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# CYANOBACTERIAL ACCLIMATION TO CHANGING ENVIRONMENTAL CONDITIONS

## Roles for group 2 sigma factors in *Synechocystis* sp. PCC 6803

by

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## LIST OF ORIGINAL PUBLICATIONS

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II **Pollari M. and Tyystjärvi T.** (2008) The SigB sigma factor of the cyanobacterium *Synechocystis* sp. PCC 6803 is necessary for adaptation to high salt stress. In: Photosynthesis. Energy from the Sun. 14th International Congress on Photosynthesis Research, Glasgow 22-27 July 2007. Allen, J.F., Gantt, E., Golbeck, J.H., Osmond, B. (eds.). Springer, Heidelberg.

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V **Pollari M., Ruotsalainen V., Tyystjärvi E. and Tyystjärvi T.** (2008) Simultaneous inactivation of the sigma factors B and D interferes with light acclimation of the cyanobacterium *Synechocystis* sp. PCC 6803. Manuscript.

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# ABBREVIATIONS

| | |
|---|---|
| $A_{730}$ | absorbance at 730 nm |
| ATP | adenosine triphosphate |
| cAMP | cyclic adenosine 3’,5’-monophosphate |
| Chl | chlorophyll |
| $Cm^r$ | chloramphenicol resistance |
| CS | control strain |
| DCBQ | 2,6-dichloro-*p*-benzoquinone |
| DCMU | 3-(3’,4’-dichlorophenyl)-1,1-dimethylurea |
| Hik | histidine kinase |
| kDa | kilodalton |
| $Kn^r$ | kanamycin resistance |
| Mbp | million base pairs |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NCD | non-conserved domain |
| PCC | Pasteur Culture Collection |
| PCR | polymerase chain reaction |
| PPFD | photosynthetic photon flux density |
| ppGpp | guanosine tetraphosphate |
| PSI | photosystem I |
| PSII | photosystem II |
| qRT-PCR | quantitative real-time PCR |
| RNAP | RNA polymerase |
| Rre | response regulator |
| RT-PCR | reverse-transcription PCR |
| Å | Ångstrom, 0.1 nm |
| $\Omega$ fragment | spectinomycin and streptomycin resistance |

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# ABSTRACT

In cyanobacteria gene expression is mainly controlled at the level of transcription initiation. The RNA polymerase holoenzyme (RNAP) is composed of a catalytic core and a sigma factor ($\sigma$) that recognizes promoter elements. Bacterial genomes encode several different $\sigma$ factors and the exchange of one factor to another is thought to be a major determinant in changing the gene expression pattern. The cyanobacterium *Synechocystis* sp. PCC 6803 encodes nine $\sigma$ factors. SigA is the group 1 $\sigma$ factor, SigB, SigC, SigD and SigE are group 2 $\sigma$ factors and SigF, SigG, SigH and SigI belong to group 3.

Structural modelling of the RNAP revealed that the three-dimensional structures of the group 2 $\sigma$ factors are very similar. Single, double and triple inactivation strains were constructed to study the roles of the group 2 $\sigma$ factors. Similar growth and photosynthesis in all inactivation strains under standard conditions (BG-11 medium pH 7.5, 32 $^{o}$C, continuous light 40 $\mu$mol photons $m^{-2}$ $s^{-1}$, air level $CO_2$) indicated that the group 2 $\sigma$ factors are nonessential for growth under standard conditions.

Group 2 $\sigma$ factors were found to be important for acclimation to environmental stress conditions. SigB and SigC have key roles in acclimation to heat stress. The SigB factor was required for survival in short extreme heat stress and for acquired thermotolerance. SigB was a positive regulator of the *hspA* gene, which encodes a heat shock protein. SigC in contrast was crucial both in long- and short-term heat stress and for acquired thermotolerance. High temperatures diminish the availability of inorganic carbon. DNA microarray analysis and physiological measurements suggest a role for the SigC factor in the regulation of carbon metabolism during heat acclimation. Optimal acclimation to salt and hyperosmotic stress involved many group 2 $\sigma$ factors. SigB was crucial for growth in both salt and hyperosmotic stress while SigC played a minor role. The SigE factor had a role only in salt-induced osmotic stress. Acclimation to different light conditions was regulated by the SigB and SigD factors together. Strains lacking the SigD factor were sensitive to light on agar plates. When growth light intensity was doubled, all strains with simultaneously inactivated *sigB* and *sigD* genes were not able to take full advantage of a greater light availability. In addition, the ΔsigBD strain was more vulnerable to high light stress than the control or single inactivation strains due to a deficient photosystem II repair cycle. The SigB and SigD factors have partial functional redundancy probably because they have highly similar structures.

*Don’t lose your panic*

# 1. INTRODUCTION

## 1.1. Cyanobacteria

Cyanobacteria are a group of eubacteria capable of oxygen-evolving photosynthesis. This group of ancient autotrophs, more mundanely known as blue-green algae, have an oxygen-evolving photosynthetic apparatus similar to plants and they are considered to be the ancestors of plant chloroplasts (Rodriguez-Ezpeleta et al. 2005). In as early as 1905 a Russian scientist, Konstantin Mereschowsky, suggested that chloroplasts might originally have been free cyanobacteria living symbiotically inside a eukaryotic cell (Martin and Kowallik 1999). His idea was, however, ignored for decades until the development of new techniques allowed the comparison of chloroplasts and cyanobacterial cells on ultra-structural, molecular and genetic level (reviewed by Bhattacharaya and Medlin 1998, McFadden 2001). According to current estimates, the primary endosymbiosis event involving the engulfment of a cyanobacterium by a primitive eukaryotic cell occurred circa 1.5 billion years ago (Hedges et al. 2004, Yoon et al. 2004).

The origin of cyanobacteria has been traced back to the Archaean period (López-García et al. 2006). Without a protective ozone layer UV radiation razed all surfaces, making the environment hostile against the emergence of life forms. Despite the unwelcoming conditions, fossil records in sedimentary rocks provide evidence for the existence of photosynthetic microbial mats and colonies from 2.5 to 3.5 billion years ago (Tyler and Barghoorn 1954, Schopf 1996, Westall 2005, Lopez-García 2006). The cyanobacterial clade has been very successful over its existence. They are both abundant and widespread. Morphologically diverse species occupying a vast number of different niches are found from aquatic to terrestrial habitats. Some cyanobacteria are unicellular while others form filaments of adjacently attached cells. Furthermore, cells with specialized purposes, such as heterocysts, hormogonia or akinetes, can differentiate from vegetative cells (Rippka et al. 1979). In addition cyanobacterial metabolism is flexible, some species being capable not only of photoautotrophy but also of photoheterotrophy (Stanier et al. 1971, Rippka 1972) and even heterotrophy in a light-activated manner (Anderson and McIntosh 1991, Kurian et al. 2005). Cyanobacteria are considered to be among the smallest organisms to exhibit endogenous circadian rhythms in their physiology and gene expression (Kondo et al. 1993, Lakin-Thomas 2006). The circadian clock of *Synechococcus* sp. PCC 7942, is set to a roughly 24 h period by the products of the *kaiABC* gene cluster (Kondo and Ishiura 2000). While cyanobacteria are the only group with all three genes, homologues of *kaiA* and *kaiB* have been found in other bacteria as well (Dvornyk et al. 2003).

Being the major primary producers in the oceans cyanobacteria are responsible for nearly one half of the net primary production on Earth (Field et al 1998, Bryant 2003). Some cyanobacteria, such as *Anabaena* sp. PCC 7120 and *Nostoc punctiforme,* form a crucial link in the nitrogen cycle because they are capable of fixing atmospheric nitrogen. Nitrogen-fixing cyanobacteria are used to fertilize fields in the cultivation of rice and thus have a role in global food production (Vaishampayan et al. 2001). Cyanobacteria are often environmentally hazardous. Serious health risks for humans and animals arise when toxic cyanobacteria are present in water used for consumption, agriculture or recreational purposes (Codd et al. 2005). Human activities promote harmful cyanobacterial growth because we contribute a great deal to the eutrophication of water bodies (Elmgren, 2001, Finni et al. 2001).

### 1.1.1. Structural and genetic features of cyanobacteria

Cyanobacteria are generally categorized among Gram-negative prokaryotes. This assignment is based on similarities in cell wall composition and structure. The cell is surrounded by an outer membrane consisting of lipopolysaccharides, lipids and transport proteins, a peptidoglycan layer and a selectively permeable plasma membrane (reviewed by Liberton and Pakrasi 2008). Peptidoglycan is a complex polymer consisting of cross-linked chains of carbohydrates and amino acids. Its role is to give the cell mechanical support. A surface layer of polysaccharides, the glycocalyx, serves to give additional protection from desiccation. Thylakoid membranes, located in the cytoplasm, are the sites for photosynthetic electron transfer reactions. Cyanobacterial thylakoids are loosely organized into networks and layers of stacked sheets instead of the grana structures present in plant chloroplasts (Nevo et al. 2007). According to van de Meene et al. (2006) they are continuous with the plasma membrane in the model species *Synechocystis* sp. PCC 6803 although Liberton et al. (2006) and Schneider et al. (2007) claim the contrary. Other structural features of a cyanobacterial cell include hexagonal carboxysomes, pili, gas vesicles, phycobilisome antennae, ribosomes and a variety of storage granules consisting of glycogen, cyanophycin, lipids or polyphosphate (Nevo et al. 2007, Liberton and Pakrasi 2008).

Prokaryote genomes are small and compact. This applies to cyanobacteria as well, in which known genome sizes vary between 1.6 Mbp in *Prochlorococcus marinus* MIT 9301 and 9.01 Mbp in *Nostoc punctiforme* PCC 73102 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi August 18th 2008). A multitude of detailed genetic information on cyanobacteria is already available. As of August 2008 (August 18th) 34 cyanobacterial genomes had been completely sequenced and many sequencing projects are currently in progress (http://bacteria.kazusa.or.jp/cyanobase/). Cyanobacteria possess a single circular chromosome and may have one or more plasmids (Kaneko and Tabata 1997). The plasmids contain functional genes although the majority have unknown or hypothetical functions (Kaneko et al. 2003). In cyanobacteria the size of the genome reflects the number of genes. Gene density is high compared to eukaryotic genomes averaging at about 1 gene per 1100 bp in *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996). As is typical in bacteria, some genes in cyanobacterial genomes are arranged into operons. Even though the cyanobacterial clade is considered to be of monophyletic origin, the degree of similarity in gene organization is negligible (Kotani and Tabata 1998).

### 1.1.2. *Synechocystis* sp. PCC 6803 – a model cyanobacterium

*Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is a unicellular, non-toxic fresh-water cyanobacterium that is not capable of nitrogen fixation. This popular model organism was the first photosynthetic organism whose genome was completely sequenced and made publicly available (Kaneko et al. 1996). Work by Kaneko et al. (1996) revealed that the total size of the *Synechocystis* genome is circa 3,6 Mbp and that there are over 3000 open reading frames. The *Synechocystis* strains used in research descend from the one stored in the Pasteur Culture Collection (PCC) (Rippka et al. 1979). The primary strain was isolated from a Californian freshwater lake in 1968 (Stanier et al. 1971, Ikeuchi and Tabata 2001). Many research laboratories, however, use a glucose-tolerant strain because of its capacity to grow photoheterotrophically in the presence of glucose (Williams, 1988). An important characteristic of *Synechocystis* is its natural competence for transformation with exogenous DNA (Grigorieva and Shestakov 1982, Williams 1988, Eaton-Rye 2004). Other cyanobacterial model species include *Synechococcus elongatus* sp. PCC 7942, the filamentous and nitrogen fixing *Anabaena* sp. PCC 7120, the thermophilic *Thermosynechococcus elongatus* and the marine *Prochlorococcus marinus* MED4.

## 1.2. Photosynthesis in cyanobacteria

### 1.2.1. Photosynthetic reactions

Photosynthetic light reactions occur on the thylakoid membranes. Light reactions convert solar energy into chemical energy in the form of ATP and NADPH. Oxygen is released as a side product. Components of the photosynthetic light reactions include membrane-associated protein complexes: the phycobilisomes, photosystem I (PSI), cytochrome $b_6f$, photosystem II (PSII) and ATP synthase as well as mobile components: plastoquinones, plastocyanins and cytochrome 552 (reviewed by Nelson and Yocum 1998, DeRuyter and Fromme, 2008). Phycobilisomes are large complexes of proteins with covalently bound bilin-pigments (MacColl 1998). The phycobilisomes are mainly attached to PSIIs on the cytoplasmic side of the thylakoid membranes and may balance the distribution of captured energy depending on the prevailing light conditions (Sarcina et al. 2001). They serve as light harvesting antennae and absorb solar radiation mainly between 500 and 650 nm (Glazer 1984). Cyanobacteria can utilize most of the visible light spectrum because they have several pigments with different absorption characteristics. The chlorophyll a pigment has absorption maxima at the wavelengths 430–440 and 670 nm. In addition orange carotenoid pigments participate in light harvesting by absorbing mainly at 420–480 nm (Mimuro and Katoh 1991, DeRuyter and Fromme 2008).

ATP and NADPH from the light reactions are used to fix atmospheric carbon dioxide into sugar compounds. The carboxylation reaction of the Calvin-Benson-Bassham cycle takes place in the cytoplasm. The Ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (Rubisco), which is packed into carboxysomes in the cytoplasm, catalyzes the fixation of $CO_2$ into ribulose 1,5-bisphosphate. The primary product of the Calvin-Benson-Bassham cycle is a three-carbon compound, glyceraldehyde-3-phosphate, which is used to make more complex sugars (for review see Raines 2003). In addition to carboxylation, the Rubisco enzyme can catalyze an energetically non-productive oxygenation reaction (reviewed by Raines 2003). In natural conditions the availability of inorganic carbon is often limiting for photosynthetic carbon fixation. Cyanobacteria have evolved carbon-concentrating mechanisms (CCMs) to concentrate inorganic carbon into the cell (reviewed by Badger and Price 2003). Five active uptake systems for inorganic carbon have been identified in cyanobacteria, although not all are present in all species (reviewed by Price et al. 2008). BCT1, SbtA and BicA take up bicarbonate (Price et al. 2008). Additionally there are two systems for $CO_2$ uptake: the low-$CO_2$-inducible $NDH1_3$ and the constitutively functioning $NDH1_4$, which have high and low affinities for dissolved $CO_2$, respectively (Shibata et al. 2001). *Synechocystis* prefers to take up inorganic carbon in the form of bicarbonate (Benschop et al. 2003).

### 1.2.3. Photoinhibition

Although light is an essential source of energy for photosynthesis, it also has destructive power. High-energy UV light is especially harmful to photosynthesis (He and Häder 2002, Hollósy 2002, Murata et al. 2007). In the light the PSII reaction centre is inactivated and the D1 protein is damaged. Inactivated PSII centres constantly undergo a repair cycle, which requires the removal and de novo synthesis of the D1 protein (Baena-González and Aro, 2002). When the rate of damage to the PSII reaction centre exceeds the rate of repair, oxygen evolution capacity decreases. This process is called photoinhibition (for review see Nishiyama et al. 2005, Murata et al. 2007, Tyystjärvi 2008). The rate constant of photoinhibition is directly proportional to light intensity in both plants (Tyystjärvi and Aro, 1996) and cyanobacteria (Allakhverdiev and Murata 2004). In the PSII repair cycle the damaged D1 protein is removed from the inactive PSII, degraded and replaced with a new copy after which the PSII is reassembled and activated (Yamamoto 2001). In *Synechocystis* the three-member *psbA* gene family codes for the D1 protein. The *psbA2* and *psbA3* genes are actively expressed and encode identical D1 proteins (Mohamed and Jansson 1989) whereas the more divergent *psbA1* is virtually silent (Mohamed and Jansson 1989; Sicora et al. 2006).

Different stages of PSII repair offer a wide range of regulatory possibilities in cyanobacteria at the levels of D1 degradation (Tyystjärvi et al. 1995, Kamata et al. 2005, Cheregi et al. 2007), synthesis and degradation of *psbA* mRNA encoding the D1 protein (Mohamed and Jansson 1990, 1991, Tyystjärvi et al. 1996, Alfonso et al. 1999), translation of *psbA* mRNA (Tyystjärvi et al. 2001, Nishiyama et al. 2004), post-translational modifications of a pre-D1 form (Anbudurai et al. 1994), assembly of the D1 protein into PSII and PSII activation (Constant et al. 2000, Sakurai et al. 2003). Different cyanobacteria have slightly different strategies for coping with photoinhibition. *Synechococcus* sp. PCC 7942, for example, switches between two forms of the D1 protein, D1:1 and D1:2 (Clarke et al. 1993a, b). Under high light or UV light the proportion of the D1:2 form increases probably to facilitate acclimation to stress conditions (Schaefer and Golden 1989, Campbell et al. 1998a). The underlying mechanism of photoinhibition itself has been under debate for a long time. Several models have been suggested. The four prevailing hypotheses are the acceptor-side (Vass et al. 1992), donor-side (Anderson et al. 1998), low-light (Keren et al. 1997) and manganese models (Hakala et al. 2005, Ohnishi et al. 2005, Hakala et al. 2006).

### 1.3. Regulation of gene expression

In eubacteria the regulation of gene expression takes place on many levels: transcription initiation, transcription elongation and termination, transcript degradation, translation, post-translational modifications and protein turnover. Transcription is initiated by the RNA polymerase (RNAP) holoenzyme, which consists of a catalytically active core and a sigma ($\sigma$) factor (Murakami et al. 2002). The $\sigma$ factor mediates promoter recognition. The RNAP holoenzyme binds to the promoter forming a closed complex (Murakami et al. 2002). Next the DNA strands unwind, melt starting from the –10 region and form the open complex where the template strand is directed to the RNAP active site (Murakami et al. 2002, Djordjevic and Bundschuh 2008). Then follows a phase of abortive initiations where the RNAP repeatedly synthesizes short segments of RNA without escaping the promoter (Vo et al. 2003, Xue et al. 2008). Promoter clearance occurs when the length of the transcript exceeds 12 bp and the holoenzyme proceeds to the transcription elongation phase (Murakami and Darst 2003).

Promoter sequences and other *cis*-acting elements up- and downstream of the transcription start site regulate the expression level of that particular gene (Busby and Ebright 1994, Xue et al. 2008). Conserved sequence elements characterize bacterial promoters. The most important of these are recognized by the RNAP holoenzyme. In the model bacterium *Escherichia coli* the primary $\sigma$ factor recognizes the hexameric –10 and –35 regions, whose consensus sequences are 5’-TATAAT-3’ and 5’-TTGACA-3’, respectively (Harley and Reynolds 1987). The length of the spacer region between the –10 and –35 regions affects transcriptional efficiency and is optimally 17 bp (Dombroski et al. 1996, Shultzaberger et al. 2007). Some promoters lack the –35 element but may have an additional extended –10 region (Harley and Reynolds 1987, Minchin and Busby 1993).

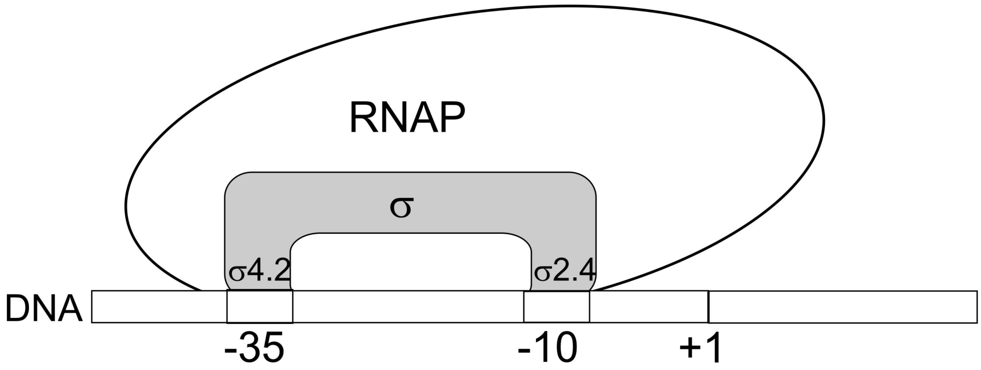

**Figure 1.** Diagram of the RNA polymerase holoenzyme attached to the DNA strand. The σ factor (light grey) domains σ2.4 and σ4.2 recognize and bind the –10 and –35 promoter elements, respectively. The transcription initiation site is marked +1.

### 1.3.1. The bacterial RNA polymerase

Cyanobacteria, like other eubacteria, have only one type of RNAP, which is responsible for the transcription of mRNA, tRNA and rRNA. RNAPs are structurally and functionally conserved among prokaryotes and eukaryotes (Allison et al. 1985) although cyanobacterial and chloroplast RNAPs have distinct features that characterize them as a group (Bergsland and Haselkorn 1991). The multi-subunit RNAP core, which is responsible for catalyzing RNA synthesis reactions, consists of 2 identical α subunits and the β, β' and ω subunits (Gruber and Gross 2003). Uniquely in cyanobacteria and their descendants, chloroplasts, the β' subunit is split into two: β' and γ (Schneider et al. 1987, Schneider and Haselkorn 1988). The structure of the RNAP has been determined with X-ray crystallography at 2.6 Å resolution in *Thermus thermophilus* (Vassylyev et al. 2002).

While the RNAP core exhibits the catalytic activity in RNA synthesis, the σ subunit of RNAP is required for the recognition of specific promoter sequences. Burgess et al. (1969) first identified σ factors as proteins stimulating transcription in *Escherichia coli*. Although the RNAP core has unspecific affinity for DNA in general, it cannot locate promoter sites and initiate transcription without a σ factor (Murakami et al. 2002, Murakami and Darst 2003). In general, bacterial genomes encode several σ factors and different σ factors recognize different promoters thus controlling the expression of distinct sets of genes (Tanaka et al. 1988, Wösten 1998). All σ factors compete for a limited amount of the RNAP core (Maeda et al. 2000, Nyström 2004, Wade et al. 2006) and the exchange of one σ factor to another is thought to be a major determinant in switching the gene expression pattern (Nyström 2004).

Bacterial σ factors are divided into two families, $\sigma^{54}$ and $\sigma^{70}$. The large $\sigma^{70}$ family derives its name from the 70 kDa-sized RpoD protein of *Escherichia coli* (Burton et al. 1981) and its members have high homology even between species (Lonetto et al. 1992). All cyanobacterial σ factors, identified on the basis of sequence homology, belong to the $\sigma^{70}$ family (Tanaka et al. 1992, Sakamoto et al. 1993, Osanai 2008). Lonetto et al. (1992) divided the $\sigma^{70}$ type σ factors into three subgroups based on sequence similarity. Group 1 σ factors are also called primary or principal σ factors. They are very conserved between different species and only one group 1 σ factor is encoded by any bacterial genome (Gruber and Gross 1997, Gruber and Bryant 1997, Osanai et al. 2008). Group 1 σ factors are essential for cell viability and are thought to be responsible for the transcription of household genes (Ishihama, 2000). Group 2 σ factors are very similar to the group 1 σ factors in amino acid sequence, but they are nonessential (Lonetto et al. 1992). The alternative, or group 3, σ factors share less sequence homology with groups 1 and 2 and are known to have specialized stress- and development-related functions (Lonetto et al. 1992). A fourth group, the extracytoplasmic function σ factors, is sometimes separated from the alternative σ factors. These factors respond to signals from the periplasm (Reviewed in Raivio and Silhavy 2001). Four regions and their sub-regions, σ1.1-σ4.2, have been distinguished in $\sigma^{70}$ type factors on the basis of amino acid sequence conservation (Malhotra et al. 1996, Gruber and Gross 2003). The extremely conserved domains σ2.4 and σ4.2 are critical for σ factor function because they recognize and bind the –10 and –35 promoter elements, respectively (Lonetto et al. 1992, Malthotra et al. 1996, Vassylyev et al. 2002).

*Synechocystis* has nine σ factors (http://bacteria.kazusa.or.jp/cyanobase/cyano.html). SigA is the primary σ factor, group 2 consists of SigB, SigC, SigD and SigE (Imamura et al 2003b, Tuominen et al. 2003) while SigF, SigG, SigH and SigI belong to group 3 (Sakamoto et al. 1993, Kaneko et al. 1996, Matsui et al. 2007, Asayama and Imamura 2008). In a phylogenetic analysis of amino acid sequences the *Synechocystis* SigA factor clusters together with the primary σ factors of *Escherichia coli* and *Bacillus subtilis* (Fig. 2). The second cluster includes RpoS, the solitary group 2 σ factor from *Escherichia coli* and the closely related SigB, SigC, SigD and SigE factors of *Synechocystis*. SigB, the general stress-responsive σ factor in *Bacillus subtilis* (Petersohn et al. 2001), clusters together with the group 3 σ factors. The abundance of σ factors varies among different cyanobacterial species: *Synechococcus* sp. PCC 7002 has five, *Thermosynechococcus elongatus* BP-1 eight, *Prochlorococcus marinus* MED4 five, *Anabaena variabilis* ATCC 29423 at least ten (http://bacteria.kazusa.or.jp/cyanobase August 10th 2008) and *Anabaena* sp. PCC 7120 twelve (Aldea et al. 2007) putative σ factor genes. Several $\sigma^{70}$ type factors are found also in plant chloroplasts (reviews Allison 2000, Kanamaru and Tanaka 2004). Plant plastids have two types of RNAP, a nuclear-encoded RNAP and a plastid-encoded, bacterial type RNAP (Reviewed in Lysenko and Kuznetsov 2005, Toyoshima et al. 2005). Plant σ factors are nuclear-encoded but transported to plastids where they participate in transcription initiation in the same fashion as in cyanobacteria (Toyshima et al. 2005). Preliminary roles have already been suggested for all six (SIG1-SIG6) σ factors identified in *Arabidopsis thaliana* (Kanamaru et al. 2001, Morikawa et al. 2001, Tsunoyama et al. 2002, Favory et al. 2005, Ishizaki et al. 2005, Zghidi et al. 2007).

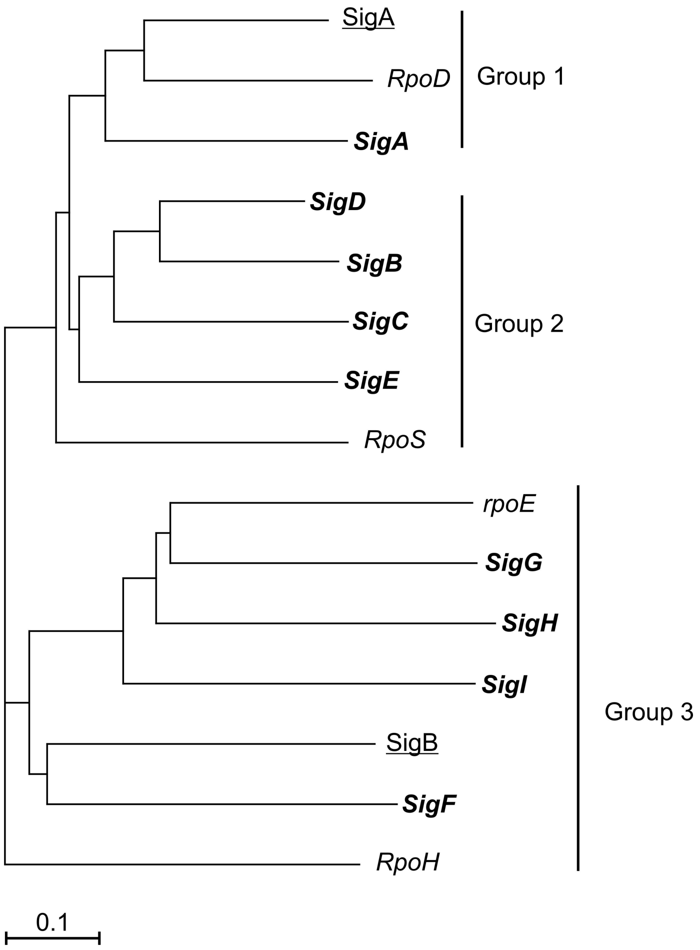

**Figure 2.** Phylogenetic tree of *Synechocystis* sigma factors (bold) together with the RpoD, RpoS, RpoH and RpoE factors of *Escherichia coli* (italics) and SigA and SigB of *Bacillus subtilis* (underlined). The amino acid sequences were obtained from NCBI and the sequence alignment was done with ClustalW2 at EMBL-EBI. The scale bar represents evolutionary distance in amino acid changes per site.

The function and activity of the σ factors themselves are under control. Anti-sigma factors are σ factor antagonists (for review see Hughes and Mathee 1998, Helmann 1999). Most information on the anti-sigma factors has been gathered from *Escherichia coli* and *Bacillus subtilis*. Guanosine-tetraphosphate, ppGpp (Campbell et al. 2008) is a global regulator involved in the bacterial stringent response (Magnusson et al. 2005). Artsimovitch et al. (2004) showed the binding of ppGpp to the transcription initiation complex in *Thermus thermophilus* and suggested that ppGpp might interfere with transcription initiation by destabilising protein-DNA interactions. On the other hand, in *Escherichia coli* transcription from $\sigma^S$ regulated promoters requires the presence of ppGpp (Kvint et al. 2000). ppGpp also interferes with the degradation of $\sigma^S$ thus prolonging its lifetime (Bougdour and Gottesman 2007). The double-stranded 6S RNA interacts with the RNA polymerase and is a regulator of $\sigma^{70}$ in the *Escherichia coli* stationary phase (Willkomm and Hartmann 2005). 6S RNA-mediated regulation can be either negative or positive depending on the target gene (Willkomm and Hartmann 2005).

## 1.4. Sensing and responding to environmental conditions

Stress responses of bacteria can be divided into short- and long-term strategies: short-term strategies include motility or taxis, the reversible modification of enzyme activities and adjustment of photosynthetic light harvesting whereas long-term processes involve profound changes in the gene expression pattern, cell metabolism, structure and morphology (reviewed in Tandeau de Marsac and Houmard 1993, Ramos et al. 2001, Marles-Wright and Lewis 2007). Stress triggers signalling cascades that lead to acclimation processes. Sensory proteins such as histidine kinases (Hik) first perceive a change in environmental conditions (Korchid and Ikura 2006). Further downstream of the signal transduction chain are σ factors and specific transcription factors (Fig. 3). Their properties affect promoter selectivity and hence influence changes in target gene expression pattern (Chung et al. 2006).

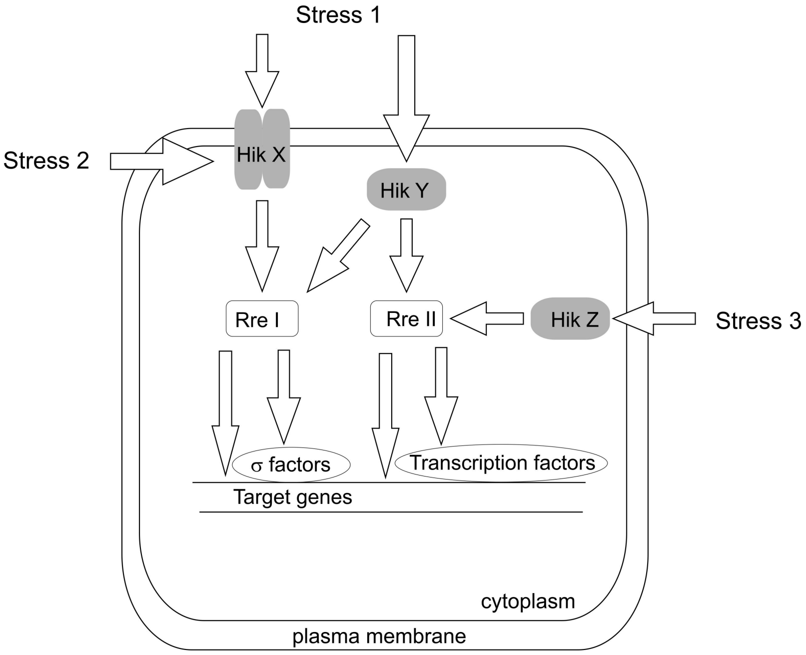

**Figure 3.** A generalized example of possible two-component signal transduction pathways in *Synechocystis*. Histidine kinases perceive stress signals and relay them to the response regulators. Some response regulators act directly as transcription factors regulating the expression of target genes, while some may function more indirectly by regulating additional regulatory factors. These regulatory factors include the σ factors and other transcription factors.

Receptor-mediated mechanisms such as two-component systems form a central part of the regulatory network in bacteria (reviewed in Ashby and Houmard 1996, Khorchid and Ikura 2006, Mascher et al. 2006). A classical two-component cognate pair consists of a sensory histidine kinase and a response regulator (Rre), which relays the signal onwards. Signal transduction by two component pairs can be initiated either on the cell membrane or in the cytoplasm (Mascher et al. 2006). The *Synechocystis* genome has a total of 91 potential two-component system genes including 47 Hiks and 42 Rres (Ashby and Houmard 2006). Mikami et al. (2002) and Murata and Suzuki (2006) used microarray analysis to study *Synechocystis* two-component systems. Their analysis of a large collection of *hik* and *rre* inactivation strains demonstrated that one Hik can sense signals originating form several types of stress: Hik33, for example, is involved in the perception of not only cold but also osmotic and redox stress signals (Mikami et al 2002, Murata and Suzuki 2006, Kanesaki et al. 2007). In addition to receptor-mediated systems, a variety of other sensing mechanisms function in cyanobacteria. Membrane fluidity has a significant role in the sensing of environmental signals, especially of temperature and osmotic changes (Sakamoto and Murata 2002, Mikami and Murata 2003, Los and Murata 2004). Also the synthesis of intracellular second messengers such as ppGpp (Magnusson et al. 2005), reactive oxygen species (He and Häder 2002) and cAMP (Botsford and Harman 1992) can be rapidly enhanced by stress.

Also a variety of non-coding RNAs regulate gene expression in bacteria (Wassarman 2007, Windbichler et al. 2008). Two types of 6S RNA have been reported from *Prochlorococcus* MED4 (Axmann et al. 2007). Moreover, Nakamura et al. (2007) and Voss et al. (2007) have identified a non-coding, 65-nucleotide-long RNA, Yfr1, from cyanobacteria. The analysis of an Yfr1 mutant strain revealed that it is involved in the regulation of stress-induced genes (Nakamura et al. 2007). In addition, Dühring et al. (2007) reported that an antisense RNA, IsrR, regulates the iron stress inducible *isiA* gene. σ factors lie at the end of the signal transduction chain together with specific transcription factors. The best-characterized transcription factor in *Synechocystis* is NtcA, which is together with the PII sensory protein involved in nitrogen metabolism (Muro-Pastor et al. 2005).

### 1.4.1. Temperature

*Synechocystis* is a mesophilic cyanobacterium with an optimal growth temperature at 32 $^{o}$C. *Synechocystis* cells can grow within the temperature range of 15 and 45 $^{o}$C, but below 25 and above 43 $^{o}$C it suffers from severe stress symptoms (Stanier et al. 1971, Inoue et al. 2001). Temperatures above 45 $^{o}$C are considered to be lethal for *Synechocystis* although cells can acquire thermotolerance through pre-exposure to sub-lethal temperatures (Lehel et al. 1993). During the first phase of the heat response, cells produce rapidly large amounts of heat shock proteins which act as molecular chaperones that enable the proper folding of proteins to their active state or solubilize aggregates of misfolded proteins (Braig 1998). Furthermore, some heat shock proteins are proteases that degrade damaged proteins (Ramos et al. 2001, Suzuki et al. 2006). The up-regulation of the heat shock proteins is a transient phenomenon and their levels decline to a steady state level (Braig 1998). Cyanobacterial heat shock proteins include HspA (Nakamoto et al. 2000, Nitta et al. 2005), HtpG (Tanaka and Nakamoto 1999), ClpB (Schlee and Reinstein 2002, Lee et al. 2003), GroES and GroEL (Goloubinoff et al. 1997), DegP (Skorko-Glonek et al. 2007) and DnaK (Varvasovszki et al. 2003, Siegenthaler et al. 2004). Inactivation of the *hspA* gene made the cells more sensitive to heat (Lee et al. 2000) while over expression enhanced heat tolerance (Nakamoto et al. 2000, Nitta et al. 2005). Suzuki et al. (2006) found a total of 113 heat-inducible genes and 90 proteins in the *Synechocystis* genome. In their DNA microarray analysis most of the induced genes fell into the category “hypothetical and unknown”, which means that it is not known how and why their activation affects heat acclimation. In *Synechocystis* light has a great influence on the heat stress response, as shown by Asadulghani et al. (2003).

High temperatures tend to make lipid bilayer membranes increasingly fluid and labile, which may affect the function of membrane-bound protein complexes. Hence another important aspect of the heat response is the stabilization of membranes by increasing the degree of fatty acid saturation (Mikami and Murata 2003, Balogi et al. 2005). In *Synechocystis* the membrane-bound histidine kinase Hik33 relays signals of the state of the lipid bilayer to regulatory proteins and brings about changes in the gene expression pattern resulting in the rigidification of the membranes (Suzuki et al. 2001). Moreover, Suzuki et al. (2005) showed that Hik34 is a negative regulator of the heat shock protein genes *htpG* and *GroESL1*. Several bacterial species, including the model organisms *Escherichia coli* and *Bacillus subtilis*, are known to have specific σ factors that are responsible for the positive regulation of genes involved in high temperature stress (Arséne et al. 2000, Helmann et al. 2001). In *Escherichia coli* the heat shock response is mediated by the $\sigma^{32}$ and $\sigma^{24}$ factors (Grossman et al. 1984, Jishage et al. 1996). $\sigma^{32}$ controls the expression of the "heat shock regulon", a set of genes involved specifically in the heat shock response (for review see Yura and Nakahigashi 1999, Arséne et al. 2000). In contrast, no exclusive heat shock σ factors have been defined in cyanobacteria although some σ factors are known to respond to heat stress (Huckauf et al. 2000, Imamura et al. 2003b, Tuominen et al. 2003). The heat response can also be negatively regulated by a *cis*-acting DNA element, CIRCE, and the HrcA protein (Naberhaus 1999, Servant and Mazodier 2001). CIRCE elements have been found on the promoters of several heat-inducible genes in *Synechocystis* and they are found in many other bacteria as well (Servant and Mazodier 2001, Nakamoto et al. 2003) HrcA is a repressor that binds the CIRCE inverted repeat sequence thus preventing transcription initiation from that promoter (Minder et al. 2000). The expression of the *groESL* and *groEL2* genes is the best known examples of CIRCE-HrcA mediated negative regulation in *Synechocystis* (Nakamoto et al. 2003, Singh et al. 2006).

Research on temperature stress in cyanobacteria has mainly focused on heat stress while less attention has been paid to low temperature. Nevertheless, some mechanisms of cold responses and acclimation have been identified (reviewed by Panoff et al. 1998, Phadtare 2004). At low temperatures chaperones act to protect proteins that are susceptible to damage. Lower temperatures make cell membranes more rigid, and as a consequence the *desA* and *desB* genes encoding desaturase-enzymes in *Synechocystis* are activated (Sakamoto et al. 1997, Sakamoto and Murata 2002). Desaturases increase the number of double bonds in fatty acid hydrocarbon chains thus increasing membrane fluidity. While its role in cellular sensing encompasses a variety of stresses, the membrane-bound Hik33 histidine kinase also perceives the degree of membrane fluidity and regulates the expression of the desaturases (Suzuki et al. 2000, Suzuki et al. 2002, Kanesaki et al. 2007).

### 1.4.2. Light

Electromagnetic radiation from the sun ranges from very high-energy, short-wavelength ultra-violet-light to low-energy infrared radiation. In between the extremes is the spectrum of visible light. Cyanobacteria can sense both light quantity and quality (Grossmann et al. 2001, Montgomery 2007). Proposed cyanobacterial photoreceptors include the photosynthetic pigments (phycobilins, chlorophylls and carotenoids), cryptochromes (Hitomi et al. 2000, Ng and Pakrasi 2001, Braatsch and Klug 2004) and phytochromes (Yeh et al. 1997, García-Domínguez et al. 2000, Park et al. 2000). Recently a novel green light receptor, CcaS, with a histidine kinase domain was identified in *Synechocystis* (Hirose et al. 2008). In addition, green light can be sensed by the rhodopsins (Jung et al. 2003). Besides the actual photoreceptors, the redox-state of the photosynthetic electron transport chain might act as a light sensory system because it reflects the prevailing light conditions (Sippola and Aro 1999, Mullineaux 2001).

Several mechanisms have been suggested to operate simultaneously to enable acclimation to different light conditions. State transitions of light-harvesting antennae may balance the distribution of light energy between photosystems I and II (Campbell et al. 1998b), chromatic adaptation involves adjustments in pigment composition (Kehoe and Gutu 2006, Schagerl and Müller 2006) and the PSII/PSI ratio may change (Hihara et al. 1998, Sonoike et al. 2001). Some cyanobacterial strains may move closer to or further away from the light source by phototaxis (Bhaya 2004, Yoshihara and Ikeuchi 2004). It has been proposed that cyclic adenosine monophosphate (cAMP), which mediates light signal transduction, is needed for phototaxis of a motile *Synechocystis* strain (Terauchi and Ohmori 2004, Bhaya et al. 2006).

### 1.4.3. Salt and osmotic stress

Cyanobacteria perceive salt and hyperosmotic stress as different kinds of stress. Firstly, salt- and hyperosmotic stress induce distinct sets of genes whose products enable successful acclimation (Kanesaki et al. 2002). Secondly, distinct physiological responses to salt- and sorbitol-induced osmotic stresses have been reported (Marin et al. 2006). Dissolved salts like sodium chloride (NaCl) cause simultaneously two distinct types of stress: ionic and hyperosmotic stress. Other solutes such as sorbitol and disaccharides cause non-ionic hyperosmotic stress. *Synechocystis* is moderately halotolerant and can withstand up to 1.2 M NaCl concentrations (Reed and Stewart 1985). High external salt concentrations result in dehydration due to the efflux of water from the cell. During the first minutes of salt shock, $Na^+$ ions enter the cell via $K^+$ channels (Reed et al. 1985). To balance ion concentrations, $Na^+/H^+$ antiporters on the plasma membrane remove excess $Na^+$ ions from the cytoplasm and K+ transporters import potassium cations to compensate for the extrusion of sodium (Reed et al. 1985, Karandashova and Elanskaya 2005). In addition to the action of ion channels cyanobacteria produce compatible solutes (Reed and Stewart 1985). Glucosylglycerol, for example, is synthesized in large quantities in response to NaCl-induced osmotic stress (Karandashova and Elanskaya 2005). Inactivation of the gene encoding the glucosylglycerol-phosphate synthase enzyme disabled the production of glucosylglycerol (Marin et al. 1998). As a consequence, *Synechocystis* cells became sensitive to salt-induced osmotic stress and were not able to divide properly (Marin et al. 1998, Ferjani et al. 2003). Furthermore, sucrose, trehalose (Mikkat et al. 1997) and, in *Synechococcus*, glycine betaine (Lu et al. 2006) are synthesized or actively up-taken in order to protect cellular components.

Photosynthesis is vulnerable to salt stress (Sudhir and Murthy 2004). High internal salt concentrations disrupt photosynthesis by damaging the sensitive oxygen-evolving complex of PSII (Allakhverdiev et al. 2000). As a result growth and productivity decrease. Osmoprotectants are known to stabilize photosynthetic membranes during osmotic stress (Hincha and Hagemann 2004). Chaperones have roles in osmotic stress and their responsibilities in salt- and sorbitol-induced stress are similar to those in heat stress: to facilitate and maintain the folding of proteins, to stabilize cellular structures and –in the case of proteases- to degrade irreversibly damaged proteins (Kanesaki et al. 2002, Asadulghani et al. 2004). The HspA heat shock protein, for example, participates in osmotic stress tolerance, as an *hspA* mutant strain is more sensitive to salt stress than a control strain (Asadulghani et al. 2004). Microarray analysis of salt and osmotic stress responses in *Synechocystis* showed that although a large number of genes are salt-induced, their transient up-regulation lasted less than 24 h (Marin et al. 2004). This indicates that full acclimation to salt stress conditions can be achieved within 24 hours. Marin et al. (2003) showed that several histidine kinases, Hik16, Hik33, Hik34 and Hik41, are involved in sensing stress signals under salt and osmotic stress, and in transducing signals to the gene regulation machinery. However, these same histidine kinases are involved in sensing many other stresses as well, and thus cannot be considered to be exclusively salt specific.

### 1.4.4. pH

In the laboratory cyanobacteria are grown at a pH close to neutral but they are able to acclimate to a range of different pH conditions (Huang et al. 2002b, Eaton-Rye et al. 2003, Ohta et al. 2005, Kurian et al. 2006). If the growth medium is not buffered, pH tends to increase over growth time (Huang et al. 2002, Thomas et al. 2005, Kurian et al. 2006). In natural habitats cyanobacteria prefer alkaline environments (Brock 1973). They did not tolerate pH below 5, whereas eukaryotic algae were present in as low pH as 1.9 (Brock 1973). Mapping of the transcriptome (Ohta et al. 2005) and proteome (Kurian et al. 2006) of acid stressed *Synechocystis* cells showed that the carbon concentrating mechanisms are intimately connected with acid stress tolerance. At a higher pH the concentration of bicarbonate is high and the availability of inorganic carbon is less likely to limit cyanobacterial growth. Sensitivity to low pH might thus be in part due to the effects of carbon limitation. The most prominent changes in the proteome were restricted to the periplasm and the cytoplasmic fraction remained relatively untouched by pH stress (Kurian et al. 2006). This implies that the cell surface moderates the effect of pH stress before intracellular components are severely affected.

### 1.4.5. Nutrient balance

A balance in nutrient availability is important because deprivation reduces productivity but on the other hand excesses may have toxic effects on cell metabolism. Homeostatic acclimation mechanisms maintain an internal balance amidst changes in nutrient availability (Schwarz and Forchhammer 2005). Non-nitrogen-fixing cyanobacteria uptake nitrogen preferably in the form of ammonium (Herrero et al. 2001). Ammonia taken up by ammonium permeases is first incorporated into glutamate to produce glutamine. Glutamine and 2-oxoglutarate are then used to generate two glutamates. This pathway is the best known, although alternative assimilation pathways are known to exist (reviewed by Luque and Forchhammer 2008). Many cyanobacterial species are capable of fixing atmospheric nitrogen (reviewed by Zhang et al. 2006). The nitrogenase enzyme is oxygen-sensitive and nitrogen fixation is separated from photosynthesis. In filamentous cyanobacteria nitrogen fixation takes place in heterocysts (Zhang et al. 2006). In contrast, unicellular nitrogen-fixing species photosynthesize during the day and nitrogen fixation accompanied by vigorous respiration occurs in darkness (Tsygankov 2007). The intracellular nitrogen balance is sensed by the PII and NtcA regulators, which relay signals to the gene expression machinery (Frías et al. 1994, Herrero et al. 2001, Forchhammer 2004). Phosphorus is another important macronutrient since it is the building block of for example membrane lipids, nucleic acids and ATP. A two-component signalling system that perceives phosphorus limitation is conserved among bacteria and homologs (SphoS and SphoR) have been identified also in *Synechocystis* (Hirani et al. 2001, Suzuki et al. 2004).

# 2. AIM OF THE STUDY

The aim of my work was to study the roles of *Synechocystis* group 2 σ factors. I approached the research question by constructing and analyzing a complete set of single, double and triple inactivation strains of the group 2 σ factors. The importance of different σ factors was investigated by measuring the performance of the inactivation strains under a variety of different environmental conditions. In addition to physiological characteristics, gene expression was analyzed. Particular attention was paid to the investigation of regulatory mechanisms involving σ factors in acclimation to heat and light stress.

# 3. METHODOLOGY

## 3.1. Strains and standard growth conditions

The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used as the control strain (CS). *Synechocystis* cells were grown photoautotrophically in BG-11 medium (Rippka et al. 1979) supplemented with 20 mM Hepes-NaOH pH 7.5 at 32 $^{o}$C. The continuous photosynthetic photon flux density (PPFD) was 40 μmol $m^{-2}$ $s^{-1}$ and the cells grew in ambient $CO_2$ concentration. Liquid cultures were shaken 90 rpm. These will be referred to as standard growth conditions. BG-11 agar plates for maintaining the single inactivation strains were supplemented with kanamycin, plates for double inactivation strains with kanamycin, spectinomycin and streptomycin and plates for triple inactivation strains with chloramphenicol, kanamycin, spectinomycin and streptomycin.

## 3.2. Construction of σ factor inactivation strains

All inactivation strains (Table I) were made by interrupting the chosen σ factor gene or genes with antibiotic resistance cassettes. The *sig* genes in the single inactivation strains were interrupted with a kanamycin resistance cassette to obtain the strains ΔsigB, ΔsigC, ΔsigD and ΔsigE. The second *sig* gene was interrupted with the Ω fragment (Prentki et al. 1984) conferring resistance to spectinomycin and streptomycin. The *sigC* gene was inactivated in the ΔsigB strain to obtain ΔsigBC, the *sigD* gene was inactivated in the ΔsigB and ΔsigC strains to obtain ΔsigBD and ΔsigCD and the *sigE* gene was inactivated in the ΔsigB, ΔsigC and ΔsigD strains to obtain ΔsigBE, ΔsigCE and ΔsigDE, respectively (Paper I). The triple inactivation strains were generated by interrupting a third *sig*-gene with a chloramphenicol resistance cassette. The *sigC* gene was inactivated in ΔsigBD to obtain ΔsigBCD and the *sigE* gene in ΔsigBC, ΔsigBD and ΔsigCD to obtain ΔsigBCE, ΔsigBDE and ΔsigCDE, respectively (Paper V). *Synechocystis* cells were transformed according to Williams (1988). The selection processes for the inactivation strains are described in papers I, III, IV and V as indicated in Table I.

## 3.3. Structural modelling of the *Synechocystis* RNA polymerase

For structural modelling the amino acid sequences of the *Synechocystis* RNA polymerase subunits were obtained from CyanoBase (www.kazusa.or.jp/cyanobase). The sequences were aligned with their corresponding counterparts from the 2.6 Å *Thermus thermophilus* RNA polymerase structure (Protein Data Bank code 2A6E, Artsimovitch et al. 2005) using the MALIGN program and manual adjustment. Secondary structures were predicted with PredictProtein (Rost and Liu 2003) and final three-dimensional modeling was done with the MODELLER software (Šali and Blundell 1993). The computer-generated models were examined visually and their overall stereo-chemical quality was assessed with PROCHECK (Laskowski et al. 1993).

## 3.4. Physiological characterization of the inactivation strains

### 3.4.1. Growth experiments

Light absorbance of liquid cell culture at 730 nm ($A_{730}$) gives a measure of cell density. When $A_{730}$ is 0.1, the cell density is circa 3.6 x $10^{6}$ $ml^{-1}$. The cell count was determined with flow cytometry at the Turku Centre for Biotechnology (Paper IV). Growth rate was measured under standard conditions (Papers I and V), at 80 μmol of photons $m^{-2}$ $s^{-1}$ (Paper V) and under a $CO_2$

enriched atmosphere (3 % $CO_2$ in air) (Paper I). Growth was followed also at 43 $^oC$ at 35 µmol of photons $m^{-2}$ $s^{-1}$ in ambient $CO_2$ concentrations at pH 7.5 (Papers III and IV), at pH 6.7 and pH 8.3 (Figure 3.) or in 3 % $CO_2$ at pH 7.5 (Paper IV). In Papers I and II cells were grown in BG-11 medium supplemented with 0.7 M NaCl or 0.5 M sorbitol. Furthermore, we compared the growth of the control and ΔSigBD strains in very dilute cultures by setting the $A_{730}$ to 0.01 or 0.001 and growing them under standard conditions (Paper I). To study the behaviour of the cells in a diurnal light rhythm, they were grown under a 12 h light and 12 h dark rhythm. The PPFD was 40 and 10 photons $m^{-2}$ $s^{-1}$. In addition to liquid cultures, the growth of the control and inactivation strains was observed on agar plates under the PPFD of 20, 40 or 80 µmol $m^{-2}$ $s^{-1}$ (Paper I). $A_{730}$ of the cell suspensions was adjusted to 0.1 and 5 µl aliquots were spotted onto BG-11 agar plates. The density-dependent growth of the ΔsigBD strain was studied by spotting 5 µl of culture with an $A_{730}$ of 10, 1, 0.1 or 0.01 on agar plates and growing the cells under standard conditions (Paper I).

### 3.4.2. Survival in high temperature

The survival of *Synechocystis* cells in heat stress was examined with a viability assay (Papers III and IV). Cell culture was incubated for 15 min at 48 $^oC$ under the PPFD of 5 µmol $m^{-2}$ $s^{-1}$ with or without a 1 h pre-treatment at 43 $^oC$ under 35 µmol of photons $m^{-2}$ $s^{-1}$. After the heat treatments the samples were diluted 1000 fold and 10 µl aliquots were spotted onto BG-11 agar plates. The cells were grown under standard growth conditions for one week after which colonies were counted. The percentage of surviving cells was calculated by dividing the number of colonies from treated samples by the number of colonies from untreated control samples.

### 3.4.3. Oxygen evolution measurements

Light saturated rate of photosynthetic oxygen evolution was measured *in vivo* from cells grown under standard growth conditions (Papers I and V), at 80 µmol of photons $m^{-2}$ $s^{-1}$ (Paper V) or after incubation at 48 $^oC$ (Paper IV). Oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech, UK) at 32 $^oC$ in the presence of 10 mM $NaHCO_3$ (Papers I and V). In addition, photosynthetic activity was measured from cells grown for two days in standard growth light (40 µmol of photons $m^{-2}$ $s^{-1}$) or at 80 µmol of photons $m^{-2}$ $s^{-1}$ using the same light intensity as in the growth conditions (Paper V).

Light saturated rate of PSII electron transfer was measured in the presence of 0.5 mM 2,6-dichloro-*p*-bentsoquinone (DCBQ), an artificial electron acceptor. 0.7 mM ferricyanide was added to keep DCBQ in an oxidized form. Measurements of PSII capacity were done from cells grown under standard conditions (Paper I) or in BG-11 medium supplemented with salt or sorbitol (Paper II). PSII capacity was also measured from cells grown at double light intensity (80 µmol of photons $m^{-2}$ $s^{-1}$) and after 0 min, 15 min, 30 min and 45 min of illumination at 1500 µmol of photons $m^{-2}$ $s^{-1}$ high light. The high light treatments were done also in the presence of lincomycin, a translation inhibitor (Paper V).

### 3.4.4. Biophysical measurements

Flash-induced increase and subsequent decay of chlorophyll fluorescence was measured with a FL2000 fluorometer (PS Instruments) from cells grown under standard conditions (Paper V). A 2 ml sample of cell suspension containing 10 µg chl $ml^{-1}$ was dark adapted for 5 min in the fluorometer cuvette to ensure that the $Q_A$ electron acceptor of PSII was in an oxidized state. $Q_A$

was reduced with a short, strong flash and reoxidation was then followed with a series of weak probe flashes. Fluorescence relaxation was also measured in the presence of 10 µM DCMU (3-(3',4'-dichlorophenyl)-1,1-dimethylurea), a quinone analogue, which inhibits the transfer of electrons from $Q_A$ to $Q_B$.

77 K Fluorescence emission spectra were measured with an Ocean Optics S2000 spectrometer (Paper V). Cells were grown for two days in liquid culture under standard growth conditions (40 µmol of photons $m^{-2}$ $s^{-1}$) or at double light (80 µmol of photons $m^{-2}$ $s^{-1}$) and concentrated to 40 µg chl $ml^{-1}$. The sample was excited with blue (450 nm low-pass filter, Corion, FL, USA) or orange (580 nm narrow-band filter, Corion) light. The spectra were normalized to the PSI emission peak value at 723 nm.

Thermoluminescence was measured with a home-made luminometer (Paper V). The $A_{730}$ of the cell suspension was adjusted to 1.0, concentrated 300 times and mixed with glycerol to a final concentration of 30 %. To measure the Q-band 20 µM DCMU was added. Before measurement, the cell suspension was dark incubated. The temperature was lowered to -20 °C and a 4 µs Xenon flash was fired. Heating at the rate of 1 °C $s^{-1}$ was started 30 s after the flash.

## 3.5. Gene expression

### 3.5.1. Stress conditions and treatments

Mild heat stress was induced by incubating the cells at 43 $^oC$ at 35 µmol of photons $m^{-2}$ $s^{-1}$ (Papers III and IV). More severe heat stress was induced by short 15 min treatments at 48 $^oC$ under the PPFD of 5 µmol $m^{-2}$ $s^{-1}$ with or without triggering acquired thermotolerance with a 1 h pre-treatment at 43 $^oC$ (Papers III and IV). Salt stress was induced by supplementing the BG-11 medium with 0.7 M NaCl (Papers I and II). Hyperosmotic stress was induced by supplementing the growth medium with 0.5 M sorbitol (Papers I and II). Cells were treated for 10 min, 30 min and 1 hour in otherwise standard conditions. High light stress was induced by illuminating cell culture under the PPFD of 1500 µmol $m^{-2}$ $s^{-1}$ (Paper V). After the treatments cells were harvested by centrifugation at 4 $^oC$.

### 3.5.2. RNA analysis

*RNA isolation and Northern blot.* Total RNA from 15 ml samples of cell culture containing 10 µg chl $ml^{-1}$ was isolated by extracting with acidic phenol according to (Tyystjärvi et al. 2001). 10 µg of RNA was separated on 1.2 % agarose-glyoxal gels and Northern blot hybridizations were performed as in (Tyystjärvi et al. 2001). Gene specific probes were amplified by PCR from *Synechocystis* genomic DNA. The probes were labelled with $^{32}$P-dCTP (Paper IV) or with digoxigenin-dUTP (Papers I, II and III), which is less sensitive but non-radioactive.

*Reverse-transcription PCR.* The expression of the *sigA* and *sigC* genes was studied with RT-PCR (paper IV). Isolated RNA was treated with RQ1-RNase-Free DNase (Promega). Gene specific primers and a total of 1 µg of RNA were used in cDNA synthesis with the You-Prime First-Strand Beads kit (Amersham Biosciences). PCR was performed and samples of the reactions were separated with agarose gel electrophoresis. RT-PCR is a semi-quantitative method, which gives an estimation of the relative amount of the template used in the reaction.

*Quantitative real-time PCR*. The *psbA2* and *psbA3* genes are two very similar members of the *psbA* gene family. The sensitive qRT-PCR technique was used to study the expression of these two genes in high intensity light (Paper V). After isolation RNA samples were treated with Turbo DNase (Ambion) to eliminate contaminating genomic DNA prior to cDNA synthesis with the iScript kit (BioRad). A total of 1 µg of RNA was used for cDNA synthesis. Primers were designed for the *psbA2* and *psbA3* genes and two reference genes: *rrn16Sa* and *rnpB*. The same reverse primer was used for both *psbA* genes. Specific forward primers were designed to the less similar upstream regions of the *psbA2* and *psbA3* genes. All primers were first subjected to a temperature gradient and a melt curve analysis to determine optimal primer melting temperature $^{o}$C and to confirm the specificity of the PCR product. Quantitative RT-PCR was performed on a BioRad iCycler. The PCR efficiencies of each reaction were estimated with the LineReg-program (Ramakers et al. 2003) and the expression of the *psbA* genes was calculated relative to the reference genes.

*DNA microarray*. (Paper IV) Global gene expression profiling was performed as in Eisenhut et al. (2007) with an oligonucleotide DNA microarray (Agilent). The used *Synechocystis* oligonucleotide microarray contained 8091 probes, each reading frame being represented from two to four times on the chip.

### 3.5.3. Protein analysis

Translation in high light stress was investigated by labelling the cells with radioactive methionine (Paper V). $^{35}$[S]-methionine (Perkin Elmer) is incorporated into *de novo* synthesized proteins, which can be visualized by autoradiography. Membrane proteins were isolated and chlorophyll content was measured as in Tyystjärvi et al. (1995). The membrane proteins were separated on a commercially available sodium docedyl sulphate polyacrylamide gel (NEXT-GEL, Amresco) and blotted onto Immobilon P-membrane (Millipore). Equal loading was confirmed by staining the membranes with 0.1 % Ponceau S solution. The radioactive proteins were visualized by autoradiography. To determine the amount of the D1 protein, we performed Western blot on these membranes. The D1 antibody used in immunodetection was purchased from Agrisera.

# 4. RESULTS AND DISCUSSION

## 4.1. Structural features of the *Synechocystis* RNA polymerase

Comparative modelling is a useful tool for studying the three-dimensional structures of proteins for which X-ray crystal structures are not available. In the case of the bacterial RNAP holoenzyme, crystal structures have been determined for *Thermus thermophilus* (Vassylyev et al. 2002) and *Thermus aquaticus* (Zhang et al. 1999) at 2.6 Å and 3.3 Å resolutions, respectively. Crystallization of the RNAP has succeeded only in these two thermophilic bacteria. The structures of RNAP subunits are conserved in all bacteria and therefore it was possible to use the *Thermus thermophilus* structure as a template for the modelling of the *Synechocystis* RNAP holoenzyme.

All *Synechocystis* RNAP subunits except ω showed 40-50 % sequence identity with the template, which is high enough to ensure reliable modelling. The sequence identity of the ω subunit was only 20 % and therefore it was excluded from the model (Paper I). *Synechocystis* RNAP has the split β’ subunit specific to cyanobacteria (Schneider and Haselkorn 1988). The β’ subunit corresponds to the C-terminal and γ to the N-terminal sequence of the β’ subunits of other eubacteria. According to the model (Paper I) the first and last amino acids of the β’ and γ subunits, respectively, are located on the surface of the RNAP and therefore the split doesn’t have a significant effect on overall structure. Another specific feature is that the *Synechocystis* β’ subunit contained a large, 635 amino acid insertion (Paper I). The structure of the insertion could not be determined because of the lacking template. The accuracy of our model was examined by superimposing the model on the template structure. Ramachandran plots showed that circa 90 % of amino acid residues were in the most favourable regions and less than 1 % were in disallowed regions (Paper I) indicating good overall reliability. Previously bacterial RNAPs have been modelled in *Bacillus subtilis* (MacDougall et al. 2005) and *Mycobacterium tuberculosis* (Josa et al. 2008) using the *Thermus aquaticus* and *Thermus thermophilus* structures as templates, respectively.

## 4.2. Group 1 and group 2 σ factors in *Synechocystis*

Attempts to inactivate the *sigA* gene have failed indicating that SigA is essential for cell viability (Imamura et al. 2003b, Lemeille et al. 2005a). The SigA protein is constitutively expressed in exponentially growing cells (Imamura et al. 2003b) and *sigA* transcripts dominate the *sig* transcript pool under standard conditions (Tuominen et al. 2003). Furthermore, in phylogenetic analyses the *sigA* gene clusters together with the primary σ factors of other cyanobacteria (Gruber and Bryant 1998b, Goto-Seki et al. 1999, Khudyakov and Golden 2001, Yoshimura et al. 2007). These results have led to the conclusion that SigA is the group 1 primary σ factor in *Synechocystis*. The primary σ factor has been identified and characterized also in other cyanobacterial species, including *Synechococcus* sp. PCC 7002 (Caslake and Bryant 1996), *Microcystis aeruginosa* K-81 (Asayama et al. 1996) and *Anabaena* sp. PCC 7120 (Bramsha and Haselkorn 1991).

Amino acid alignment of the group 1 and group 2 σ factors showed that their sequences were similar (Paper I). According to comparisons of three-dimensional models, also the three-dimensional structure of SigA resembles closely the structures of the group 2 σ factors (Paper I). High similarity in the regions binding to the –10 and –35 promoter elements suggests that the group 2 σ factors might have overlapping functions. The three-dimensional structures of the group 2 σ factors differed significantly only in the non-conserved domain (NCD) located between regions 1.2 and 2.1 (Paper I). SigA and SigC had the longest NCDs, 86 and 84 amino acids respectively, while the length of the domain was only 42 in SigB, 43 in SigD and 44 in SigE (Paper I). Although the length of the NCD was similar in SigA and SigC, their sequence identities were low and thus the structures of the domains cannot be considered similar. The only significant identity, 47 %, was found in the NCDs of SigB and SigD (Paper I). It is tempting to speculate that the dissimilar NCD may be a potential candidate for the regulation of promoter selectivity. The NCD lies close to the σ2.4 domain that recognizes the –10 promoter region (Paper I). The position of the NCD suggests that it can possibly bind transcription factors that further specify promoter selection. Interaction with different proteins is known to regulate σ factor function and stability (Campbell et al. 2008). An example of such regulation is provided by the AsiA-protein of the T4 bacteriophage, which binds the *Escherichia coli* $\sigma^{70}$ and specifically inhibits transcription form certain promoters (Colland et al. 1998). The specific roles of the group 2 σ factors were investigated using inactivation strains.

Four σ factor single inactivation strains (ΔsigB, ΔsigC, ΔsigD and ΔsigE), six double inactivation strains (ΔsigBC, ΔsigBD, ΔsigBE, ΔsigCD, ΔsigCE and ΔsigDE) and four triple inactivation strains (ΔsigBCD, ΔsigBCE, ΔsigBDE and ΔsigCDE) were constructed and characterized. The strains that were constructed and used in the studies are listed in Table I. All inactivation strains segregated completely. First we monitored growth in standard conditions (+32 $^{o}$C, 40 µmol photons $m^{-2}$ $s^{-1}$ continuous light, BG-11 medium pH 7.5 at air level $CO_2$ concentration and 90 rpm shaking) and found that all inactivation strains grew as well as the control strain (Papers I and V). Our results indicated that one, two or three group 2 σ factors can be inactivated simultaneously in any possible combination and, apparently, *Synechocystis* can grow under standard conditions with only one group 2 σ factor present in the cell. The simultaneous inactivation of all four group 2 sigma factors has not been attempted yet. In addition to *Synechocystis*, single inactivation strains of group 2 σ factor genes have been constructed in *Anabaena* sp. PCC 7120 (Bramsha and Haselkorn 1992, Khudyakov and Golden 2001) and *Synechococcus elongatus* sp. PCC 7942 (Caslake et al. 1997, Gruber and Bryant 1998a, Nair et al. 2002). All of these studies reported that the inactivation of group 2 σ factor genes did not affect growth under standard conditions. Also in agreement with our results, previously constructed ΔsigBD, ΔsigBE and ΔsigDE strains in *Synechocystis* (Summerfield et al. 2007) and a double inactivation strain in *Anabaena* sp. PCC 7120 (Khudyakov and Golden 2001) grew as well as the control strain. In addition to our complete set of triple inactivation strains Summerfield and Sherman (2007) have recently constructed a ΔsigBDE triple inactivation strain in *Synechocystis*. Similarly to our results, they found no differences in growth under standard conditions between the control and the inactivation strain.

**Table I** *Synechocystis* strains constructed and used in the studies.

| Strain | Inactivated genes and antibiotic resistance | Description | Studies |
|---|---|---|---|
| ΔsigB | *sll0306*::Kn$^r$ | Paper III | Papers I-V |
| ΔsigC | *sll0184*::Kn$^r$ | Paper IV | Papers I and IV |
| ΔsigD | *sll2012*::Kn$^r$ | Paper III | Papers I, III and V |
| ΔsigE | *sll0189*::Kn$^r$ | Paper I | Paper I |
| ΔsigBC | *sll0306*::Kn$^r$<br>*sll0184*::Spc$^r$/Str$^r$ | Paper I | Papers I and IV |
| ΔsigBD | *sll0306*::Kn$^r$<br>*sll2012*::Spc$^r$/Str$^r$ | Paper III | Papers I, III and V |
| ΔsigBE | *sll0306*::Kn$^r$<br>*sll1689*::Spc$^r$/Str$^r$ | Paper I | Paper I |
| ΔsigCD | *sll0184*::Kn$^r$<br>*sll2012*::Spc$^r$/Str$^r$ | Paper I | Paper I |
| ΔsigCE | *sll0184*::Kn$^r$<br>*sll1689*::Spc$^r$/Str$^r$ | Paper I | Paper I |
| ΔsigDE | *Sll2012*::Kn$^r$<br>*sll0189*::Spc$^r$/Str$^r$ | Paper I | Paper I |
| ΔsigBCD | *sll0306*::Kn$^r$<br>*sll0184*::Cm$^r$<br>*sll2012*::Spc$^r$/Str$^r$ | Paper V | Paper V |
| ΔsigBCE | *sll0306*::Kn$^r$<br>*sll0184*::Spc$^r$/Str$^r$<br>*sll1689*::Cm$^r$ | Paper V | Paper V |
| ΔsigBDE | *sll0306*::Kn$^r$<br>*sll2012*::Spc$^r$/Str$^r$<br>*sll1689*::Cm$^r$ | Paper V | Paper V |
| ΔsigCDE | *sll0184*::Kn$^r$<br>*sll1202*::Spc$^r$/Str$^r$<br>*sll1689*::Cm$^r$ | Paper V | Paper V |

Bhaya et al. (1999), Huckauf et al. (2000) and Imamura et al. (2003b) have inactivated *Synechocystis* group 3 σ factors, which differ considerably from the group 1 and 2 factors. Bhaya et al. (1999) used a motile *Synechocystis* strain and inactivated the *sigF* gene. They found that the *sigF* inactivation strain did not form pili-structures and as a result had lost its capacity for movement. More recently, Asayama and Imamura (2008) showed that SigF specifically recognizes the promoter of the *pilA* gene, which is involved in establishing motility. Inactivated *sigF*, *sigH* and *sigI* genes segregated completely, but the segregation of the *sigG* gene remained incomplete despite of a vigorous selection process (Huckauf et al. 2000, Matsui et al. 2007). The group 3 σ factor gene *sigG* seems to be essential for cell viability. Huckauf et al. (2000) reported similar growth rates in the *sigF*, *sigH* and *sigI* inactivation strains compared to the control strain.

## 4.3. Physiological roles of group 2 σ factors

### 4.3.1. Photosynthesis

Photosynthetic and PSII capacities were measured with a Clark-type oxygen electrode from cells grown under standard growth conditions. Light-saturated rates of oxygen evolution in all inactivation strains were similar to that of the control strain (Paper I and V). Clearly the inactivation of one, two or three group 2 σ factors did not affect photosynthetic oxygen evolution capacity nor cause defects in the function of the PSII electron transport chain. Photosynthesis is fundamental process in cyanobacteria and one could assume that under standard conditions the primary housekeeping σ factor, SigA, is responsible for the transcription of photosynthetic genes. Shibato et al. (1998) and Imamura et al (2003b) showed that the SigA factor may recognize *in vitro* the promoter of the *psbA2* gene encoding the D1 protein. Although previous studies of cyanobacterial group 2 σ factor inactivation strains have not presented data on photosynthetic oxygen evolution, the inactivation of the group 3 σ factor genes *sigF*, *sigH* and *sigI* did not significantly affect photosynthesis (Huckauf et al. 2000).

### 4.3.2. Growth phase transfer

Growth under standard conditions was similar to the growth of the control strain in all single, double and triple inactivation strains (Paper I and V). The growth pattern of bacterial cell cultures is divided into three stages: the lag phase, the exponential phase and the stationary phase (Buchanan et al. 1997). According to our results, *Synechocystis* growth was exponential only during the first day, after which the cultures grew linearly for a few days before they reached a stationary phase of very slow growth (Papers I and V). The *Synechocystis* growth pattern was not a typical bacterial one. In our experiments, samples were taken once every 24 h. With that sampling rate a lag phase was not detected and the exponential phase was very short. In the case of *Synechocystis*, a stage of linear growth is known to follow a short exponential phase (Foster et al. 2007).

When very dilute cell culture was spotted onto agar plates, the ΔsigBD inactivation strain grew slower than the control strain but when denser cultures were spotted on the plates, the strains grew similarly (Paper I). The same phenomenon was observed also in liquid cultures. If the initial $A_{730}$ was set to 0.001, the ΔsigBD strain had a lengthy lag phase that was not evident in the control strain. In contrast to the control strain, ΔsigBD grew very slowly during the first day after which its growth rate increased significantly. After three days the differences in growth had disappeared as cell densities of the ΔsigBD strain had reached those of the control strain (Paper I). Our results show that the ΔsigBD double inactivation strain is not able to transfer normally from the lag phase to the exponential growth phase. On the other hand, cells in very dilute cultures experience higher light intensities than in cells denser cultures. The lengthy lag phase might thus be an indirect result of light-sensitivity. The effect, however, persisted when a lower light intensity was used in the growth experiments. Next we tested if the effect might be caused by chemical signals that cells secrete into the growth medium. We grew ΔsigBD cells in medium filtered from control strain cultures and vice versa. The ΔsigBD strain always exhibited a very slow transfer from the lag phase to the exponential phase. Thus, chemical communication signals are unlikely to be responsible for the slow growth phase transfer in the ΔsigBD strain. The slow transfer from the lag to the exponential phase was observed only in the ΔsigBD double inactivation strain and not in the ΔsigB or ΔsigD strains. This indicates that the SigB and SigD σ factors, which are very similar in structure (Paper I), may compensate for each other. A ΔsigB strain has been studied earlier with regard to transfer from the exponential to the linear growth phase (Foster et al. 2007). Similarly to our results, no growth rate differences between the control and ΔsigB strains were reported in this study although many differences were detected in transcription profiles. Foster et al (2007) showed that fewer genes were differentially expressed in the ΔsigB strain than in the control strain and that the expression of the *sigB* gene itself was up-regulated upon transition to the exponential phase.

Although all of our σ factor inactivation strains behaved like the control strain in the stationary phase under standard conditions, Asayama et al. (2004) showed that a ΔsigC strain entered the stationary phase slightly earlier than the control strain in their standard conditions. In addition, they measured less viable cells in the ΔsigC strain than in the control strain at the stationary phase. They propose that the SigC σ factor regulates the expression of the *glnB* gene encoding the PII transcription factor and as a result is important in the stationary phase (Asayama et al. 2004, Imamura et al. 2006). Differences in growth light, temperature, inorganic carbon concentration and other conditions may influence the role of the SigC factor in the stationary phase. We noticed that under high $CO_2$ and high temperature conditions ΔsigC enters the stationary phase earlier than the control strain (Paper IV). The gradual slowing of growth may depend on the exhaustion of nutrient resources, cell density, decreased light availability due to shading or, most likely, a combination of many factors (Tandeau de Marsac and Houmard 1993). The SigC factor may possibly have different roles depending on the cause of transfer into the stationary phase. A SigC homologue in *Synechococcus* sp. PCC 7002 seems to have a role in the stationary phase (Gruber and Bryant 1998a).

### 4.3.3. Acclimation to high temperature stress

Fluctuations in temperature are among the most important environmental factors affecting physiological processes in cyanobacteria. I studied the roles of the SigB, SigC and SigD factors in both long- and short-term heat acclimation. Acclimation to long-term heat stress was studied by subjecting the cells to moderate heat stress at 43 $^{o}$C for several days. At 43 $^{o}$C, the ΔsigBD strain grew more slowly than then the control strain (Paper III). Neither of the single inactivation strains ΔsigB or ΔsigD showed retarded growth, which strengthens the idea of partial functional redundancy of the SigB and SigD factors. Moreover, both the ΔsigC and ΔsigBC strains lacking the SigC σ factor were not able to grow at 43 $^{o}$C (Paper IV). The ΔsigCD strain behaves like the ΔsigC strain (data not shown). These results indicate that the SigC σ factor plays a key role in acclimation to long-term moderate heat stress. Under heat stress conditions the expression of the *sigA* gene decreased in general but declined even more in the ΔsigBD, ΔsigC and ΔsigBC strains compared to the control strain (Papers III and IV). Lower amounts of the sigA transcript may be one possible reason for slower growth at high temperature. It has been shown that lower amounts of the primary σ factor slow down the growth rate in *Escherichia coli* (Magnusson et al. 2003)

Viability experiments were conducted to investigate acclimation to short-term extreme heat stress. Cells were subjected to a 15-minute heat stress treatment at 48 $^{o}$C and then spotted onto BG-11 plates. After one week of growth under standard conditions the number of colonies was counted and used to calculate survival percentage. 20 % of the control and ΔsigD cells survived, while only 2 % of the ΔsigB and ΔsigBD and 4 % of ΔsigC cells did (Papers III and IV). Only 0,1 % of ΔsigBC cells survived which indicates that the double inactivation strain is more sensitive than either of the single inactivation strains. Photosynthesis is a highly heat-sensitive process (Mamedov et al. 1993), which is why I investigated the effect of high-temperature stress on the photosynthetic activity. Only 2 % of photosynthetic activity remained in the ΔsigBC strain after 45 minutes at 48 °C whereas the control strain retained 21 % of its original activity (Paper IV). A similar, although slightly less dramatic effect was observed in the other inactivation strains as well (Papers III and IV). The ΔsigD strain maintained its photosynthetic activity at the same level as the control for the first five minutes but finally it, too, lost its activity faster than the control strain (Paper III). Thus, both the SigB and SigC σ factors are required for short-term tolerance of extreme high temperatures whereas SigD has only a minor role.

Pre-treatment in sub-lethal heat stress conditions allows the cells to acclimate so that they can tolerate more severe stress. This phenomenon is known as acquired thermotolerance (Lee et al. 2000). We tested the capacity of the strains to acquire thermotolerance by incubating cells for 1 hour at 43 °C before subjecting them for 15 minutes to 48 °C. In this experiment nearly all of the control and ΔsigD strain cells survived, 45 % of ΔsigB and ΔsigBD cells (Paper III), 60 % of ΔsigC but only 25 % of ΔsigBC cells survived (Paper IV). Based on these results, the SigC and SigB factors are required for full acquired thermotolerance. The abundance of heat shock gene transcripts was investigated under standard conditions and after 1 h treatment at 43 °C (Papers III and IV). Under standard conditions expression levels were similar and no differences were observed between the control and inactivation strains indicating that under standard conditions the inactivation strains are capable of producing normal amounts of heat shock gene transcripts..

The expression of the *sigB* gene is rapidly but only transiently up-regulated in response to heat stress (Tuominen et al. 2003, Imamura et al. 2003b). The expression pattern of the sigB gene under heat stress resembles that of *Synechocystis* heat shock genes. Since their products play central roles in the heat stress response, we analysed the expression of some *Synechocystis* heat shock genes. The ΔsigB and ΔsigBD strains produced less *hspA* transcripts after 1 hour of heat treatment at 43 °C and after a 15-minute treatment at 48 °C, indicating that the SigB factor regulates positively the *hspA* gene in both long- and short-term heat stress (Paper III). The *hspA* gene is highly induced at high temperatures (Suzuki et al. 2006) and its product is involved in the protection of phycobilisomes and thylakoids during heat stress (Nakamoto et al. 2000, Török et al. 2001, Nitta et al. 2005, Nakamoto and Honma 2006). Thus, lower amounts of the HspA protein may explain why photosynthesis in the ΔsigB and ΔsigBD strains is more susceptible to heat stress than in the control strain. The inactivation of the *hspA* (also called *hsp16.6* and *hsp17*) gene causes similar effects as observed in our ΔSigB and ΔSigBD strains, although the phenotype is more dramatic (Lee et al. 2000, Asadulghani et al. 2004). Furthermore, Singh et al. (2007) reported that in addition to *hspA*, many other heat shock genes, including g*roEL1*, g*roES* and *dnaK*, were less induced in a ΔsigB strain than in the control strain at 45 °C. These results suggest that the normal activation of heat shock genes requires the SigB factor.

Unlike *sigB*, the expression of the *sigC* gene was not up-regulated in the control strain in response to high temperature stress at 43 °C (Paper IV). Surprisingly, the amount of *sigC* transcripts decreased upon the onset of high temperature stress, but after 2 days at 43 °C, *sigC* transcript levels returned to those measured under standard conditions (Paper IV). In agreement with these results neither Tuominen et al. (2003) nor Imamura et al. (2003b) observed any induction of sigC expression in heat stress. To find out why the SigC factor is extremely important in heat acclimation we studied the gene expression pattern in the control and ΔsigC strains after 24 hours at 43 °C with a DNA microarray and Northern blot analysis (Paper IV). In contrast to the effects of the inactivation of the sigB gene, the expression of heat shock protein genes was unaffected in the ΔsigC strain (Paper IV). Comparison of the two strains showed that three genes (*sll0108*, *ssl2501* and *ssl2384*) were less expressed in ΔsigC than in the control strain, while the expression level of 15 genes was higher in ΔsigC than in the control strain (Paper IV). Eleven out of these 15 are genes that were shown to be up-regulated also when *Synechocystis* cells were transferred from high (3 %) to low (air level) $CO_2$ (Wang et al. 2004). Among these were *stbA*, *stbB* and the *ndhF3-ndhD3-cupA* operon, which are genes involved in the uptake of inorganic carbon. The microarray results point to a possible role of the SigC factor in the regulation of carbon metabolism. Higher temperatures decrease the solubility of $CO_2$ to water, and therefore cyanobacteria exposed to high temperatures have less inorganic carbon available for photosynthesis. When the control and ΔsigC strains were grown at 43 $^oC$ under air supplied with 3 % $CO_2$, the growth of the ΔsigC strain was improved considerably compared to growth in air level $CO_2$ at 43 $^oC$ (Paper IV). The ΔsigC strain was able to grow as well as the control strain for one day after which its growth stalled. The control strain, on the other hand, continued to grow for two days (Paper IV). This result provides some support for the proposition that a SigC mediated mechanism of high temperature acclimation is connected with the low availability of inorganic carbon at high temperatures. To obtain higher growth rates, some research groups grow cyanobacteria under an enriched $CO_2$ atmosphere even under optimal growth temperature, usually 3 % or 5 % in air. We found that at 32 $^oC$ 3 % $CO_2$ increased the growth of *Synechocystis* 1.6 fold during the first day and that there were no differences between the control and inactivation strains (Paper I). If the concentration of $CO_2$ is elevated slowly, some cyanobacterial species such as *Anabaena* sp. PCC 7120 and *Synechococcus* 7942 and can tolerate culture media equilibrated with 100 % $CO_2$ although such high concentrations inhibit active growth (Thomas et al. 2005). Interestingly, our recent experiments provide a new link for SigC involvement in the carbon concentrating mechanism. We have observed that even at 32 $^oC$ the ΔsigC strain did not grow as well as the control strain if the $CO_2$ concentration was lowered to 1/10 of that in normal air. Although also the control strain grew slower under low $CO_2$ than under standard or high (3 %) $CO_2$ conditions, the ΔsigC strain showed a clear defect in acclimating to low $CO_2$.

Not only temperature, but also the pH of the growth medium affects the amount and form of available inorganic carbon. To examine the effect of pH on high-temperature growth, we tested the growth of the cells at 43 $^oC$ in slightly acidic and alkaline pH. The control strain grew twice as fast in pH 8.3 than in pH 6.7. The ΔsigC strain did not grow at 43 $^oC$ in pH 6.7 but in a more alkaline pH 8.3 it was able to grow as well as the control strain for three days (Fig 4). After four days the growth of the ΔsigC strain finally slowed down slightly compared to the control strain. This result illustrates a previously unobserved phenomenon that the growth of *Synechocystis* at high temperature is dependent on the pH of the growth medium and that the effect is very pronounced in the ΔsigC inactivation strain.

The pH optimum of *Synechocystis* is slightly alkaline and if the pH of the growth medium is lowered to 6.0 at optimal temperature, growth is significantly repressed (Kurian et al. 2006). The pH dependence of growth has been previously reported for some inactivation strains under optimal temperature. *Synechocystis* strains ΔNdhB with a non-functional NAD(P)H dehydrogenase complex, and ΔNdhD3/NdhD4 with an inactivated $CO_2$ uptake system grow in BG-11 medium at pH 8.3 but not at pH 7.5 (Wang et al. 2004). Furthermore, a strain lacking proteins of the oxygen-evolving complex of PSII was able to grow in alkaline pH but not in neutral or acidic pH (Eaton-Rye et al. 2003). Neutral or slightly acidic pH, however, does not *per se* inhibit the growth of the ΔsigC strain, as similar growth rates were measured for the control and ΔsigC strains at 32 $^oC$ at pH 6.7, 7.5 and 8.3 (data not shown).

Ohta et al. (2005) studied gene expression at low pH. We noticed that seven of the genes that were more up-regulated in ΔsigC than in the control strain at 43 °C (Paper IV) are down-regulated in low pH (Ohta et al. 2005). Using the dissociation constant of bicarbonate (4.3 x $10^{-7}$ $mol^{-1}$), the equilibrium carbon concentrations ($CO_2$ and $HCO_3^-$) of the growth medium at pH 6.7 and 8.3 were calculated to be 0.2 and 6 times the carbon concentration at pH 7.5, respectively. At alkaline pH most carbon is in the form of $HCO_3^-$, while at pH 6.7, 46 % of the dissolved carbon is $CO_2$. Cyanobacteria can uptake both forms using distinct inorganic carbon uptake systems, some of which function constitutively and some are up-regulated specifically when the availability of carbon is low (Benschop et al. 2003). The pH dependency of growth at 43 °C together with gene expression data suggests that one component of high temperature acclimation is acclimation to low inorganic carbon concentration. According to our results, the SigC factor has a central role in this process.

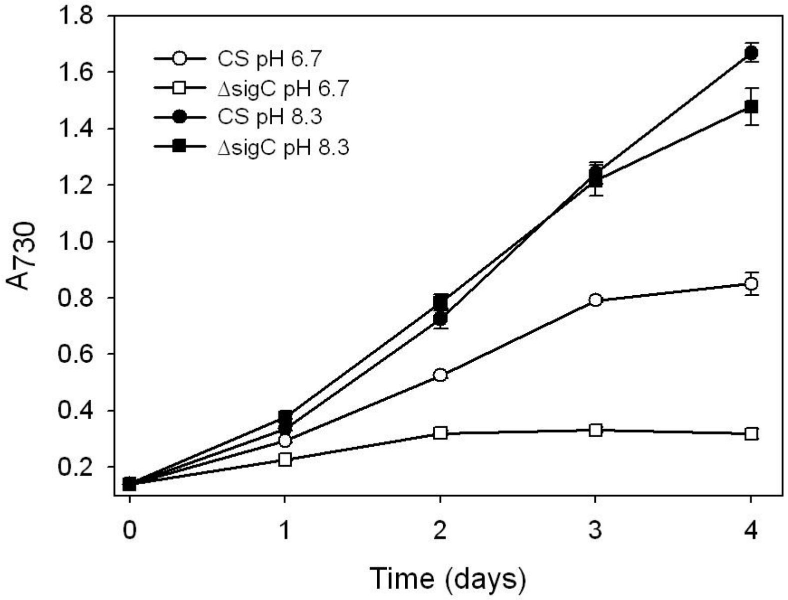

**Figure 4.** Growth of the control (CS) and ΔsigC strains at 43 °C in BG-11 medium pH 6.7 (open symbols) and in pH 8.3 (solid symbols).

Based on our results we conclude that both SigB and SigC σ factors mediate the short-term heat stress response in *Synechocystis* and that SigC is absolutely necessary for long-term heat acclimation. These σ factors regulate high temperature stress responses via different mechanisms. The ΔsigBC double inactivation strain was extremely vulnerable to high temperature stress probably because it lacks both the SigB and SigC mediated acclimation mechanisms.

### 4.3.4. Osmotic stress tolerance

We studied the roles of the group 2 σ factors in two types of osmotic stress: high-salt stress induced by the addition of 0.7 M NaCl to the growth medium and hyperosmotic stress induced by 0,5 M sorbitol (Papers I, II). These concentrations were chosen because both slowed down the growth of the control strain by 30 % (data not shown). It has been shown earlier that *Synechocystis* is more sensitive to sorbitol- than to salt-induced osmotic stress and that these stresses have different physiological effects (Marin et al. 2006). None of the strains with an inactivated *sigB* gene (ΔsigB, ΔsigBC, ΔsigBD and ΔsigBE) grew well in either salt- or sorbitol-induced osmotic stress (Papers I and II) indicating that the SigB σ factor is crucial for acclimation to both salt- and sorbitol-induced osmotic stress. Furthermore, a pronounced and rapid increase in the expression of the *sigB* gene was observed at the onset of both stresses (Papers I and II). This result agreed with earlier studies reporting an induction of *sigB* expression in high-salt (Kanesaki et al. 2002, Tuominen et al. 2003, Shoumskaya et al. 2005) and hyperosmotic stress (Kanesaki et al. 2002, Mikami et al. 2002, Paithoonrangsarid et al. 2004). The transient induction of the *sigB* gene in the control strain reached a peak after 10 minutes of salt treatment after which the amount of *sigB* transcripts decreased so that only traces could be detected after 24 hours (Paper I). Under sorbitol-induced stress, on the other hand, the peak in sigB transcripts was observed after 6 hours (Paper I). Thus, the dynamics of *sigB* activation were different in salt- and sorbitol-induced osmotic stresses. Based on DNA microarray experiments Marin et al. (2004) also commented on the dynamic nature of gene expression changes in response to salt stress. They found that different genes are induced in a very different manner and on different time scales. However, the *sigB* gene was not among their “top ten” lists of up-regulated genes in salt stress, which included *ggps* (glucosylglycerol phosphate synthase), *hliB* (high light-inducible polypeptide), *hsp17* (16.6 kDa small heat shock protein) and several genes of unknown function (Marin et al. 2004).

In order to study the effect of high-salt stress on photosynthesis the growth medium was supplemented with 0.7 M NaCl and the light-saturated PSII electron transfer rate was measured. The control strain lost 70 % and the ΔsigB strain more than 80 % of PSII electron transfer in two days (Paper II). While the control strain was able to acclimate to salt stress and had re-established 100 % of its PSII capacity by the third day, the ΔsigB strain was unable to reach its original PSII capacity (Paper II). According to Nitta et al. (2005) and Nakamoto and Honma (2006), the heat shock protein HspA is involved in the protection of photosynthetic membranes and phycobilisomes during stress and the *hspA* inactivation strain was demonstrated to be salt-sensitive (Asadulghani et al. 2004). The *hspA* inactivation strain had less phycobilisomes than the control strain suggesting that its role is connected to the stabilization of the light-harvesting antenna during salt stress (Asadulghani et al. 2004). The Northern blot analysis showed that the expression of the *hspA* gene was reduced and lasted a shorter time in the ΔsigB strain than in the control strain in salt stress (Paper II). The incapability to produce enough *hspA* transcripts may be related to the sensitivity of the ΔsigB strain to salt-induced osmotic stress. Similarly to heat stress, salt-stress causes the denaturation of proteins. The cytoplasmic histidine kinase Hik34 has been shown to be involved in salt-stress related regulation of heat shock proteins in *Synechocystis* (Marin et al. 2003). The induction of *sigB* and some heat shock protein genes in response to salt stress was poor in a salt-sensitive Δhik34 inactivation strain (Marin et al. 2003). Marin et al. (2003) further proposed that also Hik16 and Hik33 may be a part of the signal transduction pathway responding to salt stress.

In addition to the strains with an inactivated *sigB* gene, growth in salt-induced osmotic stress was poor also in the strains lacking SigC, SigE or both. Furthermore, the ΔsigD strain grew slightly more slowly than the control strain (Paper I). In sorbitol-induced osmotic stress, on the other hand, ΔsigE grew as well as the control strain although slower growth was observed in strains lacking SigC or SigD (Paper I). Thus, all group 2 σ factors have roles in acclimation to salt-induced osmotic stress and all except the SigE factor participate in acclimation to hyperosmotic stress. Shoumskaya et al. (2005) reported that the *sigD* gene was up-regulated in response to both salt- and sorbitol-induced osmotic stress although Tuominen et al. (2003) did not detect any effect under short-term salt stress. The experiments by Shoumskaya et al (2005) were, however, performed under a higher light intensity (70 μmol photons $m^{-2}$ $s^{-1}$) than those of Tuominen et al. (2003), who used the PPFD of 50 μmol $m^{-2}$ $s^{-1}$. Osmotic and high light stress, among other conditions, cause oxidative stress via the production of reactive oxygen species. During acclimation to osmotic stress, Hik33 together with its response regulator 31 relays signals to the SigD factor (Marin et al. 2003, Shoumskaya et al. 2005). Genes involved in this signal cascade were also up-regulated in response to UV and high light stress (Huang et al. 2002) and, furthermore, down-regulated by hydrogen peroxide-induced oxidative stress in a Δhik33 inactivation strain (Kanesaki et al. 2007). All of these conditions and treatments cause oxidative stress via the production of reactive oxygen species. Therefore we tested how the ΔsigD inactivation strain grows in mild oxidative stress induced with a chemical agent. We grew control and ΔsigD cells in BG-11 medium supplemented with 0.1 μM methyl viologen. Methyl viologen causes the production of superoxide, which leads to the generation of other reactive oxygen species. In the presence of methyl viologen the growth of the ΔsigD strain was retarded compared to that of the control strain (Paper I). This result suggests that the *Synechocystis* SigD factor is involved in acclimation to oxidative stress. Imamura et al. (2003a) have commented on the redox-responsiveness of the SigD protein. They showed that in the presence of DCMU the amount of the SigD protein increased (Imamura et al. 2003a). Moreover, the addition of DCMU has been shown to increase the amounts of *sigD* transcripts nearly three-fold (Hihara et al. 2003).

### 4.3.5. Acclimation to different light conditions

Research groups grow *Synechocystis* under very different light conditions. The definition of "low" and "high" light thus depends on the standard conditions used in each particular laboratory. Our standard growth light is 40 μmol photons $m^{-2}$ $s^{-1}$, low light 20 μmol photons $m^{-2}$ $s^{-1}$ and for moderate light we doubled the light intensity to 80 μmol photons $m^{-2}$ $s^{-1}$. We tested the growth of single and double inactivation strains on plates under different light conditions and found that all strains with an inactivated *sigD* gene (ΔsigD, ΔsigBD, ΔsigCD and ΔsigDE) grew poorly at 80 μmol photons $m^{-2}$ $s^{-1}$ (Paper I). Following this we grew cells of the control and triple inactivation strains in liquid cultures at 80 μmol photons $m^{-2}$ $s^{-1}$. In this experiment the overall growth of the control strain improved. The triple inactivation strains lacking both SigB and SigD (ΔsigBCD and ΔsigBDE) did not grow as well as the control strain (Paper V). We further investigated the roles of the SigB and SigD factors in light acclimation by testing the growth performances of ΔsigB, ΔsigD and the ΔsigBD double inactivation strain. All strains grew faster at 80 than at 40 μmol photons $m^{-2}$ $s^{-1}$ but the increase in growth rate was poor in the ΔsigBD strain. Also the ΔsigD single inactivation strain showed slower growth compared to the control strain (Paper V). It was surprising that although the ΔsigBD strain was extremely sensitive to 80 μmol photons $m^{-2}$ $s^{-1}$ on plates, it was nevertheless able to grow in liquid. On agar plates the cells are subjected to quite a different environment from liquid cultures. For example, the availability of carbon dioxide is different. The conditions the cells experience on plates may be harsher than those in liquid, which may explain why the ΔsigBD strain was more sensitive on plates than in liquid. Next we measured oxygen evolution to discover possible differences in photosynthetic activities between cells grown at 40 and 80 μmol photons $m^{-2}$ $s^{-1}$. Photosynthetic activity was measured with the same light intensity in which the cells were grown. Oxygen evolution was similar in all strains under the standard growth light (40 μmol photons $m^{-2}$ $s^{-1}$) which corresponds well with the equal growth rates. At 80 μmol photons $m^{-2}$ $s^{-1}$, the photosynthetic activity was almost twice as high than at standard growth light in the control and ΔsigB strains (Paper V). In ΔsigBD, photosynthetic activity at 80 μmol photons $m^{-2}$ $s^{-1}$ was only 60 % of that measured in the control strain. Activity in the ΔsigD strain was 20 % smaller than in the control strain (Paper V). These results indicate that the ΔsigD and ΔsigBD strains were unable to enhance their photosynthetic activity normally and thus to take full advantage of the greater availability of light energy at 80 μmol photons $m^{-2}$ $s^{-1}$.

We compared the 77 K fluorescence emission spectra from control and ΔsigBD cells grown at 40 and 80 μmol photons $m^{-2}$ $s^{-1}$. No significant differences were observed between the strains at 40 or 80 μmol photons $m^{-2}$ $s^{-1}$ when blue light, exciting mainly chlorophyll, was used as actinic light (Paper V). Higher PSII emission peaks were measured from cells grown at 80 than at 40 μmol photons $m^{-2}$ $s^{-1}$ when orange light was used to excite the samples. Also emission from the phycobilisome antenna was greater in cells grown at 80 than at 40 μmol photons $m^{-2}$ $s^{-1}$. The increase in emission from phycobilin at 80 μmol photons $m^{-2}$ $s^{-1}$ was more prominent in the control than in the ΔsigBD strain (Paper V). Furthermore, the ratio of the 695 to 685 nm emission increased in the control strain upon transfer to higher growth light. This change was not observed in the ΔsigBD strain (Paper V). These results indicated that while the photosystem stoichiometry was not differentially affected in cells grown at 40 and 80 μmol photons $m^{-2}$ $s^{-1}$, the light harvesting antenna of the ΔsigBD strain probably does not adjust to brighter light normally

Previous studies have proposed that SigD is a light-responsive σ factor (Hihara et al. 2001, Huang et al. 2002, Imamura et al. 2003b). Our results from physiological experiments support the hypothesis that the SigD factor is involved in acclimation to different light conditions. In addition our results revealed that optimal acclimation requires also the contribution of the SigB factor. Upon illumination, SigD expression is up-regulated both at transcript (Hihara et al. 2001, Huang et al. 2002) and protein level (Imamura et al. 2003b). Expression of the *SigD* gene is induced also after a dark-to-light shift (Gill et al. 2002, Imamura et al. 2003a). Moreover, its expression is enhanced by UV-B light (Huang et al. 2002). In addition to the *sigD* gene, also the *sigB* gene was induced by high light and UV irradiation (Huang et al. 2002). In *Synechococcus elongatus* sp. PCC 7942 a *sigD* homologue, *rpoD3*, was identified as a light-activated gene (Seki et al. 2007). The inactivation of *rpoD3* in this species resulted in sensitivity to high light (Seki et al. 2007). Taken together, the accumulated evidence underlines the role of the SigD factor in acclimation to different light conditions.

To further study the roles of SigB and SigD factors in light acclimation we subjected the cells to high light stress by illumination under intense light (1500 μmol photons $m^{-2}$ $s^{-1}$) and measured light-saturated rate of PSII electron transport after 15 min, 30 min and 45 min. In our photoinhibition experiments the ΔsigBD strain lost its PSII capacity faster than the control, ΔsigB and ΔsigD strains (Paper V). The experiment was repeated with lincomycin, an antibiotic that inhibits the repair of PSII by blocking protein synthesis required for *de novo* synthesis of the reaction centre D1 protein. In the presence of lincomycin, PSII capacities declined similarly in all strains (Paper V). This indicates that while photoinhibitory damage occurs at an equal rate in the control and ΔsigBD strains, the ΔsigBD strain is more sensitive due to a deficient PSII repair cycle. Fluorescence and thermoluminescence measurements showed that PSII functions normally in the ΔsigBD strain under standard conditions (Paper V).

We continued to investigate PSII repair under high light stress by measuring the abundance of the D1 protein in the control and ΔsigBD strains. The amount of the D1 protein remained constant in the control strain, but decreased slightly in ΔsigBD at 1500 μmol photons $m^{-2}$ $s^{-1}$ (Paper V). In *Synechocystis*, three *psbA* genes, *psbA1*, *psbA2* and *psbA3* encode the D1 protein. The p*sbA2* and *psbA3* genes are highly similar and are actively expressed, whereas the more divergent *psbA1* gene is virtually silent under low and high light conditions (Mohamed and Jansson 1989, Mohamed et al. 1993, Sicora et al. 2006). Recent data has suggested that the *psbA1* gene is activated under anaerobic conditions (Sicora and Aro, personal communication). Because the *psbA1* gene is not actively expressed in the conditions used in this study, only the expression of the *psbA2* and *psbA3* genes was analyzed in the control and ΔsigBD strains. Illumination under high light induced *psbA* gene expression significantly (Paper V), which is a well-known phenomenon (Mohamed and Jansson 1989).

Quantitative real-time PCR analysis revealed that after 30 and 45 minutes of illumination at the PPFD of 1500 μmol $m^{-2}$ $s^{-1}$ ΔsigBD had in less *psbA* transcripts than the control strain (Paper V). The expression of the *psbA2* gene was less up-regulated in ΔsigBD than in the control strain. Although the expression of the *psbA3* gene occurred initially as in the control strain, its activation stalled after 30 minutes of illumination. After 45 minutes of illumination, the ΔsigBD strain had circa 40 % less *psbA3* transcripts than the control strain (Paper V). We then calculated the percentage fractions of the *psbA2* and *psbA3* transcripts and found that under standard conditions *psbA2* contributed 90 % and *psbA3* 10 % to the total *psbA* transcript pool. Earlier studies with *Synechocystis* have revealed a similar ratio (Mohamed et al. 1993). Upon illumination under intense light (1500 μmol photons $m^{-2}$ $s^{-1}$), the ratio of the two transcripts changed so that *psbA2* now made up 60 % and *psbA3* 40 % of the transcript pool. The ratios were similar in both the control and ΔsigBD strains (Paper V). Imamura et al (2003a) studied the expression of the *psbA* genes in a ΔsigD inactivation strain using the primer extension method. They reported that the light-induced expression of *psbA2* and *psbA3* genes was significantly reduced in a ΔsigD strain (Imamura et al 2003a). We found that while the amount of the D1 protein remained constant in the control strain, it decreased slightly in the ΔsigBD strain during high light illumination (Paper V). Protein synthesis, especially the translation elongation phase, is sensitive to oxidative stress (Nishiyama et al. 2004, Nishiyama et al. 2005). Excess light excitation of the photosynthetic apparatus results in oxidative stress (Krieger-Liszkay 2005). In vivo labelling with radioactive methionine, however, did not reveal any differences in the overall translation activity during high light treatment (Paper V). Thus, inefficiency of the ΔsigBD strains in PSII repair does not seem to be a result of decrease in overall translation activity under high light stress.

All group 2 σ factors have been shown to affect the circadian rhythms of gene expression in *Synechococcus* sp. PCC 7942 (Tsinoremas et al. 1996, Nair et al. 2002). The SigB and SigD σ factors are light regulated and claimed to have roles in light-dark transitions (Imamura et al. 2003a). Transcripts of the *sigB* gene were found to accumulate after 24 h in darkness (Gill et al. 2005), although Tuominen et al. (2003) did not detect any increase in *sigB* mRNA after 18 h of darkness. Instead, they showed a rapid but transient activation of *sigB* transcription when cells were transferred from dark to light (Tuominen et al. 2003). We found that under a diurnal light rhythm, 12h light (40 photons $m^{-2}$ $s^{-1}$) and 12 dark, the ΔsigBD strain did not grow as well as the control or ΔsigB and ΔsigD strains (Fig. 5A). This effect was observed also when the light intensity during the light phase was only 10 photons $m^{-2}$ $s^{-1}$ (Fig. 5B). Thus it seems that the presence of either SigB or SigD is necessary for optimal growth in a diurnal light rhythm. Also Summerfield and Sherman (2007) reported that the inactivation of *sigB* or *sigD* alone had no effect on growth under a diurnal light rhythm. Interestingly, they noticed that the inactivation of either the *sigB* or *sigD* gene had distinct effects on gene expression in light/dark transitions (Summerfield and Sherman 2007). Inactivation of the *sigD* gene influenced mostly gene expression in the light, whereas the inactivation of the *sigB* gene affected gene expression in the dark (Summerfield and Sherman 2007). Although the inactivation of the *sigB* or *sigD* genes has an effect on the circadian gene expression pattern, the only *Synechocystis* group 2 σ factor gene to show circadian rhythm in its expression *per se* was *sigE* (Kucho et al. 2005). Taken together, these results suggest roles for the SigB and SigD factors in acclimating the cell to light-dark transitions.

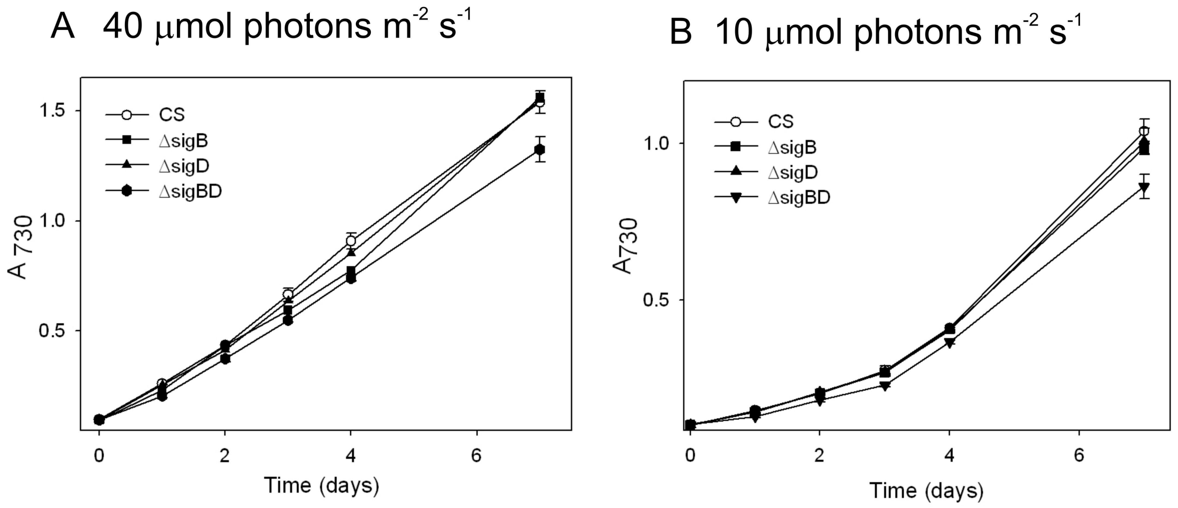

**Figure 5.** Growth of the control (CS), ΔsigB, ΔsigD and ΔsigBD strains under a diurnal light rhythm, 12 hours light and 12 hours dark in 32 °C, BG-11 medium pH 7.5 and ambient $CO_2$ concentration. A) The PPFD during the light phase was 40 μmol $m^{-2}$ $s^{-1}$. B) The PPFD during the light phase was 10 μmol $m^{-2}$ $s^{-1}$.

### 4.3.6. Other roles for group 2 σ factors

Cyanobacterial group 2 σ factors have been assigned many other roles in addition to the environmental stress conditions discussed above. *Synechocystis* SigC and SigE participate in nitrogen metabolism and acclimation to nitrogen deficiency (Bramsha and Haselkorn 1992, Caslake et al. 1997, Muro-Pastor et al. 2001, Imamura et al. 2006). In *Anabaena* sp. PCC 7120, up-regulation of the group 2 σ factor genes *sigC*, *sigE* and *sigG* occurred in connection with nitrogen deficiency and heterocyst development (Aldea et al. 2007). Gene expression related to sugar catabolism, on the other hand, seems to be under SigE mediated positive regulation (Osanai et al. 2005, Osanai et al. 2006, Summerfield 2007). Summerfield et al (2007) also reported that the simultaneous inactivation of the *sigB* and *sigE* genes completely disables the capacity for photoheterotrophic growth. In *Synechococcus* sp. PCC 7942, group 2 σ factors are involved in the regulation of circadian gene expression (Tsinoremas et al. 1996, Nair et al. 2002).

### 4.3.7. Functional redundancy of cyanobacterial σ factors

Group 2 σ factors form a part of a complex interacting network regulating gene expression patterns (Goto-Seki et al. 1999, Lemeille et al. 2005a, 2005b, Matsui et al. 2007). The simultaneous inactivation of the SigB and SigD factors caused in many cases a more severe phenotype than observed in either the ΔsigB or ΔsigD strains. For example the transfer from the lag phase to the exponential growth phase was slow only in the ΔsigBD strain (Paper I). The ΔsigBD strain was also more sensitive to high temperature and high light stress than ΔsigB or ΔsigD alone (Papers III and V). According to the three-dimensional models SigB and SigD are the most similar pair of the group 2 σ factors (Paper I), supporting the idea that they may have overlapping functions. The SigB and SigD factors can probably recognize similar promoters contributing to the expression of at least partially the same genes although conclusive evidence for this is still lacking. The SigB and SigC factors were found to control different acclimation routes but their simultaneous inactivation caused an emphasized phenotype in only one of the studied conditions, heat stress (Paper IV). In heat stress the double inactivation strain ΔsigBC was more sensitive than either ΔsigB or ΔsigC and it remains to be elucidated why the effect was limited to only one condition. *In vitro* experiments in *Synechococcus* sp. PCC 7942 revealed that the promoter of the *rrnA* gene was recognized by at least three group 2 σ factors (Goto-Seki et al. 1999). Moreover, Nair et al. (2001) showed that all group 2 σ factors in this cyanobacterium are capable of recognizing the *psbAI* promoter, supporting the idea of widespread redundancy in promoter recognition between the group 2 σ factors. Partial functional redundancy occurred between the group 2 σ factors of *Anabaena* sp. PCC 7120, in which the phenotype of a ΔsigDE double inactivation strain could be complemented by a wild type copy of either *sigD* or *sigE* genes (Khudyakov and Golden 2001). Summerfield and Sherman (2007) studied inactivation strains in different light-dark periods and found that their ΔsigBDE and ΔsigBE strains were not able to grow in an 8 h-16 h light-dark period while the single inactivation strains could grow. When the dark phase was shorter, 12 h, the double inactivation strain could grow and in continuous light also the triple inactivation strain grew well (Summerfield and Sherman 2007).

Functional redundancy, σ factor interactions and competition for a limited amount of RNAP are aspects complicating research with the aim of solving σ factor roles. This is illustrated by the enormous complexity of σ factor networks (Goto-Seki et al. 1999, Lemeille et al. 2005a, 2005b, Matsui et al. 2007). Relating physiological information to mathematically generated network models is problematic in many ways. For example, the basic assumption in Lemeille et al. (2005b) was that the contribution of a σ factor to transcription is proportional to its own expression level. We have, however, noticed that the transcript abundance of a group 2 σ factor does not always correlate with its physiological importance. For example, the *sigB* gene was highly expressed in heat stress but its absence did not affect growth at 43 $^{o}$C (Paper III). In contrast, the expression of the *sigC* gene was not up-regulated in response to heat stress but the SigC factor was required for growth at 43 $^{o}$C (Paper IV). In addition to interactions among themselves, group 2 σ factors are probably regulated also on other levels as well. While the analysis of inactivation strains gives valuable information, it is difficult to distinguish between direct and indirect effects of gene inactivations. Further research will be needed to determine which σ factors are bound to the RNAP in a given situation *in vivo*. Even more interesting would be to find out the promoters each σ factor binds. It remains unclear, however, if σ factors indeed are exclusive in promoter selection. Immunoprecipitation together with DNA tiling-arrays might be one way to address this question. In immunoprecipitation formaldehyde-fixed RNAP-DNA complexes are collected and western blot is used to detect the σ factor bound to RNAP. Upon reversing the fixation, the DNA can be purified and used to hybridize a tiling microarray. The results will reveal where the RNAP holoenzyme was bound. The work presented in this thesis establishes σ factor connections to physiological stress acclimation and may serve as a starting point for further investigations.

# 5. CONCLUSIONS

The studies included in this doctoral thesis show that group 2 σ factors have important functions in cyanobacterial acclimation processes. Based on the results I can draw the following general conclusions about the roles of the group 2 σ factors in *Synechocystis*:

The *Synechocystis* group 1 and 2 σ factors have a very similar overall three-dimensional structure. SigB and SigD are the most similar pair. All single, double and triple inactivation strains segregated completely demonstrating that the group 2 σ factors can be deleted singly, in pairs and even in triplets. Therefore it is evident that the group 2 σ factors are not essential for cell viability and *Synechocystis* can grow in standard conditions with only one group 2 σ factor. Group 2 σ factors may have overlapping functions in some conditions. The reason can be that different σ factors control overlapping sets of genes making them functionally redundant. Another possibility is that they control completely different regulatory routes affecting the same physiological phenomenon.

SigB factor

- Expression is activated rapidly but transiently by a variety of stresses
- Is important in short-term heat acclimation and for acquired thermotolerance
- Is required for growth in salt- and sorbitol-induced osmotic stress
- Is a positive regulator of the *hspA* heat shock gene
- Participates in light acclimation together with the SigD factor

SigC factor

- Has an important role in both short- and long-term heat stress acclimation
- Is involved in the regulation of carbon metabolism under heat stress
- Is required for efficient acclimation to salt- and sorbitol induced osmotic stress

SigD factor

- Is needed for optimal acclimation to different light conditions
- Has a minor role in acclimation to salt- and sorbitol induced osmotic stress

SigE factor

- Is involved in optimal acclimation to salt-induced osmotic stress

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Towards new adventures!

Turku November 2008

Maija

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