobacteria, not only making them evolutionary highly interesting organisms, but also explaining their ubiquitous distribution on Earth till this very day. A schematic representation of the cyanobacterial cell is shown in Figure 2.

The name 'cyano' (blue, in Greek) refers to a typical attribute of the light harvesting antennae of cyanobacteria. Phycobilisomes are attached to the thylakoid membrane, and bear the phycocyanin pigment that is responsible for the blue-green pigmentation of most cyanobacteria. Phycobilisomes play a role in light harvesting for Photosystem II. Other

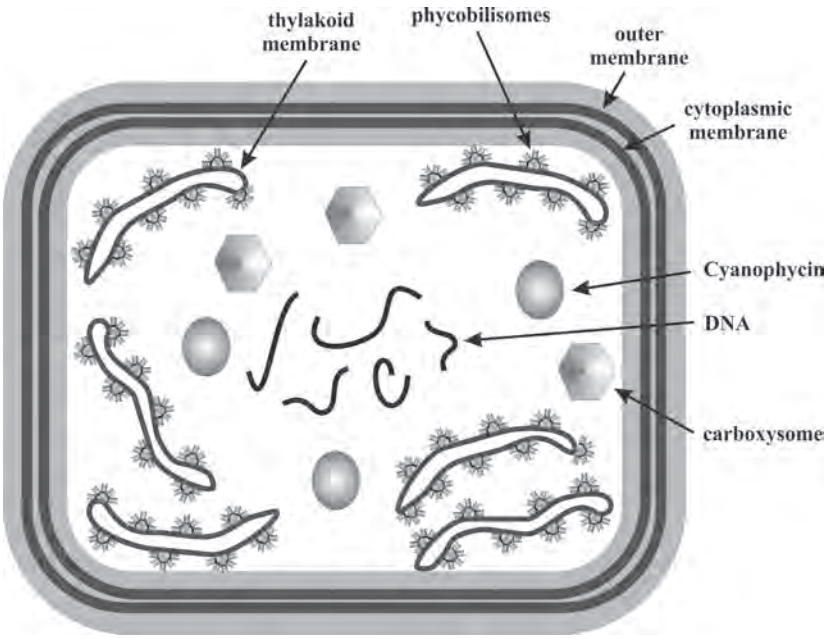

Figure 2. Diagram of the cyanobacterial cell.

pigments may be present as well, such as phycoerythrins (also located in phycobilisomes) and carotenoids, introducing red, brown and yellow palettes. Differences in relative proportion of chlorophyll *a*, phycocyanin, phycoerythrin and other accessory pigments give cyanobacteria a plethora of color variations: yellow, red, violet, pink, green, deep blue and blue-green cyanobacteria are known (Haverkamp *et al.*, 2009). The light spectrum also influences the pigment composition of cyanobacteria, and this way cells can maximize the use of available light for photosynthesis. In particular, through a process known as complementary chromatic adaptation, some cyanobacteria adapt their pigment composition to complement the prevailing light spectrum. Accordingly, these cyanobacteria appear bluish green by accumulating phycocyanin in red light, while cells turn reddish by producing phycoerythrin in green light (Stomp *et al*., 2004 and 2008).

Marine cyanobacteria are the most abundant photosynthetic organisms in the oceans. *Prochlorococcus* and *Synechococcus* are prevalent in the picoplanktonic fraction (with cell diameter less than 2 μm). The *Prochlorococcus* group dominates the nutrient-poor subtropical gyres of the world's oceans, and it is speculated to be the most abundant photosynthetic organism on the planet (Partensky *et al*., 1999). In the subtropical Pacific, for example, it often represents 50% of the total chlorophyll. These tiny cyanobacteria are also the smallest known photosynthetic organisms with a genome size of approximately 2 Mb. While *Synechococcus* is less abundant on a global scale, it has a more cosmopolitan distribution and is both genetically and morphologically more diverse than *Prochlorococcus*. *Synechococcus* cells can bloom in nutrient-rich coastal waters, and are also often found in freshwater ecosystems.

1.2. *Synechocystis* sp. PCC 6803

Model cyanobacterium *Synechocystis* sp. PCC 6803 is a small unicellular picocyanobacterium isolated from a freshwater lake in 1968 (Fig. 1i). The genome of *Synechocystis* sp. PCC 6803 is the first completely sequenced genome of a photosynthetic organism (Kaneko *et al.,* 1996). *Synechocystis* offers the advantage of an easily accessible genomic database with a relatively small genome size. *Synechocystis* can also be grown photo- and chemoheterotrophically, enabling decoupling of the light and dark reactions of photosynthesis. Moreover, *Synechocystis* can integrate foreign DNA into its own DNA via homologous recombination (Shestakov and Khyen, 1970), which makes cloning procedures for *Synechocystis* sp. PCC 6803 straightforward and thus provides researchers with an easy tool for targeted mutagenesis. Hence, *Synechocystis* has been one of the most popular organisms for genetic, biochemical and physiological studies, particularly for the analysis of oxygenic photosynthesis.

2. Current advances in transcriptomics and proteomics of cyanobacteria

2.1. "…omics"

Recently, the biological sciences have been revolutionized by a series of "omes": the genome (the complete genetic information of an organism), the transcriptome (all messenger RNAs expressed at a given time in a cell), the proteome (the protein equivalent), and the latest addition to the family is the metabolome (all small molecules that are the product of enzymatic and chemical activity within the cell). In contrast to the genome, which is fairly inert, the latter three molecular entities are highly dynamic and vary greatly according to endogenous and exogenous conditions; they also vary throughout the life cycle of an organism. The dynamic expression of genes as mRNA (the transcriptome) can be followed in a quantitative and qualitative manner using binding assays based on DNA arrays (Schena *et al*., 1995). Alternatively, sequencing methods based on either differential-display polymerase chain reactions (PCR) (Liang and Pardee, 1992 and 1995) or tagged-DNA approaches for serial analysis of gene expression (Velculescu *et al*., 1995) can be used. DNA-microarrays thus provide the technology to decipher how organisms exploit their genetic makeup in different environments. It has become an indispensable tool for analyzing the information encoded in the genome and transcriptome.

2.2. Cyanobacterial genomic projects

The complete genome sequence of the cyanobacterium *Synechocystis* sp. PCC 6803 was determined in 1996 by the Kazusa DNA Research Institute (http://genome.kazusa.or.jp/cyanobase). It was the first genome completely sequenced in Japan and the fourth in the world following the three genomes (*Haemophilus influenzae*, *Mycoplasma genitalium*, and *Methanococcus jannaschii*) sequenced earlier by TIGR. In comparison to other bacterial species, such as *Escherichia coli* and *Bacillus subtilis*, the *Synechocystis* genome contained a larger proportion of unknown genes, because cyanobacteria and their genes had not been well studied despite their importance in the evolution of life and the maintenance of the biosphere. Thus, with the availability of the genome sequence, gene annotation became a great spurt to cyanobacterial scientists, accelerating their research and resulting in a large number of publications.

Since 1996, more than 50 cyanobacterial genomes have been sequenced including freshwater and marine, unicellular and filamentous model organisms and ecologically relevant species. Some of the most noteworthy sequenced species are enlisted in Table 1.

2.3. Cyanobacterial transcriptomics

Since the appearance in Japan of the first microarray platform for the cyanobacterium *Synechocystis* sp. PCC 6803, in 2001 (Hihara *et al.*, 2001), researchers performed numerous experiments that made use of the transcriptome approach based on cDNA microarray platforms. The first oligo-microarray platform for the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC

Table 1. A selection of published cyanobacterial genomes (http://genome.kazusa.or.jp/cyanobase).

Organism	Genome size *, bp	Year	Description	Reference
Synechocystis sp. PCC 6803	3,573,471	1996	naturally transformable freshwater unicellular cyanobacterium; can be grown photoheterotrophically	Kaneko *et al.* (1996)
Anabaena sp. PCC 7120	6,413,771	2001	freshwater nitrogen-fixing filamentous, heterocyst-forming cyanobacterium; obligate photoautotroph	Kaneko *et al.* (2001) Ohmori *et al.* (2001)
Thermosynechococcus elongatus BP-1	2,593,857	2002	formerly *Synechococcus elongatus*; thermophilic (optimum ca. 55 °C), unicellular	Nakamura *et al.* (2002)
Gloeobacter violaceus PCC 7421	4,659,019	2003	unicellular cyanobacterium, which lacks thylakoids and phycobilisomes are attached to the plasma membrane	Nakamura *et al.* (2003)
Prochlorococcus marinus MED4	1,657,995	2003	ecotype adapted to high light	Rocap *et al.* (2003)
Prochlorococcus marinus MIT9313	2,404,274	2003	ecotype adapted to low light	Rocap *et al.* (2003)
Prochlorococcus marinus SS120	1,751,080	2003	small genome size, marine; ecotype adapted to low light	Dufresne *et al.* (2003)
Synechococcus sp. WH8102	2,434,428	2003	marine, unicellular, swimming motility	Palenik *et al.* (2003)
Prochlorococcus marinus MIT9312	1,709,204	2004	marine high-light adapted ecotype	JGI[a]
Synechococcus elongatus PCC 7942	2,695,905	2004	freshwater unicellular	JGI
Synechococcus sp. CC9605	2,510,659	2004	marine oligotrophic	JGI
Synechococcus sp. CC9902	2,234,828	2004	marine coastal	JGI
Trichodesmium erythraeum IMS101	7,750,108	2004	marine filamentous, nitrogen fixing	JGI
Anabaena variabilis ATCC29413	6,365,727	2005	filamentous heterocyst-forming; intensively studied for its hydrogen production	JGI
Synechococcus sp. CC9311	2,606,748	2006	marine coastal	Palenik *et al.* (2006)
Synechococcus sp. JA-2-3B	3,046,682	2006	thermophilic *Synechococcus*, nitrogen fixing	Allewalt *et al.* (2006)
Synechococcus sp. JA-3-3Ab	2,932,766	2006	thermophilic *Synechococcus*, nitrogen fixing	Allewalt *et al.* (2006)
Microcystis aeruginosa NIES-843	5,842,795	2007	Bloom-forming toxic	Kaneko *et al.* (2007)
Synechococcus elongatus PCC 6301	2,696,255	2007	freshwater unicellular	Sugita *et al.* (2007)
Prochlorococcus marinus NATL2A	1,842,899	2007	marine low-light adapted ecotype	JGI
Prochlorococcus marinus MIT9215	1,738,790	2007	marine high-light adapted ecotype	JGI
Prochlorococcus marinus AS9601	1,669,886	2007	marine high-light adapted ecotype	Moore[b]
Prochlorococcus marinus MIT9515	1,704,176	2007	marine high-light adapted ecotype	Moore
Prochlorococcus marinus MIT9303	2,682,675	2007	marine low-light adapted ecotype	Moore
Prochlorococcus marinus NATL1A	1,864,731	2007	marine low-light adapted ecotype	Moore
Prochlorococcus marinus MIT9301	1,641,879	2007	marine high-light adapted ecotype	Moore
Synechococcus sp. RCC307	2,224,914	2007	marine	Genoscope[c]
Synechococcus sp. WH7803	2,366,980	2007	marine	Genoscope
Synechococcus sp. PCC7002	3,008,047	2008	marine	Penn[d]
Acaryochloris marina MBIC11017	6,503,724	2008	chlorophyll d as major (95%) pigment	TGen[e]
Nostoc punctiforme ATCC29133	8,234,322	2008	freshwater nitrogen-fixing filamentous, heterocyst-forming cyanobacterium	JGI
Prochlorococcus marinus MIT9211	1,688,963	2008	marine low-light adapted ecotype	Moore
Cyanothece sp. ATCC51142	4,934,271	2008	aerobic, unicellular, nitrogen-fixing cyanobacterium	Welsh *et al.* (2008)
Cyanothece sp. PCC7424	5,942,652	2008	unicellular isolated from rice fields	JGI
Cyanothece sp. PCC7425	5,374,574	2008	unicellular isolated from rice fields	JGI
Cyanothece sp. PCC8801	4,679,413	2008	unicellular isolated from rice fields	JGI
Arthrospira platensis NIES-39	6,788,435	2010	filamentous	Fujisawa *et al.* (2010)

***** genome size is given for the main chromosome only excluding plasmids; **a** published online by Joint Genome Institute (www.jgi.doe.gov); **b** The Gordon and Betty Moore Foundation; Marine Microbiology Initiative (www.moore.org); **c** Genoscope (www.cns.fr); **d** published by Penn State University; **e** TGen Sequencing Center.

7120 appeared in 2005 (Ehira *et al.*, 2005) and the first oligo-microarray for *Synechocystis* appeared in 2007 (Eisenhut *et al.*, 2007). Publication of many new cyanobacterial genomes facilitated fast appearance of new microarray platforms for various cyanobacteria, and this promoted more complete transcriptome analysis and the reconstruction of genomic networks in cyanobacteria. These studies investigated how the entire transcriptome responded to, amongst others, salt, osmotic, cold, high light and oxidative stress (Table 2).

Despite these advances, remarkably few studies have investigated the transcriptome response of cyanobacteria to nitrogen limitation. Sato *et al.* (2004) and Flaherty *et al.* (2011) investigated the transcriptome response of *Anabaena* PCC 7120 to nitrogen deprivation. This is a somewhat special case, however, since *Anabaena* can replenish its cellular nitrogen by atmospheric nitrogen fixation. Osanai *et al.* (2006) presented the response of the entire transcriptome of *Synechocystis* PCC 6803 to 4 hours of nitrogen starvation. These authors found induction of many sugar catabolic genes and repression of sugar anabolic genes. They also found induction of several genes related to nitrogen assimilation, and repression of photosynthetic genes and ribosomal protein genes. However, since their study covered only a limited time span of 4 hours, the persistence of these short-term responses or additional long-term responses to nitrogen starvation remained unknown. Hence, a better understanding of nitrogen limitation at the transcriptome level is one of the key challenges to be tackled in this PhD thesis.

2.4. Cyanobacterial proteomics

Although transcriptome studies have gained many new insights, there are several objections against the reduction of biological studies to monitoring of the transcriptome (mRNA) only: (1) the level of mRNA does not allow accurate prediction of the level of protein expression (Anderson and Seilhamer, 1997; Gygi *et al*., 1999); (2) protein function is controlled by many post-translational modifications; and (3) protein maturation and degradation are dynamic processes that dramatically alter the final amount of active protein, independent of the mRNA level. Correlation of the mRNA levels with the expression, modification and activity of proteins requires a systematic method for separating and visualizing the protein components of a cell that allows: (1) extraction and high-resolution separation of protein components, including membrane, extreme-pI and low-copy-number proteins; (2) identification and quantification of each component; and (3) comparison, analysis and visualization of complex changes in expression patterns.

In one of the first proteome studies of *Synechocystis* PCC 6803 proteins have been investigated by two-dimensional gel electrophoresis (2DGE) and *N*-terminal amino acid sequencing. In this study 234 protein spots could be identified (Sazuka *et al*., 1999). 2DGE aided by mass spectrometry identification of resolved spots has been successfully applied in qualitative characterization of the protein content of purified cellular sub-fractions of outer and cytoplasmic membranes (Huang *et al.,* 2002a and 2004), for thylakoid membranes (Wang *et al*., 2000; Herranen *et al.,* 2004), as well as for a total proteome of *Synechocystis*

(Gan *et al*., 2005). Numerous efforts have been undertaken for quantitative proteomics in cyanobacteria. However, only a limited number of proteins were found to be differentially expressed in the acclimation to high salinity (Fulda *et al.,* 2000 and 2006), high light (Choi *et al.,* 2000), or high pH stress (Zhang *et al*., 2009). The proteome of *Synechocystis* acclimated to low CO_2 was measured with the iTRAQ technique (isobaric Tag for Relative and Absolute Quantification; Ross *et al*., 2004). In this study, 19% of the *Synechocystis* proteome was identified and expression changes were quantified for 86% of the identified proteins (Battchikova *et al*., 2010). New trends in high-throughput proteomics of cyanobacteria are rapidly developing (reviewed by Ow and Wright, 2009).

3. Assimilatory nitrogen metabolism and its regulation

3.1. Nitrogen assimilation

Nitrogen is one of the key elements of life, and an important limiting nutrient in many terrestrial, freshwater and marine ecosystems (Vitousek and Howarth, 1991; Sterner and Elser, 2002). Microorganisms and plants incorporate nitrogen through assimilation processes. As sources of nitrogen they may utilize ammonium, nitrate and nitrite ions, sometimes dinitrogen gas, and organic compounds like urea, amino acids and some nitrogen-containing purine and pyrimidine bases. Uptake of these nitrogen-containing compounds takes place through permeases that are located in the cytoplasmic membrane (Fig. 3). For instance, ABC-type uptake transporters have been shown to be involved in the uptake of nitrate and nitrite (Omata *et al.,* 1993; Luque *et al*., 1993 and 1994) in several cyanobacteria. ABC-type permeases are also required for the uptake of amino acids like arginine and glutamine (Quintero *et al*., 2001). These permeases use ATP to drive active nitrogen uptake.

For most microorganisms ammonium is the preferred source of nitrogen, because ammonium can be easily incorporated into amino acids. The transport of ammonium is mediated by secondary permeases of the Amt family (Fig. 3). It has been shown that [^{14}C] methylammonium is accumulated in cells of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 to a level that suggests a membrane potential-driven transport (Montesinos *et al*., 1998; Vazquez-Bermudez *et al*., 2002). Intracellular ammonium is incorporated into carbon skeletons through the GS/GOGAT pathway (Fig. 3). In cyanobacteria, 2-oxoglutarate (2-OG) provided by the tricarboxylic acid (TCA) cycle is mainly used as carbon skeleton for the incorporation of nitrogen (Vazquez-Bermudez *et al*., 2000; Muro-Pastor *et al*., 2001). It was generally accepted that cyanobacteria lack 2-oxoglutarate dehydrogenase, an enzyme of the TCA cycle (Stanier and Cohen-Bazire, 1977). However, a recent finding has provided evidence that 2-oxoglutarate may be converted to succinate by an alternative enzyme, thus closing the TCA cycle (Zhang and Bryant, 2011). The enzyme glutamine oxoglutarate aminotransferase (GOGAT) combines one molecule of 2-oxoglurate with one molecule of glutamine to produce two molecules of glutamate. Subsequently, the two glutamate molecules incorporate two ammonium ions and are converted in tandem into two glutamine molecules

Table 2. Key studies featuring cyanobacterial transcriptomics.

Strain	Research question	Reference
Synechocystis sp. PCC 6803 [1]	Acclimation to high light	Hihara *et al.* (2001)
	Cold stress; cold-regulated genes by Hik33 kinase	Suzuki *et al.* (2001)
	Salt stress and hyperosmotic stress	Kanesaki *et al.* (2002)
	Screening for the targets of cAMP receptor protein	Yoshimura *et al.* (2002)
	Mn2+-sensing system	Yamaguchi *et al.* (2002)
	Osmotic stress and cold stress	Mikami *et al.* (2002)
	Salt stress; high light stress	Allakhverdiev *et al.* (2002)
	Cold stress	Inaba *et al.* (2003)
	Analysis of redox-responsive genes	Hihara *et al.* (2003)
	Perception of salt stress	Marin *et al.* (2003)
	Phosphate limitation	Suzuki *et al.* (2004)
	Oxidative stress	Kobayashi *et al.* (2004)
	High salinity stress	Hihara *et al.* (2004)
	High salinity stress	Marin *et al.* (2004)
	Osmotic stress, histidine kinases	Paithoonrangsarid *et al.* (2004)
	Salt stress	Asadulghani *et al.* (2004)
	Response to red and far-red light	Hubschmann *et al.* (2005)
	Salt and hyperosmotic signals	Shoumskaya *et al.* (2005)
	Regulation of sugar catabolic pathways	Osanai *et al.* (2005a)
	Heat shock	Suzuki *et al.* (2005)
	Transcription profiling of the delta-sll1961 mutant	Fujimori *et al.* (2005)
	Glucose sensitivity	Kahlon *et al.* (2006)
	Heat shock response	Suzuki *et al.* (2006)
	Nitrogen starvation	Osanai *et al.* (2006)
	Hydrogen peroxide perception	Kanesaki *et al.* (2007)
	Low-temperature stress	Prakash *et al.* (2010)
Synechocystis sp. PCC 6803 [2]	Light-to-dark transition	Gill *et al.* (2002)
	Response to irradiation with UV-B and white light	Huang *et al.* (2002b)
Synechocystis sp. PCC 6803 [3]	Response to iron deficiency and iron reconstitution	Singh *et al.* (2003)
	High and low light conditions	Tu *et al.* (2004)
	Expression in response to hydrogen peroxide	Li *et al.* (2004)
	Redox control of gene expression	Singh *et al.* (2004)
	Expression in delta-isiA mutant	Singh *et al.* (2005)
	Heat shock	Singh *et al.* (2006)
	Growth at alkaline conditions	Summerfield and Sherman (2008)
	Iron and oxidative stress	Shcolnick *et al.* (2009)
	Low-oxygen conditions	Summerfield *et al.* (2011)
Synechocystis sp. PCC 6803 [4]	Response to inorganic carbon limitation	Eisenhut *et al.* (2007)
	Heat acclimation	Tuominen *et al.* (2008)
	Coordination C/N metabolism	Schriek *et al.* (2008)
	Photorespiration	Hackenberg *et al.* (2009)
	Low-carbon acclimation	Hackenberg *et al.* (2012)
	Nitrogen starvation	This thesis (Chapter 5)
	Transitions from nitrogen to light limitation	This thesis (Chapter 6)
Anabaena sp. PCC 7120 [5]	Gene expression under desiccation	Katoh *et al.* (2004)
	Nitrogen deprivation, low temperature and drought stress	Sato *et al.* (2004)

Table 2 (continued). Key studies featuring cyanobacterial transcriptomics.

Strain	Research question	Reference
Anabaena sp. PCC 7120 [6]	Low-temperature regulated expression	Ehira *et al.* (2005)
	Sodium chloride, cAMP concentration	Imashimizu *et al.* (2005)
	Genes for trehalose metabolism in dehydration tolerance	Higo *et al.* (2006)
	Heterocyst development	Ehira and Ohmori (2006)
Fremyella diplosiphon [7]	Genes regulated by light color	Stowe-Evans *et al.* (2004)
	Chromatic adaptation	Shui *et al.* (2009)
Thermosynechococcus elongatus BP-1 [8]	Circadian clock expression	Kucho *et al.* (2004)
Prochlorococcus MED4 [9]	Light sensing	Steglich *et al.* (2006)
Prochlorococcus MIT9313 [9]	Host-phage interaction	Lindell *et al.* (2007)
	Light-dark cycle	Zinser *et al.* (2009)
	Iron availability	Thompson *et al.* (2011)
Cyanothece sp. ATCC 51142 [10]	Light-dark and continuous light growth	Toepel *et al.* (2008)
	Diurnal oscillation	Stöckel *et al.* (2008)
	Short day/night cycles	Toepel *et al.* (2009)
Microcystis aeruginosa PCC 7806 [11]	Light/dark cycle	Straub *et al.* (2011)
Synechococcus sp. WH7803 [12]	Oxidative stress	Blot *et al.* (2011)

Microarray platform: 1, CyanoCHIP – PCR-amplified DNA microarray (TaKaRa, Japan); 2, PCR-amplified DNA microarray (Gill *et al*., 2002); 3, PCR-amplified DNA microarray (Postier *et al*., 2003); 4, Agilent 60-mer oligonucleotide microarray; 5, Long chromosomal DNA fragments spotted as microarray (Katoh *et al*., 2004); 6, Complete oligo microarray (Ehira *et al*., 2005); 7, Long chromosomal DNA fragments representing half of the genome (Stowe-Evans *et al*., 2004); 8, 45-mer oligonucleotide microarray (Kucho *et al*., 2004); 9, Affymetrix high-density array MD4-9313; 10, Agilent 60-mer oligonucleotide microarray; 11, Agilent 4x44K 60-mer oligomicroarray; 12, 60-mer oligonucleotide.

by the enzyme glutamine synthetase (GS). One of these glutamines goes back into the GS/GOGAT cycle, while the other can be used for further amino acid synthesis. The GS/GOGAT pathway thus plays a key role in the C to N balance of the cells.

Two types of nitrate transport systems prevail in cyanobacteria, a prokaryotic ABC-type transporter known as NrtABCD (Omata *et al.*, 1993) and a nitrate transporter more similar to those found in Eukaryotes known as NrtP (Sakamoto *et al.*, 1999). NrtABCD effectively binds both nitrate and nitrite, whereas the affinity of NrtP is higher for nitrate than nitrite. In addition, a secondary transporter of the major facilitator superfamily has been identified as nitrate-nitrite transporter in some marine cyanobacteria (Sakamoto *et al*., 1999).

The ABC-type transporter NrtABCD is composed of four subunits (Fig. 4): (i) NrtA, a high-affinity periplasmic solute-binding lipoprotein, (ii) NrtB, an integral membrane permease, (iii) NrtC, a unique ATPase solute-binding fusion protein, and (iv) NrtD, a cytoplasmic ATPase (Omata, 1995). The 3D structure of NrtA from *Synechocystis* sp. PCC 6803 was revealed at a resolution of 1.5 Å (Koropatkin *et al*., 2006). NrtA is the most abundant protein in the plasma membrane of cyanobacteria, and necessary for cell survival when nitrate is the primary nitrogen source (Maeda and Omata, 1997). NrtA represents a unique class of transport proteins as it is significantly larger than other oxyanion-binding proteins. Nitrate is scavenged in the periplasmic space by NrtA and then delivered to the cytoplasmic membrane permease NrtB. The transfer of nitrate through the transmembrane pore is assisted

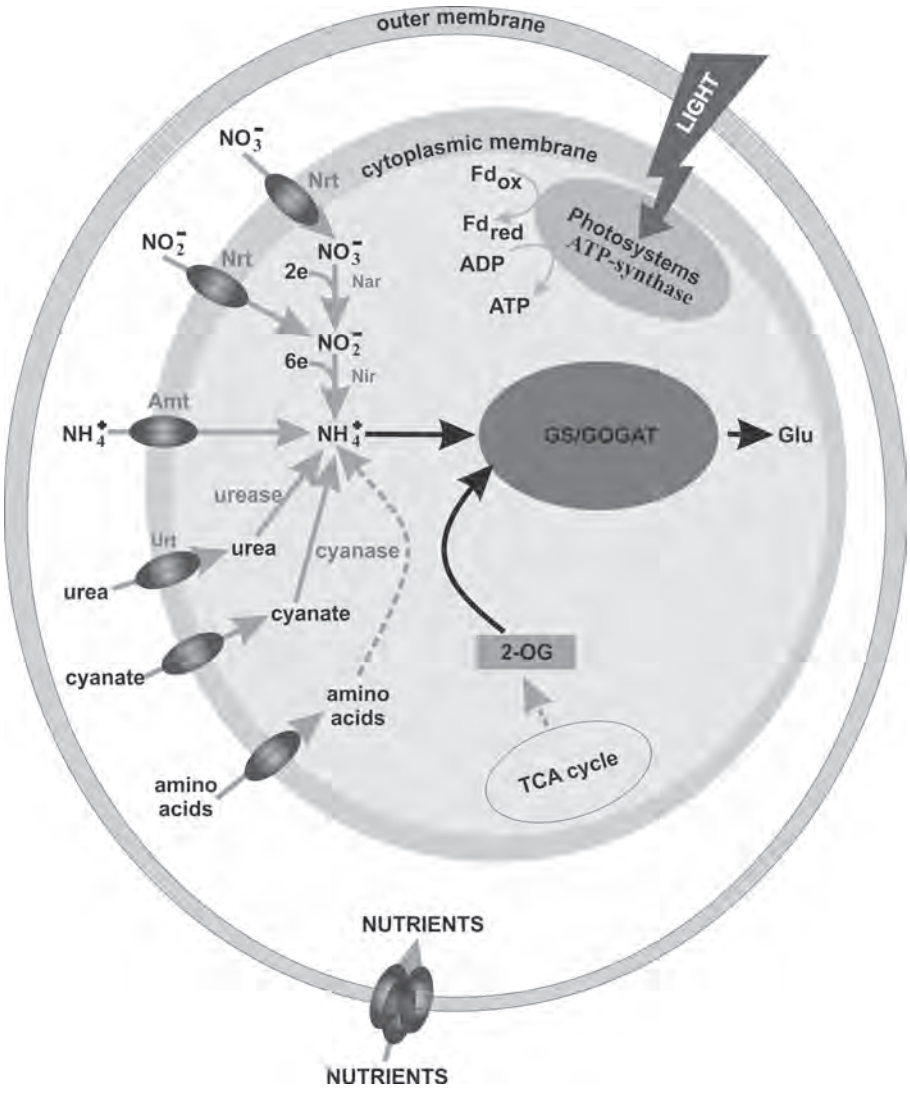

Figure 3. Main nitrogen assimilation pathways in cyanobacteria. Combined nitrogen sources are taken up through permeases and metabolized to ammonium, which is incorporated into carbon skeletons through the glutamine synthetase–glutamate synthase pathway (GS/GOGAT). Nitrogen is then distributed from glutamine or glutamate to the other nitrogen-containing organic compounds. Amt, ammonium permease; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; GOGAT, glutamate synthase; GS, glutamine synthetase; Nar, nitrate reductase; Nir, nitrite reductase; Nrt, ABC-type nitrate/nitrite transporter; 2-OG, 2-oxoglutarate; TCA cycle, tricarboxylic acid cycle; Urt, ABC-type urea transporter.

by ATPase activity of NrtC and NrtD. NrtD consists of a single ATPase domain, whereas NrtC contains both an ATPase domain and a C-terminal solute-binding domain that shares 50% amino acid sequence similarity with NrtA. This domain is required for the ammonium-mediated inhibition of nitrate transport (Kobayashi *et al*., 1997 and 2005). Another feature of the cyanobacterial NrtABCD transporter is its homology to the bicarbonate transporter CmpABCD. For instance, almost 50% of the amino acids in the subunit NrtA, which is the receptor subunit of the transporter, are identical to the subunit CmpA in the bicarbonate transporter. Koropatkin *et al*. (2006) present a model, based on the structure and sequence alignments of bicarbonate and nitrate transporters, that suggests that regulatory domains of both bicarbonate and nitrate transport systems bind nitrate. Comparison of the NrtA and putative NrtC and CmpC binding sites shows that all three proteins might bind nitrate. This similarity might enable cyanobacteria to perform synergetic coordination of their nitrogen and carbon assimilation.

Intracellular nitrate is reduced to nitrite and then to ammonium by nitrate and nitrite reductases (Fig. 3), which are products of the *nar*B and *nir*A genes respectively (Rubio *et al*., 1996; Luque *et al*., 1993). Cyanobacterial nitrate reductase is homologous with Mo-containing bacterial oxidoreductases but is unique in that it uses ferredoxin as an electron donor (Hirasawa *et al*., 2004). In this enzyme system, electrons flow from reduced ferredoxin

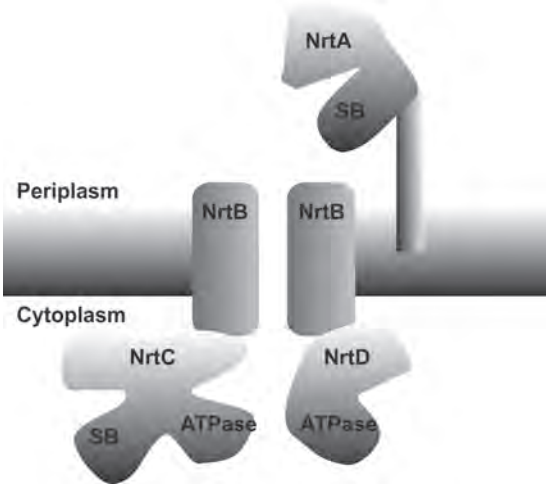

Figure 4. Assembly of the NrtABCD nitrate transporter from *Synechocystis* sp. PCC 6803. NrtA is attached to the periplasmic membrane by a flexible linker. It captures nitrate/nitrite in the periplasm by a solute-binding domain (SB) and subsequently delivers this nitrogen to the transmembrane pore created by the NrtB-dimer. NrtC and NrtD are ATPases that couple ATP hydrolysis to nitrate/nitrite transport through the pore; NrtC contains a C-terminal solute-binding domain (SB) homologous to NrtA.

to the iron-sulphur cluster and then to the Mo cofactor, where nitrate is reduced to nitrite (Rubio *et al*., 1998 and 1999; Jepson *et al*., 2004). Cyanobacterial nitrite reductase is homologous with the ferredoxin-dependent nitrite reductase of plants, and contains a [4Fe-4S] cluster and haem as prosthetic groups. Electrons from reduced ferredoxin are transferred to the iron-sulphur cluster and then to sirohaem, where nitrite is reduced to ammonium. This ammonium can subsequently be incorporated into carbon skeletons through the GS/GOGAT pathway as described above.

Cyanobacteria may also use organic compounds as sources of nitrogen (Fig. 3). For instance, urea is degraded to ammonium and CO_2 by a standard bacterial Ni^{2+}-dependent urease. Another organic nitrogen source is arginine, which is catabolized by an unusual pathway that combines the urea cycle and the arginase pathway rendering ammonium and glutamate as final products (Quintero *et al*., 2000).

3.2. Chlorosis and nitrogen limitation

Lack of nitrogen leads to a series of general responses in cyanobacteria: inhibition of cell division, loss of photosynthetic membranes, accompanied by loss of photosynthetic pigments (chlorophyll, phycobiliproteins, and all carotenoids except zeaxanthin), and accumulation of glycogen and inclusion bodies. The effect of nitrogen depletion on the abundance of pigment molecules has been reported in a wide range of cyanobacterial strains including *Anacystis nidulans* (Allen and Smith, 1969), *Synechococcus* spp. (Yamanaka *et al*., 1978; Sauer *et al.*, 2001), *Anabaena* (Foulds and Carr, 1977; Wood and Haselkorn, 1980) and *Synechocystis* strain PCC 6803 (Elmorjani and Herdman, 1987). The common result is massive and rapid decrease of the chlorophyll and phycobilisome content, which leads to a dramatic change in color from deep blue-green in nitrogen-replete cells to light yellow-green in nitrogen-deplete cells. This process is historically referred to as "chlorosis" (Allen and Smith, 1969). In some cyanobacterial strains, chlorosis also occurs in response to starvation by sulfur (Schmidt *et al*., 1982; Jensen and Rachlin, 1984), phosphorus (Ihlenfeldt and Gibson, 1975), carbon (Miller and Holt, 1977), and iron (Sherman and Sherman, 1983). Schwarz and Grossman (1998) suggested that chlorosis under stress conditions is required to decrease light absorption by the photosynthetic machinery of the cell. The general and specific stress responses of cyanobacteria to macronutrient deficiencies, including modification of the photosynthetic apparatus and phycobilisome degradation, are reviewed by Schwarz and Forchhammer (2005). In cyanobacteria, phycobilisomes can constitute up to 50% of the total cellular protein. Hence, they act as an important internal nitrogen store for the cell, providing building blocks for protein synthesis at times when extracellular nitrogen availability is low. In *Synechococcus* PCC 7942, knock-out mutagenesis has been used to identify genes required for degradation of the phycobilisomes. The knock-out mutants produce a non-bleaching phenotype during nitrogen and sulfur starvation (Schwarz and Grossman, 1998; Dolganov and Grossman, 1999). The *nblB* gene is constitutively expressed, whereas transcription of the *nblA* gene initiates phycobilisome degradation during nutrient starvation (Dolganov and

Grossman, 1999; Baier *et al.*, 2001). It encodes the NblA protein, which is a small polypeptide of 59 amino acids that binds to the phycobilisome and serves as an adaptor to the ClpC-ClpP protease complex, thus inducing proteolytic degradation of the phycobilisomes (Karradt *et al.*, 2008).The *nblR* gene product, which exhibits strong similarity to the response regulator OmpR (Schwarz and Grossman, 1998), controls the induction of expression of the *nbl*A gene. It has been suggested that NblR integrates diverse environmental signals that lead to phycobilisome degradation (Schwarz and Grossman, 1998).

3.3. The nitrate assimilation genes

Recent sequencing of numerous cyanobacterial genomes has permitted an extensive analysis of their nitrate assimilation genes. The distribution of these genes among different cyanobacteria reflects their evolutionary adaptation to various ecological niches (Bird and Wyman, 2003; Palenik *et al*., 2006; Scanlan *et al*., 2009).

Of the active nitrate/nitrite transporters, the prokaryotic ABC-type transporter NrtABCD is encoded by the *nrt*ABCD-gene cluster (Omata *et al*., 1993), while the eukaryotic-like permease NrtP is encoded by the *nrt*P gene (Sakamoto *et al*., 1999). In addition, a putative nitrite transporter encoded by the *focA* gene is present in some cyanobacteria (Martiny *et al.*, 2009; Ohashi *et al.*, 2011). Nitrate reductase (NarB) and nitrite reductase (NirA), the two key enzymes involved in the reduction of nitrate to ammonium, are encoded by the *narB* gene and *nir*A gene, respectively. Other genes related to nitrate assimilation, such as *narM* (Maeda and Omata, 2004), *cnaT* (Frias *et al*., 2003) and *nirB* (Suzuki *et al*., 1995), are often found in the proximity of the nitrate and nitrite transporter and reductases genes. Mutagenesis has shown that these three genes can modulate the activity of the major nitrogen assimilation genes, but their exact functions have remained elusive.

Cyanobacteria are classified in two groups by having Rubisco form 1A and 1B and α- and β-carboxysomes (Badger *et al*., 2002). β-cyanobacteria include diverse freshwater and marine strains, while the α-cyanobacteria group contains solely marine picoplanktonic strains (Ohashi *et al*., 2011). Most freshwater β-cyanobacteria bear the *nrt*ABCD gene cluster and are capable to assimilate nitrate, some freshwater β-cyanobacteria possess additionally the *nrt*P transporter gene (Table 3). Conversely, α-cyanobacteria such as the marine *Synechococcus* strains bear the *nrt*P gene, and some of them also have *foc*A (Palenik *et al*., 2003 and 2006). *Prochlorococcus* strains lack both the *nrt*ABCD and *nrt*P gene, although some of the strains possess *focA* (Table 3). Nitrate and nitrite reductases are present in all β-cyanobacteria and in some α-cyanobacteria. However, *Prochlorococcus* lacks the *nar*B gene, and only some *Prochlorococcus* strains bear the *nir*A gene (Dufresne *et al*., 2003; Rocap *et al*., 2003). Hence, in contrast to most other cyanobacteria, *Prochlorococcus* strains are unable to grow on nitrate.

The nitrate assimilation genes are commonly clustered near each other on the chromosomes of cyanobacteria. They often form a so-called *nirA* operon, with genes arranged in the order *nirA-nrtABCD-narB*, as illustrated for, e.g., *Synechococcus elongatus* PCC 7942 (Omata *et*

Table 3. Nitrate assimilation genes (adapted from Ohashi *et al*., 2011)**.**

	Nitrate/nitrite transporters			Reductases		Regulators				Unknown function		
Strains	***nrtABCD***	***nrtP***	***focA***	***narB***	***nirA***	***ntcB***	***ntcA***	***glnB***	***pipX***	***narM***	***cnaT***	***nirB***
Freshwater α-cyanobacteria												
Gloeobacter violaceus PCC7421	+	-	-	+	+	+	+	+	+	+	+	-
Synechocystis sp. PCC6803	+	-	-	+	+	+	+	+	+	+	+	-
Microcystis aeruginosa NIES-843	+	-	-	+	+	+	+	+	+	+	+	+
Synechococcus elongatus PCC6301	+	-	-	+	+	+	+	+	+	+	+	+
Synechococcus elongatus PCC7942	+	-	-	+	+	+	+	+	+	+	+	+
Synechococcus sp. JA-3-3Ab	+	-	-	+	+	+	+	+	+	+	+	-
Synechococcus sp. JA-2-3B'a	+	-	-	+	+	+	+	+	+	+	+	-
Thermosynechococcus elongatus BP1	+	-	-	+	+	+	+	+	+	+	+	-
Anabaena sp. PCC7120	+	-	-	+	+	+	+	+	+	+	+	+
Anabaena variabilis ATCC29413	+	-	-	+	+	+	+	+	+	+	+	+
Cyanothece sp. PCC7425	+	-	-	+	+	+	+	+	+	+	+	-
Cyanothece sp. PCC7424	+	+	-	+	+	+	+	+	+	+	+	+
Cyanothece sp. PCC8801	+	+	-	+	+	+	+	+	+	+	+	+
Cyanothece sp. PCC8802	+	+	-	+	+	+	+	+	+	+	+	+
Arthrospira platensis NIES-39	+	+	-	+	+	+	+	+	+	+	+	+
Nostoc punctiforme ATCC29133	-	+	-	+	+	+	+	+	+	+	+	+
Marine β-cyanobacteria												
Cyanothece sp. ATCC51142	-	+	-	+	+	+	+	+	+	+	+	+
Trichodesmium erythraeum IMS101	-	+	-	+	+	+	+	+	+	+	+	+
Acaryochloris marina MBIC11017	-	+	-	+	+	+	+	+	+	+	+	+
Synechococcus sp. PCC7002	-	+	+	+	+	+	+	+	+	+	+	+
Marine β-cyanobacteria												
Synechoccocus sp. WH8102	-	+	-	+	+	-	+	+	+	+	+	-
Synechoccocus sp. CC9605	-	+	+	+	+	-	+	+	+	+	+	-
Synechoccocus sp. CC9902	-	+	+	+	+	-	+	+	+	+	+	-
Synechococcus sp. CC9311	-	+	+	+	+	-	+	+	+	+	+	-
Synechoccocus sp. WH7803	-	+	+	+	+	-	+	+	+	+	+	-
Synechococcus sp. RCC307	-	+	+	+	+	-	+	+	+	+	+	-
Prochlorococcus marinus NATL1A	-	-	+	-	+	-	+	+	+	-	+	-
Prochlorococcus marinus NATL2A	-	-	+	-	+	-	+	+	+	-	+	-
Prochlorococcus marinus MIT9303	-	-	+	-	+	-	+	+	+	-	+	-
Prochlorococcus marinus MIT9313	-	-	+	-	+	-	+	+	+	-	+	-
Prochlorococcus marinus MIT9211	-	-	-	-	-	-	+	+	+	-	+	-
Prochlorococcus marinus SS120	-	-	-	-	-	-	+	+	+	-	+	-
Prochlorococcus marinus MED4	-	-	-	-	-	-	+	+	+	-	-	-
Prochlorococcus marinus MIT9515	-	-	-	-	-	-	+	+	+	-	-	-
Prochlorococcus marinus MIT9215	-	-	-	-	-	-	+	+	+	-	-	-
Prochlorococcus marinus MIT9301	-	-	-	-	-	-	+	+	+	-	-	-
Prochlorococcus marinus MIT9312	-	-	-	-	-	-	+	+	+	-	-	-
Prochlorococcus marinus AS9601	-	-	-	-	-	-	+	+	+	-	-	-

al., 1993) and *Anabaena* sp. strain PCC 7120 (Frias *et al*., 1997) in Figure 5. This gene arrangement ensures higher expression levels for upstream genes in the operon, and suggests the production of a balanced amount of the different proteins of the pathway (Frias *et al*., 1997). In several other species, the *nirA* operon contains the *nrtP* gene instead of the *nrtABCD* gene cluster (Fig. 5). Genes *narM*, *cnaT*, *nirB*, and *ntcB* are often found close to the *nir*A operon. Quite often various gene insertions are observed between genes of the *nir*A operon, and in some cases the *nirA* gene is distinctly separated from the other genes. Fast progress in cyanobacterial genomics also revealed other arrangements of the nitrate assimilation genes. For example, *nir*A, *nrt*ABCD and *nar*B genes are scattered on the chromosome of *Microcystis aeruginosa* NIES-843 (Fig. 5).

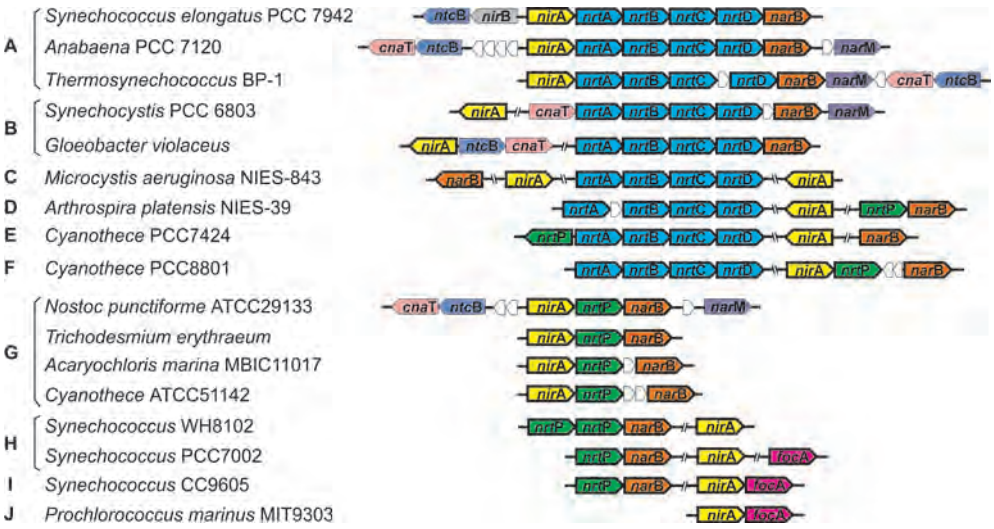

Figure 5. Nitrogen assimilation genes in different cyanobacteria. Bold pentagons represent core nitrate/nitrite assimilation genes (*nrt*ABCD, *nrt*P, *foc*A, *nar*B, and *nir*A). Genes *cna*T, *nar*M, *nir*B and *ntc*B are also shown in some cases, where they are located in close proximity to the core genes. The orientation of genes and relative position on the chromosome with respect to each other is shown. White pentagons refer to open reading frames encoding proteins with unknown function.

3.4. Nitrogen control

The activation or suppression of different pathways for nitrogen assimilation is known as nitrogen control. Nitrogen control systems have been well characterized in various microorganisms, including the NtrB-NtrC two-component regulatory system in proteobacteria (Merrick and Edwards, 1995), the GATA family of global nitrogen control transcription factors in yeast and some fungi (Marzluf, 1997), the GlnR-TnrA system in *Bacillus subtilis* (Fisher, 1999), and the AmtR master regulator of nitrogen assimilation in *Corynebacterium glutamicum* (Jakoby *et al.*, 2000). In cyanobacteria, several key regulators and a number of accessory players tightly control nitrogen assimilation, by exerting control at both the transcriptional and post-transcriptional level.

Transcriptional control is mainly exerted by the NtcA global nitrogen regulator, and is supported by the NtcB transcriptional regulator. NtcA activates transcription of nitrogen assimilation genes when nitrogen becomes limiting. Moreover, NtcA also acts as a sensor for the intracellular 2-oxoglutarate (2-OG) concentration, thus linking the C and N assimilation of cells. NtcA binds to the DNA of nitrogen assimilation genes, and its binding is enhanced by 2-OG (Vazquez-Bermudez *et al*., 2002). The consensus sequence of promoters targeted by NtcA is GTA-N_8-TAC-$N_{22/23}$-TAN_3T, where GTA-N_8-TAC corresponds to the NtcA binding motif and TAN_3T is the -10 element (Luque *et al*., 1994; Herrero *et al*., 2001). NtcA bound to the NtcA-binding site of the promoter recruits RNA polymerase via interaction with its σ subunit, thus facilitating gene transcription (Barnard *et al*., 2004). The transcriptional regulator NtcB is involved in activation of some of the nitrate assimilation genes and requires the presence of nitrite (Aichi and Omata, 1997, Aichi *et al*., 2001). In particular, activation of the *nirA* operon is achieved by dual action of NtcA and NtcB, with nitrite and 2-OG as co-activating factors. While NtcA is essential for the activation of gene transcription, NtcB by itself is not sufficient to promote gene expression (Maeda *et al*., 1998). The molecular basis of the NtcB action remains unclear. However, a conserved pair of the NtcB-binding motifs (ATGC-N_7-GCAT) is essential in promoters of nitrate assimilation genes in β-cyanobacteria (Ohashi *et al*., 2011). The *ntcB* gene is absent in the genomes of α-cyanobacteria, and its binding motifs are absent in the promoters of the associated genes.

Post-transcriptional control of nitrogen assimilation genes in cyanobacteria is mainly provided by the P_{II} protein, which is encoded by the *glnB* gene (Forchhammer 2004). Its key role is highlighted by the fact that the *gln*B gene is present in all cyanobacterial strains sequenced so far. The P_{II} protein binds to the central metabolites ATP, ADP, and 2-oxoglutarate (2-OG), and modulates activities of key enzymes in response to the energetic status and carbon/nitrogen balance of the cyanobacterial cell. Under conditions of nitrogen limitation, cyanobacterial P_{II} is phosphorylated at the conserved Ser^{49} residue. Dephosphorylation in turn is performed by the PphA protein phosphatase (Kloft and Forchhammer, 2005). This covalent modification of P_{II} plays a critical role in the regulation of N-acetylglucomate kinase (NAGK) activity (Burillo *et al*., 2004; Heinrich *et al*., 2004). NAGK is a key enzyme in arginine biosynthesis, and it catalyses conversion of N-acetylglutamate to N-acetylglutamate

phosphate. Low nitrogen content (corresponding to high 2-OG levels) or low energetic status (corresponding to high ADP levels) promotes dissociation of the P_{II}-NAGK complex. Without P_{II}, NAGK is highly sensitive to feedback inhibition by arginine. Conversely, in conditions of nitrogen and energy excess, P_{II} forms a stable complex with NAGK, thus promoting NAGK catalytic activity and an induced level of arginine biosynthesis. High arginine levels lead in turn to intracellular storage of nitrogen in cyanophycin.

Recent studies have highlighted the role of PipX in NtcA activity regulation (Espinosa *et al*., 2006, 2007, 2009). PipX is a small P_{II}-and NtcA-binding protein, and its gene is also present in all cyanobacterial strains. Its binding capacity to NtcA is affected by the nitrogen status of the cell (2-OG levels). When the 2-OG concentration is low, PipX preferentially binds to P_{II}. With increasing 2-OG concentration, PipX dissociates from P_{II}, binds to NtcA and enhances NtcA activity (Fig. 6). Hence, P_{II} perceives the nitrogen, carbon and energetic status of the cell by sensing the 2-OG, ATP and ADP levels and by interaction with its partners PipX, PphA, NAGK and PamA. The latter is a putative membrane protein of unknown function; it interacts with P_{II} in a 2-OG and ATP-sensitive manner (Osanai *et al*., 2005b) and influences transcription levels of several NtcA-dependent genes by a not yet identified mechanism.

The interplay between NtcA, P_{II} and PipX thus yields a remarkable spectrum of regulatory functions in cyanobacterial cells, controlling carbon and nitrogen metabolism at the gene expression level as well as at the protein activity level.

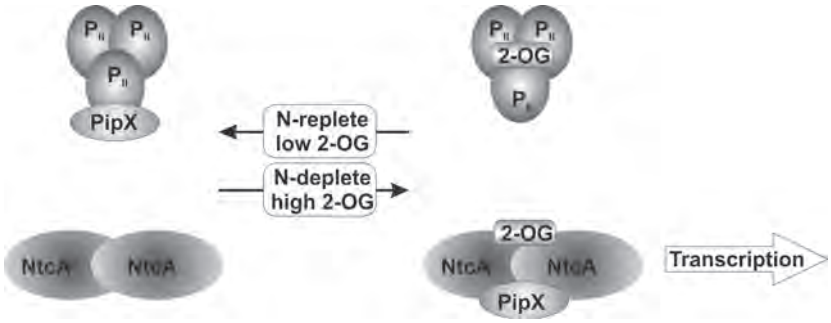

Figure 6. Regulation of NtcA activated transcription by P_{II} and PipX. Under N-replete conditions (low 2-OG levels), PipX binds preferentially to a P_{II}-trimer. In case of N-deplete conditions (high 2-OG levels), PipX dissociates from P_{II} and interacts with NtcA, resulting in enhanced transcription of NtcA-dependent genes.

4. Scope and outline of this thesis

The research presented in this thesis concentrates on the genetic, molecular and physiological aspects of nitrogen assimilation in the model freshwater cyanobacterium *Synechocystis* sp. PCC6803. In particular, I will investigate dynamic changes in gene expression across the entire transcriptome, and will compare this with changes in physiological traits, to elucidate the cascade of genetic and physiological reactions unfolding at different time scales during adaptation to nitrogen starvation.

Chapter 2 presents our initial transcriptomics approach based on macro-arrays obtained from the *Synechocystis* sequencing project. In this chapter we overlaid patterns of gene expression from three distinct stress conditions: high salt stress, nitrogen starvation and phosphorus starvation. This enabled the identification of several general as well as several stress-specific responses. Moreover, based on the results of this chapter, we selected several hypothetical open-reading frames (ORFs) for targeted gene mutagenesis and showed that ORFs *ssr2016* and *slr1208* play a role in Photosystem-I driven cyclic electron flow (Yeremenko *et al.*, 2005).

To investigate the response of *Synechocystis* to changing environmental conditions in more detail our research group designed an oligonucleotide DNA microarray (Eisenhut *et al.*, 2007; Tuominen *et al.*, 2008; Schriek *et al.*, 2008; Hackenberg *et al*., 2009 and 2012; Aguirre von Wobeser *et al.*, 2011). **Chapter 3** describes methods applied for the data analysis of microarray experiments in chapters 4 and 6 of this thesis.

Chapter 4 describes a pilot experiment on nitrogen starvation for 12 hours in batch cultures of *Synechocystis*. It investigates reliability of the designed microarray platform and the applied method for data analysis.

In **Chapter 5** we have applied our microarray platform to the large-scale investigation of dynamic changes in whole-genome expression of *Synechocystis* during the transition between nitrogen and light-limited growth in continuous culture (Aguirre von Wobeser *et al.*, 2011).

Chapter 6 of this thesis describes our detailed investigation of dynamic changes in whole-genome expression of *Synechocystis* during nitrogen starvation for 96 hours and subsequent recovery for 12 hours in batch cultures. In this chapter, we overlay transcriptomics with physiological data on photosynthetic performance of nitrogen-starved cultures and discuss the involved metabolic pathways (Krasikov *et al.*, 2012).

Finally, **Chapter 7** concludes this thesis with a general discussion and shows how the data support further understanding of the genetic and physiological adaptation of cyanobacteria to nitrogen limitation.

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