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CYANOBACTERIAL RNA POLYMERASE: STRUCTURAL FEATURES AND ACCLIMATION TO ENVIRONMENTAL CHANGE

by

Liisa Gunnelius

TURUN YLIOPISTO
UNIVERSITY OF TURKU
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From the Laboratory of Molecular Plant Biology

Department of Biochemistry

University of Turku, Finland

Supervised by

Dr. Taina Tyystjärvi

Laboratory of Molecular Plant Biology

Department of Biochemistry

University of Turku, Finland

Reviewed by

Junior Professor Ilka Axmann

Institute of Synthetic Microbiology, University of Düsseldorf, Germany

and

Dr. Tina Summerfield

Department of Botany, University of Otago, Dunedin, New Zealand

Opponent

Professor Enrique Flores

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas

University of Sevilla, Spain

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ABBREVIATIONS

ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BG-11	growth medium for *Synechocystis* sp. PCC 6803
bp	base pairs
cAMP	cyclic adenosine 3',5'-monophosphate
Chl	chlorophyll
C_i	inorganic carbon
CS	control strain
DCBQ	2,6-dichloro-*p*-benzoquinone
DCMU	3-(3',4'-dichlorophenyl)-1,1-dimethylurea
Hik	histidine kinase
IsiA	iron-stress-induced IsiA protein, CP43'
Mbp	million base pairs
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NCBI	National Center for Biotechnology Information
NCD	non-conserved domain
OD_{730}	optical density at 730 nm
PCC	Pasteur Culture Collection of cyanobacteria
PCR	polymerase chain reaction
PPFD	photosynthetic photon flux density
(p)ppGpp	guanosine tetraphosphate and/or guanosine pentaphosphate
PSI	photosystem I
PSII	photosystem II
RNAP	RNA polymerase
Rre	response regulator
RT-PCR	reverse-transcription PCR
Å	Ångström, 0.1 nm

TABLE OF CONTENTS

ABSTRACT

Bacteria acclimate to environmental changes by regulating gene expression. The first step in gene expression is transcription, which is executed by the RNA polymerase (RNAP). In bacteria, the RNAP core consists of two identical α subunits, one β and one β' subunit, which form the catalytic reaction center, and one ω subunit, which is thought to assist the β' subunit in folding and assembly to the complex. In cyanobacteria, the β' subunit has been split and the part corresponding to the N-terminal part of β' in other bacteria is called γ, while the C-terminal part retains the name β'. For transcription initiation, the RNAP core forms a holoenzyme with a sigma (σ) factor, which can bind to specific promoter sequences and unwind the DNA. σ factors are often encoded by gene families, and different σ factors compete in binding to the RNAP core.

In *Synechocystis* sp. PCC 6803, a model cyanobacterium, SigA is the primary σ factor, which is thought to be responsible for the transcription of housekeeping genes. SigB, SigC, SigD, and SigE are primary-like σ factors, which resemble the primary σ factor. Comparative modelling of *Synechocystis* RNAP holoenzyme with primary or primary-like σ factors revealed that the structures of the σ factors are very similar. It was shown that the primary-like σ factors are not essential, since the ΔsigBCDE strain, which lacks all four primary-like σ factors, grew like the control strain in standard conditions. However, further investigation showed that in addition to its previously reported roles, SigB is crucial for acclimation to both ionic and non-ionic hyperosmotic stress. SigC was found to have a key role in acclimating the carbon metabolism to prolonged heat stress, while SigD was found to be essential for acclimation to increased light intensity, together with SigB. SigE was found to play a role in initial acclimation to salt-induced stress.

The cyanobacterial ω subunit is unique compared to ω subunits in other bacteria. An inactivation strain, ΔrpoZ, was constructed to study the role of the ω subunit in cyanobacteria. The ω subunit was found to be non-essential in standard growth conditions, but lower light-saturated photosynthetic activity and accumulation of protective carotenoids and α-tocopherol were revealed as major physiological changes in *Synechocystis* without the ω subunit. Furthermore, transcriptional profiling revealed that highly expressed genes in the control strain, including many genes related to photosynthesis and carbon acquisition, were down-regulated in ΔrpoZ while genes with moderate or low expression levels in the control strain were up-regulated in ΔrpoZ. One of the down-regulated transcripts was *sigA,* and cells of the ΔrpoZ strain contained less SigA. Moreover, the ratio of RNAP-bound to free SigA was smaller in the inactivation strain, suggesting that the ω subunit might regulate the binding of the primary σ factor to RNAP. Thermal acclimation responses were severely disturbed in the ΔrpoZ strain, rendering ΔrpoZ unable to grow even in a very mild heat stress that did not affect the growth of the control strain.

TIIVISTELMÄ

Bakteerien sopeutuminen ympäristöolojen muutoksiin tapahtuu geenien ilmenemisen säätelyn kautta. Geenien ilmenemisen ensimmäinen vaihe on transkriptio, jota katalysoi RNA-polymeraasi (RNAP). Bakteereilla RNAP:n ydin muodostuu kahdesta samanlaisesta α-alayksiköstä, yhdestä β- ja yhdestä β'-alayksiköstä, jotka muodostavat katalyyttisen reaktiokeskuksen, sekä ω-alayksiköstä, jonka ajatellaan auttavan β'-alayksikköä laskostumisessa ja ydinkompleksin muodostumisessa. Syanobakteereilla β' on jakaantunut kahtia: muiden bakteerien aminopäätä vastaava osa on nimeltään γ-alayksikkö, ja karboksipäätä vastaava osa on β'. Transkription aloitusta varten ytimeen kiinnittyy sigma-tekijä, joka voi sitoutua promootterisekvenssiin ja avata DNA-kierteen. Bakteereilla on yleensä useampia σ-tekijöitä koodaavia geenejä, ja eri σ-tekijät kilpailevat RNA-polymeraasin ytimeen sitoutumisesta.

Syanobakteerin *Synechocystis* sp. PCC 6803 primaarinen σ-tekijä SigA on vastuussa ns. taloudenpitogeenien transkriptiosta normaaleissa kasvuolosuhteissa. SigB, SigC, SigD ja SigE ovat primaarisen kaltaisia σ-tekijöitä, jotka ovat hyvin samanlaisia vaikka eivät identtisiä SigA:n kanssa. *Synechocystiksen* RNA-polymeraasin vertaileva mallinnus osoitti, että σ-tekijät SigA, SigB, SgiC, SigD ja SigE ovat keskenään hyvin samankaltaisia. Primaarisen kaltaiset σ-tekijät eivät kuitenkaan ole välttämättömiä, sillä ΔsigBCDE, jolta puuttuvat kaikki primaarisen kaltaiset σ-tekijät, kasvoi standardiolosuhteissa kuten kontrollikanta. Lisätutkimukset osoittivat, että aiemmin kuvattujen roolien lisäksi SigB oli tärkeässä roolissa hyperosmoottiseen stressiin sopeutumisessa. SigC puolestaan osallistuu hiilimetabolian säätelyyn pitkän kuumastressin aikana, ja SigD oli keskeinen tekijä kirkkaaseen valoon sopeutumisessa. SigE osallistuu säätelyyn suolastressin alkuvaiheessa.

Syanobakteereiden ω-alayksikkö on erikoinen muihin bakteereihin verrattuna. ω-alayksikön roolia syanobakteereissa alettiin selvittää tekemällä inaktivaatiokanta ΔrpoZ. ω-alayksikön puuttuminen ei haitannut *Synechocystiksen* kasvua normaalioloissa, mutta ΔrpoZ:n fotosynteesiaktiivisuus oli alempi kuin kontrollikannan ja suojaavien karotenoidien ja α-tokoferolin määrä lisääntyi ΔrpoZ-soluissa. Transkriptomiikkautkimuksessa selvisi, että kontrollikannalla normaalioloissa runsaasti ilmentyvät geenit, kuten monet fotosynteesiin ja hiilen käyttöön liittyvät geenit, hiljenivät ja vähän ilmenevät geenit puolestaan aktivoituivat ΔrpoZ-soluissa. Yksi hiljenevistä geeneistä oli *sigA,* ja ΔrpoZ-soluissa havaittiin myös vähemmän SigA-proteiinia. RNAP:iin sitoutuneen ja vapaan SigA:n suhde oli pienempi ΔrpoZ-soluissa, ja ω-alayksikön puuttuminen saattaakin heikentää primaarisen σ-tekijän sitoutumista RNA-polymeraasiin. Lämpötilaan liittyvä säätely oli häiriintynyt ΔrpoZ-soluissa, eikä inaktivaatiokanta pystynyt kasvamaan edes erittäin lievässä kuumastressissä, jossa kontrollikanta kasvoi kuten normaalioloissa.

Mostly harmless

1. INTRODUCTION

The Sun and the Earth formed from the debris of ancient stars more than four and a half billion years ago (Bouvier and Wadhwa 2010). The first billion years of our planet's history were a time of violent bombardment from the outside and letting out of steam from the inside, but as soon as things settled down a bit, evidence of emerging life can be found (Schopf 2006). The conditions on early Earth were still far from what they are today, the most striking features being an oxygen-free atmosphere and oceans full of dissolved metals. The Sun was about 20% fainter than today (Ribas et al. 2010), but it was the only abundant source of energy in addition to the limited supply of reductants in the oceans and the geothermal energy from Earth's crust (Nealson and Conrad 1999). However, a rich fossil record shows that by 2.6 billion years ago, photoautotrophic organisms using oxygenic photosynthesis had already become abundant (Olson 2006). In oxygenic photosynthesis, energy of sunlight is converted to chemical energy, and oxygen is released as a by-product. The first organisms that utilized oxygenic photosynthesis were cyanobacteria, and they have indeed changed the face of the planet they inhabit. First, the released oxygen oxidized the dissolved metal ions in the oceans, and about 2.4 billion years ago the oxygen level of the atmosphere started to rise (Bekker et al. 2004). Subsequent oxidation of atmospheric methane may have abolished the greenhouse effect and triggered a global glaciation event (Kopp et al. 2005). Eventually the formation of the ozone layer and evolution of oxidative phosphorylation enabled the emergence of oxygen-consuming and land-dwelling life forms (van der Giezen and Lenton 2012). Now, some of these other life forms are studying cyanobacteria, among other things, to find out the ultimate question of life, the universe, and everything.

1.1. Cyanobacteria: flexible photoautotrophs

Cyanobacteria are one of the most diverse and widely distributed groups of eubacteria even today. It has been estimated that currently at least one third of global primary production results from cyanobacterial photosynthesis (Field et al. 1998, Bryant 2003), and there is increasing interest in harnessing the power of photosynthesis to the production of energy and valued substances for humans. In addition to carbon fixation via photosynthesis, some cyanobacteria can fix atmospheric nitrogen (N_2). Other organisms have embraced the overspill from photosynthesis and nitrogen fixation by utilising cyanobacteria as symbionts with varying degree of freedom. For example, about 12% of known lichen-forming fungi associate with cyanobacteria (Rikkinen 2002). However, the cyanobacteria are fully independent and can survive without the fungus. A more intimate relationship involving cyanobacteria is found in plants and algae. According to the endosymbiosis theory, an ancient cyanobacterium was engulfed by a eukaryotic cell and retained as an intracellular organelle for energy production. This primary endosymbiosis occurred more than a billion years ago (Embley and Martin 2006), and is thought to be the source of primary plastids (Rodríguez-Ezpeleta et al. 2005). Subsequent endosymbiosis events gave rise to the secondary and tertiary plastids found in some algae (Keeling 2004). In the process of endosymbiosis, most of the genes from the cyanobacterium were either lost or transferred to the nucleus of the host cell, rendering the plastid unable to survive without the host cell, but a set of about 120 genes is still found in the plastids of higher plants (Leister 2003). A more recent primary endosymbiosis has been observed between a cyanobacterium and the cercozoan amoeba *Paulinella chromatophora* (Yoon et al. 2006).

Cyanobacteria inhabit various aquatic and even terrestrial environments; indeed, the dominant species in North American desert soils are cyanobacteria (García-Pichel et al. 2013). When conditions are favourable for growth, the ubiquitous presence of cyanobacteria becomes visible; historically, aggregations of cyanobacteria in water bodies are referred to as "blooms of blue-green algae". This incorrect terminology persists although Stanier et al. argued already in 1971:

"The designation blue-green algae is therefore misleading, although this common name is now so firmly established that its use can probably never be eradicated. These organisms are not algae; their taxonomic association with eukaryotic algal groups is an anachronism, formally equivalent to classifying the bacteria as a constituent group of the fungi or the protozoa. In view of their cellular structure, *blue-green algae can now be recognized as a major group of bacteria, distinguished from other photosynthetic bacteria by the nature of their pigment system and by their performance of aerobic photosynthesis*."

The cyanobacterial biomass is sometimes collected and used as dietary supplement; this method of utilization has been invented independently at least twice in human history (Ciferri 1983). Crop plants also benefit from cyanobacteria. Some of the nitrogen fixed by cyanobacteria in cultivation ponds is available and crucial for rice growth (Vaishampayan et al 2001, Ariosa et al 2004). Recently it was observed that release of the plant hormone auxin from soil-dwelling cyanobacteria can enhance the growth of wheat, and the interaction can be initiated by signals from the plant (Mazhar et al. 2013). Perhaps the best known feature of cyanobacteria is, however, that some strains can produce toxins, which can cause health risks to other organisms (Merel et al. 2013, Paerl and Otten 2013). Eutrophication of water bodies mainly due to excess nutrient flow from human activities creates conditions that favour cyanobacterial growth, and raising CO_2 concentration and surface temperature will probably make things even better from the cyanobacterial perspective (Flombaum et al. 2013).

1.1.1. Morphology, basic physiology and genomic variability of cyanobacteria

Cyanobacteria are generally recognized as archetypical diderms (like most Gram-negative bacteria) i.e. their cell envelope contains two membranes (Gupta 2011). The cyanobacterial cytoplasm is enclosed in a selectively permeable plasma membrane, which in turn is surrounded by an outer membrane. The space between the plasma membrane and outer membrane, the periplasm, holds a thin, mesh-like layer of peptidoglycan, which is covalently linked to the outer membrane via lipoproteins (Typas et al. 2012). Photosynthetic electron transfer takes place in the thylakoid membrane system located in the cytoplasm. The only cyanobacteria that do not have thylakoids are the primitive Gloeobacteria, in which the photosynthetic proteins are located in specific domains of the plasma membrane (Rexroth et al. 2011). Thylakoid-containing cyanobacteria were traditionally divided into five subsections on the basis of structural complexity (Rippka et al. 1979); however, these groups are generally not supported by phylogenetic studies (Tomitani et al. 2006). Cyanobacterial thylakoids are organized loosely into networks and layers in contrast to highly compact grana structures observed in plant chloroplasts (Nevo et al. 2007). It is generally assumed that the thylakoid membrane forms an uninterrupted proton barrier needed for the pH gradient which operates the ATP synthase. Whether the plasma membrane and thylakoids are connected is still a controversial subject (Liberton et al. 2006, van de Meene et al. 2006, Schneider et al. 2007, van de Meene et al. 2012). It has been suggested that the thylakoids converge at the edges of the cell, where a specialised membrane is involved in the biogenesis of thylakoids and the photosystems

(reviewed by Nickelsen and Zerges 2013). The Vipp1 protein, which forms specific rod-like structures called thylakoid centers, seems to be important for thylakoid biogenesis (Westphal et al. 2001, Fuhrmann et al. 2009). In addition to the structures related to photosynthesis, the cyanobacterial cell contains basic structural features like ribosomes, pili, gas vesicles and storage granules for various types of metabolites (Nevo et al. 2007).

The carbon skeletons needed for the synthesis of biological molecules are built with CO_2 fixed from the atmosphere. The current atmospheric concentration of CO_2 is much lower than when cyanobacteria first emerged, and cyanobacteria have adapted and evolved effective carbon concentrating mechanisms (reviewed by Badger and Price 2003). Both CO_2 and HCO_3^- are actively taken up by cyanobacteria via distinct carbon acquisition systems, some of which function constitutively and some of which are induced in low carbon conditions (Ogawa and Kaplan 2003, Wang et al. 2004). The first reaction of carbon fixation in the Calvin-Benson cycle is catalysed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco is contained in a specialized microcompartment, the carboxysome. Cyanobacteria can be classified on the basis of carboxysomes, since there are two evolutionarily distinct forms of Rubisco: α carboxysomes are found mainly in oceanic cyanobacteria, whereas the β form is mainly found in freshwater and estuarine strains (reviewed by Rae et al 2013).

The biosynthesis of the various nitrogenous compounds, like amino acids, starts with assimilation of ammonium (Luque and Forchhammer 2008). Ammonium is taken up by ammonium permeases (encoded by *amt* genes), but is rarely present in the extracellular medium. While cyanobacteria are able to utilise various other nitrogen sources as well, ultimately they need to be converted to ammonium, which requires energy and reductive power from the light reactions of photosynthesis (Flores et al. 2005). N_2 fixation is observed in the absence of soluble nitrogen compounds when all the genes *nifHDKTEXWZ* are present in the genome (Latysheva et al. 2012). These genes encode nitrogenase(s) and regulatory factors. The nitrogenase enzyme is irreversibly inactivated by oxygen, and therefore nitrogen fixation needs to be separated from photosynthesis. Filamentous cyanobacteria can produce specialised cells, heterocysts, which are devoid of photosystem II (Zhang et al. 2006), whereas in unicellular species, nitrogen fixation can take place in darkness when photosynthesis is inactive (Tsygankov 2007). An important signal of the intracellular carbon and nitrogen status is the level of 2-oxoglutarate, which increases when carbon fixation is more active and decreases when nitrogen assimilation is more active (Forchhammer 1999, Herrero et al. 2004). The NtcA transcription factor and the P(II) signal transduction protein react to signals from the carbon, nitrogen and energy status and regulate the expression of genes involved in the nitrogen assimilatory pathways (Frías et al. 1994, Herrero et al. 2001, Forchhammer 2008).

There are currently 98 cyanobacterial genomes of the total of 4511 bacterial genomes in the National Center for Biotechnology Information (NCBI) genome database (www.ncbi.nlm.nih.gov/genome/browse) (retrieved in January 2014). The smallest cyanobacterial cells and genomes are found in the marine genus *Prochlorococcus,* yet they are the most abundant organisms in oligotrophic oceans measured in both numbers and biomass (Flombaum et al. 2013). *Prochlorococcus* genomes vary between 1.6 Mbp (strain MIT9301) and 2.7 Mbp (strain MIT9303) (Thompson et al. 2013). In the other end are the heterocyst-producing filamentous cyanobacteria: *Mastigocoleus testarum* is currently holding the top position with an estimated genome size of 15.87 Mbp. *Scytonema hofmanni* PCC 7110 has a

genome of 12.1 Mbp, comparable to the genome size of the common yeast *Saccharomyces cerevisiae,* and contains more than 12000 protein-coding sequences (Dagan et al. 2013).

1.1.2. *Synechocystis* sp. PCC 6803: a model cyanobacterium

In the field of cyanobacterial research, one of the most popular model organisms is *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which was isolated from a Californian lake (Stanier et al. 1971). *Synechocystis* is a non-toxic, unicellular cyanobacterium that can acclimate to a variety of environmental conditions and readily takes exogenous DNA into cells, making it an appealing target for molecular biology studies. *Synechocystis* is not able to fix atmospheric nitrogen. A glucose-tolerant variant of the strain (Williams 1988) can be grown heterotrophically if cell division is activated by a daily 5-minute pulse of blue light (light-activated heterotrophic growth, LAHG; Anderson and McIntosh 1991). The first draft of the *Synechocystis* genome was published in 1996 by Kaneko et al. and it was the first photosynthetic genome resolved. The approximately 3.6 billion base pairs of chromosomal DNA consist up to 87% of coding sequence organized into 3172 open reading frames (Mitschke et al. 2011). In addition to chromosomal DNA, *Synechocystis* contains seven plasmids. *Synechocystis* has multiple copies of the chromosomal genome per cell (Labarre et al. 1989).

1.2. Bacterial RNA polymerase and transcription

1.2.1. Composition and function of RNA polymerase core

In prokaryotes, one RNAP is responsible for the transcription of messenger, ribosomal, transfer, and small RNAs. The subunits of the catalytically active RNAP core are conserved in all cellular life forms: Bacteria, Archaea and Eukaryota (reviewed by Decker and Hinton 2013). The bacterial RNAP core consists of 5 subunits, encoded by 4 different genes. Structures of RNAP have been obtained by x-ray crystallography from the thermophilic bacteria *Thermus aquaticus* (Zhang et al. 1999, Murakami et al. 2002a, 2002b) and *T. thermophilus* (Vassylyev et al. 2002, Artsimovitch et al. 2004, 2005, Tuske et al. 2005), and recently also from the most widely used bacterial model organism, *Escherichia coli* (Murakami 2013; Fig. 1). The large subunits β (encoded by the *rpoB* gene) and β' (encoded by *rpoC*) form the active reaction site by clamping the DNA into a crab-claw-like pincer (Fig. 1). The β and β' subunits share some sequence and structural homology. Especially the double-psi beta-barrel fold, which binds the magnesium ions (Mg^{2+}) in the active site, is conserved in both subunits (Iyer et al. 2003). The *rpoB* and *rpoC* genes form an operon in many bacteria, and they are probably the result of a gene duplication (Iyer et al. 2003). In cyanobacteria and their descendants, plastids, the gene encoding the β' subunit has split (Schneider et al. 1987, Schneider and Haselkorn 1988). In cyanobacteria, the product of the *rpoC1* gene, corresponding to the N-terminal part of β' in other bacteria, is called the γ subunit in cyanobacteria. The product of the *rpoC2* gene, β', contains a large lineage-specific insertion that has been predicted to fold into seven sandwich-barrel-hybrid motifs (SBHMs; Iyer et al. 2003). Interestingly, the *E. coli* β' subunit also harbours an insertion, at the exactly same position, consisting of three SBHMs (Iyer et al. 2003). Recently, it was shown that in *E. coli* this domain controls the binding of the elongation factor DksA (Furman et al. 2013).

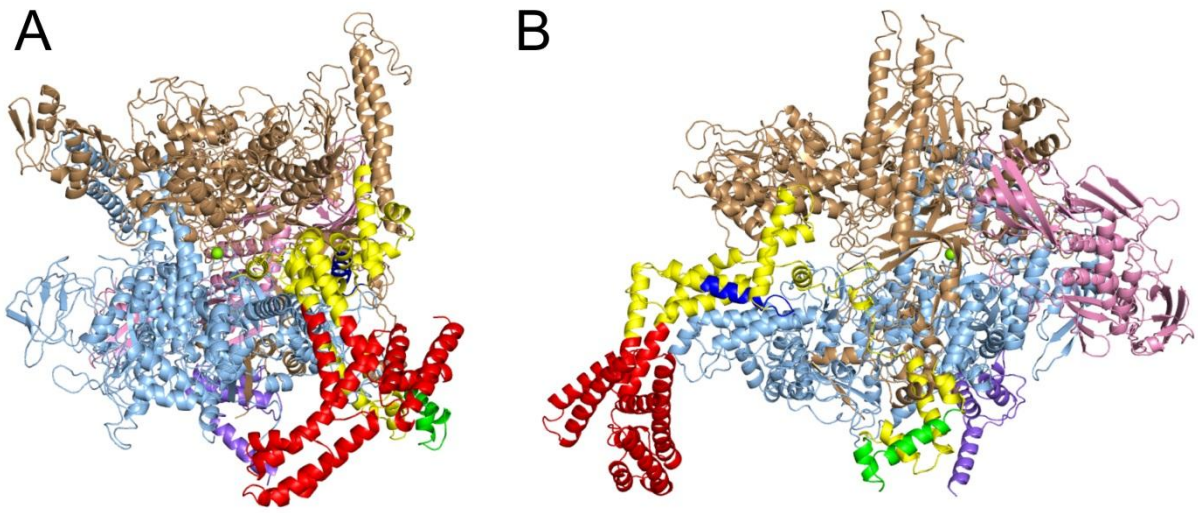

Figure 1. Structure of *E. coli* RNAP holoenzyme: visualization of Protein Data Bank entry 4IGC (Murakami 2013). (A) View towards the active site; the green sphere represents the catalytic Mg^{2+} ion. (B) View from the side. The α subunits are pink, the β subunit light brown, β' subunit light blue, ω subunit violet and the σ factor is yellow except for the non-conserved domain (red), the σ2.4 region (dark blue) and the σ4.2 region (green).

Two identical α subunits (encoded by *rpoA*) are located at the hinge end of the claw (Fig. 1). The N-terminal domain interacts with the β and β' subunits, and is required for assembly of the core and basal transcription (Zhang and Darst 1998). The C-terminal domains interact with DNA (Ross et al. 1993) and transcription factors, but this region is dispensable (Sheveleva et al. 2002). It is interesting to note that in mosses, the *rpoA* gene has been transferred from the plastome to the nuclear genome (Sugiura et al. 2003).

The tiny ω subunit (product of the *rpoZ* gene) is the only non-essential subunit of bacterial RNAP, in contrast to eukaryotic and archaeal RNAPs, where the corresponding subunit is essential (Minakhin et al. 2001). Several groups have inactivated the *rpoZ* gene in various bacteria: *E. coli* (Gentry and Burgess 1989), *Mycobacterium smegmatis* (Mathew et al. 2005), *Streptomyces kasugaensis* (Kojima et al. 2002), and *Streptomyces coelicolor* (Santos-Beneit et al. 2011). The mutants show pleiotropic phenotypes and roles of the ω subunit have remained unclear. In RNAP structures, ω is closely associated with the N- and C-terminal parts of the β' subunit (Vassylyev et al. 2002, Fig. 1). Early cross-linking experiments indicated close contacts between the ω and the β' subunit (Gentry and Burgess 1993) and later it was shown that this association promotes the final step in the assembly of the RNAP core complex from $\alpha_2\beta$ and ωβ' subassemblies (Ghosh et al. 2001). Recent studies with point-mutated ω subunits in *E. coli* also suggest that structural flexibility and the lack of secondary structure in the ω subunit are important for maintaining the catalytic centre of RNAP in active state (Sarkar et al. 2013). Overproduction of ω rescues a temperature-sensitive mutant of the β' subunit, and it has been suggested that ω latches the N-terminal and C-terminal parts of the β' subunit, thus constraining β' conformationally (Minakhin et al. 2001). In cyanobacteria, since the β' subunit is split, a similar function would mean that the ω subunit would need to make contacts with both γ and β' subunits.

1.2.2. Sigma factors

In Bacteria, the RNAP core is usually attached to DNA but for promoter recognition and transcription initiation, the core forms a holoenzyme with a σ factor (Burgess et al. 1969). The number of σ factor genes varies between bacterial genomes, the peak place held by *Streptomyces coelicolor* with currently 65 genes for σ factors identified. It is thought that the different σ factors form a pool and compete for binding to the RNAP core (Gruber and Gross 2003, Mooney et al. 2005). The σ factors can be divided into two families, σ^{70} and σ^{54}, on the basis of structural features and sequence similarity (Helmann and Chamberlin 1988). The σ^{70} family is named after the 70 kDa product of the *rpoD* gene of *E. coli*. All cyanobacterial σ factors belong to this family. The σ^{70} family can be divided into three groups (Lonetto et al. 1992). Group 1, or primary, σ factors are essential and represented by a single, irreplaceable gene in each genome. They are very conserved across the bacterial kingdom. The primary σ factor is considered to be responsible for the transcription of housekeeping genes; in rapidly growing *E. coli* cells, up to about 70% of the RNAP complexes carrying σ^{70} are transcribing ribosomal RNA genes (Raffaelle et al. 2005). In *Synechocystis,* the primary σ factor is SigA (Imamura et al. 2003b). Group 2 σ factors, sometimes called primary-like σ factors, closely resemble group 1 σ factors but are non-essential in optimal conditions. The number of group 2 σ factors varies significantly across different bacterial phyla: *E. coli* has one (σ^S), whereas cyanobacteria typically have a few group 2 σ factors (Goto-Seki et al. 1999). *Synechocystis* has four of them: SigB, SigC, SigD, and SigE, and they have been shown to be important for acclimation to environmental changes (reviewed by Osanai et al. 2008). In plants, σ factors associated with the plastid-encoded RNA polymerase have similarities with group 1 and 2 σ factors, but the genes have been transferred into the plant cell nucleus in the process of endosymbiosis (Goto-Seki et al. 1999).

Synechocystis has four additional σ factors, SigF, SigG, SigH, and SigI. These more divergent members in the σ^{70} family are classified as group 3 σ factors. SigF has been associated with the assembly of the pilus (Bhaya et al. 1999), and the consensus sequence of SigF-binding promoters has been determined (Asayama and Imamura 2008). Interestingly, while group 3 σ factors are under detection limit in *Synechocystis* cells under normal growth conditions, their transcripts are present (Imamura et al. 2003b). The *sigG* gene was found to be essential for *Synechocystis,* in contrast to *sigF, sigH,* and *sigI,* which can be inactivated (Huckauf et al. 2000, Matsui et al. 2007).

Structurally, σ factors have been divided into four conserved domains, which can be further divided into regions, on the basis of amino acid sequence homology (Lonetto et al. 1992, reviewed by Murakami and Darst 2003). The σ1 domain is very flexible and it has been impossible to obtain a crystal structure of this domain from the holoenzyme (Vassylyev et al. 2002). Conserved region σ1.1 is only found in group 1 σ factors and inhibits the binding of free σ factor to DNA (Dombroski et al. 1992, Camarero et al. 2002). Region σ1.2 interacts with the non-template DNA strand and facilitates unwinding of the DNA duplex (Bochkareva and Zenkin 2013). Between σ1.2 and σ2.1 is a non-conserved domain, σNCD (red in Fig. 1), which varies in length: in *E. coli* σ^{70}, this domain consists of 245 amino acids, whereas in the primary σ factor of *Bacillus subtilis* it is very short (Helmann and Chamberlin 1988). Regions σ2.1-σ2.4 are very conserved across a wide range of bacteria, and a recent study suggested that the -10 element of

promoter DNA interacts with regions σ1.2, σ2.1, σ2.2, σ2.3 and σ2.4 (Feklistov and Darst 2011). Specifically, the σ2.4 region (dark blue in Fig. 1) has been associated with recognition of the -10 promoter element (Gross et al. 1998), and the σ2.3 region is involved in promoter melting (Marr and Roberts 1997). Domain σ3 is a linker domain, but region σ3.0 has contacts to the extended -10 element DNA found in some promoters (Barne et al. 1997) and some residues in the σ3.1 region form contacts to the core. Region σ3.2 is involved in recruiting the initiating NTPs (Kulbachinskiy and Mustaev 2006). The σ4 domain, and especially region σ4.2 (green in Fig. 1), has a role in -35 promoter element recognition (Campbell et al. 2002).

1.2.3. Mechanistic aspects of transcription initiation

Transcription is initiated when the RNAP holoenzyme binds to the promoter forming a closed promoter complex (Murakami et al. 2002). Subsequent conformational changes in the RNAP holoenzyme then unwind and melt the DNA strands starting from the -10 region, and the open promoter complex is formed when the template strand is directed to the active site of the RNAP (Murakami et al. 2002). The σ1.1 region accelerates the formation of the open promoter complex at some promoters (Vuthoori et al. 2001). A specific tryptophan residue in the β subunit, conserved in bacteria but not in archaea or eukaryotes, interacts directly with the flipped out base of the non-template strand at the +1 site (Wiesler at al. 2013). The polymerization reaction starts, but as the RNAP holoenzyme is still strongly attached to the promoter sequence via the σ factor, the polymerization reaction is often terminated after addition of only a few nucleotides, leading to the release of the nascent RNA chain in a process called abortive initiation (reviewed by Hsu 2009). This can be repeated hundreds of times before the RNAP can unwind more DNA and synthesize a longer transcript. When the RNAP is released from the promoter, the affinity of the σ factor for the RNAP core decreases, and also the σ factor is often, but not always, released (Hsu 2002, Mooney et al. 2005).

1.2.4. Regulation of transcription

Transcription is a complicated process, and especially the initiation phase is delicately orchestrated. The promoter region and other elements of the DNA sequence near the transcription start site are important determinants of the level of gene expression (Busby and Ebright 1994, Xue et al. 2008). For the promoter elements recognized by the σ^{70} factor, frequency of each base at each position has been calculated from *E. coli* promoters. The consensus sequence for the -10 region is TATAAT and for the -35 region TTGACA (Harley and Reynolds 1987, Lisser and Margalit 1993). However, these consensus sequences are rarely found intact in promoters. Also the number of bases between the two consensus sequences affects transcriptional efficiency, the optimal length being 17 bp (Dombroski et al. 1996, Shultzaberger et al. 2007). However, the -35 element is not obligatory for transcription, and some promoters have an extended -10 element (Harley and Reynolds 1987, Minchin and Busby 1993). Some promoters contain upstream promoter element(s) that are recognized by the dimerised C-terminal domains of the α subunits of RNAP (Ross et al. 1993). Furthermore, promoter strength is influenced by abortive initiation and promoter escape (Hsu 2009). The promoter region can also contain response elements, which are binding sites for specific transcription factors. Transcription factors can act either as activators, by recruiting the RNAP to certain promoters, or repressors, inhibiting transcription. Both σ factors and transcription factors are controlled by response regulators (Rres), which in turn are controlled by sensory

proteins such as histidine kinases (Hiks). Also serine-threonine kinases (Spks) and phosphatases are involved in regulation of transcription. In fact, most Rres are transcription factors themselves as they bind to DNA with a conserved helix-turn-helix (HTH) motif. A specific aspartate residue on the Rre can be phosphorylated by the corresponding kinase, which modulates the interaction of the Rre with the RNAP (Perego and Hoch 1996). The *Synechocystis* genome encodes 45 putative Rre genes, 47 putative Hik genes, 12 putative Spk genes, at least eight putative phosphatase genes, and 57 putative DNA-binding transcription factor genes (Los et al. 2010).

The activity of RNAP is controlled by various factors. Intensive studies have been dedicated to the bacterial elongation factor NusG, which is homologous to archaeal and eukaryotic Spt5 proteins (Werner 2012). NusG binds to the clamp region of RNAP core and facilitates association of other factors, like ribosomal protein S10 and termination factor Rho. Also certain non-coding RNAs can directly interact with RNAP. In *E. coli,* the 6S RNA binds to the σ^{70}-RNAP holoenzyme by mimicking open promoter DNA and inhibits transcription by locking the RNAP holoenzyme; the inhibition is relieved by 6S RNA-dependent transcription when the cellular nucleotide triphosphate concentration rises (Steuten et al. 2014). 6S RNA genes are widespread among bacteria, and cyanobacterial 6S RNAs are functional with *E. coli* RNAP (Rediger et al. 2012).

In addition to regulation of transcription, transcript stability and availability to translation are regulated. Intensive research has been conducted on antisense-RNA (reviewed by Georg and Hess 2011) and it seems that in *Synechocystis,* two thirds of the transcripts in a cell are actually noncoding (Mitschke et al. 2011). For example, expression of the iron-stress-induced *isiA* gene is under negative control by antisense-RNA (Dühring et al. 2006). Also expression of the *flv4-2* operon, encoding flavodiiron proteins, and *psbA* genes encoding the central polypeptide of photosystem II are regulated by antisense RNAs (Eisenhut et al. 2012, Sakurai et al. 2012). Another level of regulation is exerted by nanoRNAs, extremely short (2 to 5 nucleotides) oligomers that can be used by RNAP in transcription initiation instead of single nucleotide triphosphates (Goldman et al. 2011). Environmental stress can also directly affect transcription by altering the superhelicity of DNA. For example, the *desB* gene of *Synechocystis,* encoding a fatty acid desaturase, is induced in cold stress because its promoter region is negatively supercoiled when temperature is lowered (Prakash et al. 2009).

1.3. Acclimation to heat, light and hyperosmotic stresses in cyanobacteria

When there is no immediate threat to the cellular homeostasis from the environment, the cell can allocate resources to growth and reproduction. Synthesis of macromolecules needed for this requires that the cell is provided with the chemical elements in suitable combinations. The basic building blocks are hydrocarbon skeletons, and nitrogen, oxygen, phosphorus and sulphur containing groups provide reactivity. In addition, many enzymes and other reactive molecules like pigments need certain metal ions in their active centers. Different cyanobacteria have adapted to different kinds of environments, and what are optimal conditions for one strain may be hazardous for another. Here, stress conditions will be examined in more detail with emphasis on *Synechocystis*.

Different kinds of stress share some similar responses, as the cells ultimately need to keep the proteins in their native state and maintain the intracellular homeostasis of solutes. Moreover,

the translation machinery appears to be especially sensitive to environmental stress (Murata and Suzuki 2006). Many stress conditions, including heat and high salinity, can result in the misfolding or complete unfolding, denaturation, of proteins. Hydrophobic amino acid residues, which are normally buried, become exposed in non-native folding, and trigger aggregation of proteins (Tyedmers et al. 2010). However, cells have repair proteins that prevent aggregation by capturing misfolded proteins. These repair proteins include molecular chaperones, which assist other proteins to retain their native fold, and components of the proteolytic pathway, which target and degrade misfolded proteins. Among these are the heat shock proteins (HSPs), a group of unrelated but highly conserved proteins (Lee et al. 1997). Although HSPs were first named after the response to increased temperature, they function in other stress conditions as well. Indeed, many HSPs are essential to cell viability even in standard conditions (Bukau and Walker 1989). The intracellular water potential is also influenced by a variety of environmental conditions, obviously osmotic balance and drought are important factors but also temperature has an effect. In addition to the specific transporters, intracellular homeostasis is maintained by the synthesis of compatible solutes (Klähn and Hagemann 2011). In cyanobacteria, the processes concerning the oxygenic photosynthesis must be tightly regulated because imbalances in the function of different photosynthesis reactions quickly lead to the accumulation of reactive oxygen species (ROS); however, ROS also have a role as intracellular signal molecules, as the relative content of specific ROS depends on the environmental conditions (Schmitt et al. 2014).

1.3.1. Heat

Heat stress can be broadly defined as temperatures higher than the optimum. *Synechocystis* is a mesophilic cyanobacterium with an optimal growth temperature of 30-32 °C. However, cells can be grown for a few days at 43 °C (Inoue et al. 2001, Tuominen et al. 2006), and pretreatment of cells in mild heat stress leads to acquired thermotolerance allowing longer survival in otherwise lethal temperatures up to 50 °C (Lee et al. 2000).

Photosynthesis is a heat-sensitive process (Mamedov et al. 1993), and full acclimation of the most vulnerable component, photosystem II (PSII), might take several hours (Rowland et al. 2010). The fluidity of membranes will increase in high temperatures, which can be counterbalanced by decreasing the unsaturation of fatty acid chains of membrane lipids (Balogi et al. 2005). In *Synechocystis,* the small heat shock protein HspA stabilizes thylakoid membrane lipids (Török et al. 2001). In *Synechocystis,* heat stress induces the expression of HSPs, but also other genes which mainly encode proteins with unknown function (Suzuki et al. 2006). High-temperature induced shift of the transcriptional pattern in *Synechocystis* can be roughly divided into rapid and delayed responses (Rowland et al. 2010). The group 2 σ factor SigB is rapidly up-regulated upon heat shock and up-regulates expression of *hspA* (Tuominen et al. 2006) and some other heat shock genes as well (Singh et al. 2006). The 5'-untranslated region of the *hspA* transcript acts as an inbuilt thermosensor, stimulating translation upon heat stress (Kortmann et al. 2011). The SigC factor, in turn, has been shown to be important to sustained functional photosynthesis in elevated temperatures (Tuominen et al. 2008). For heat stress, Hik34 has been recognized as an important regulator, negatively controlling the expression of important heat shock genes like *htpG* and the *groESL1* operon (Suzuki et al. 2005). In addition, the *groESL1* operon and the *groEL2* gene have CIRCE (controlling inverted repeat of chaperone expression) elements in their promoter regions, and are partially controlled by the repressor protein HrcA (heat shock regulation at CIRCE elements) (Nakamoto et al. 2003, Singh et al. 2006).

1.3.2. Light and oxidative stress

The lengths of the light and dark periods on Earth are somewhat constant, and thus cells can prepare their metabolism for the change in conditions before it happens. The circadian clock maintains cellular activities at an approximately constant rhythm of 24 hours by varying the expression of certain genes periodically (Bell-Pedersen et al. 2005). Remarkably, the periodicity persists even in the absence of environmental cues (for example, in continuous light), but the system is able to adjust its phase whenever information about the environment is available (Bell-Pedersen et al. 2005). In *Synechococcus elongatus* PCC7942, a model organism for the cyanobacterial circadian mechanisms, the core oscillator proteins KaiA†, KaiB, and KaiC (Ishiura et al. 1998) control the phosphorylation of KaiC diurnally (reviewed by Markson and O'Shea 2009). This oscillation can also be reconstituted *in vitro* when the three Kai proteins are mixed in defined amounts with ATP (Nakajima et al. 2005). The phosphorylation state of KaiC is transmitted further to the response regulator RpaA (rhythm of phycobilisome associated) via two histidine kinases, SasA and CikA (Gutu and O'Shea 2013). The phosphorylated RpaA activates the transcription of more than a hundred genes, including the *kaiBC* operon and σ factor genes (Markson et al. 2013). However, for reasons yet unknown, all cyanobacterial genomes do not contain all of the circadian clock components (Axmann et al. 2014).

Responses to light and oxidative stress are often intertwined, because excess light energy often results in the generation of ROS (Latifi et al. 2009). When high light conditions persist, the light-harvesting phycobilisome antenna and photosystem I (PSI) and PSII are down-regulated (Muramatsu and Hihara 2012) and protective carotenoid pigments accumulate (Schäfer et al. 2006, Sozer et al. 2010). The absorbed energy which cannot be utilised for electron transfer must be dissipated safely. The excitation energy can be dissipated as heat via the light-activated orange carotenoid protein in a process called non-photochemical quenching (Wilson et al. 2006, Kirilovsky and Kerfeld 2012). Sometimes the thermal dissipation can cause alterations in the light-harvesting proteins themselves (Tamary et al. 2012). The chlorophyll a (Chl a) containing protein IsiA is induced by high light, and has been suggested to have a photoprotective function (Havaux et al. 2005). *Synechocystis* has five *hli* (high light inducible) genes, and when four of them were simultaneously inactivated, cells were light-sensitive (Havaux et al. 2003). In addition, the flavodiiron proteins have been shown to have a role in the photoprotection of *Synechocystis* (Helman et al. 2003, Zhang et al. 2009, 2012, Allahverdiyeva et al. 2013, Hakkila et al. 2013, 2014). Furthermore, state transitions balance energy distribution between photosystems (van Thor et al. 1998).

The reaction center protein of PSII, D1, is damaged by light, and constantly degraded and replaced by a newly synthesized polypeptide; this process is known as the PSII repair cycle (Nishiyama et al. 2006, Mulo et al. 2009). In constant high light, repair and protection mechanisms cannot efficiently counteract the light-induced damage to PSII, which leads to lowering of the photosynthetic rate. This phenomenon, known as photoinhibition, is a major mechanism of inactivation of photosynthesis (Vass 2012, Tyystjärvi 2013).

Light-responsive regulation of gene expression is only partially understood. Cyanobacteria might have several genes encoding light receptive phytochrome family proteins. The *Synechocystis* genome contains six hik genes (*hik1, hik3*, *hik24, hik32*, *hik35,* and *hik44*), that are

† The Japanese word *kai* means 'cycle' (Ishiura et al. 1998).

at least somewhat homologous to plant phytochromes (Los et al. 2010). Of these, Hik35 has been identified as cyanobacterial phytochrome Cph1, responding to red and far-red light (Hughes et al. 1997), and Hik32 (renamed CcaS) was shown to be able to distinguish between green and red light (Hirose et al. 2008), whereas inactivation of *hik3* rendered the mutant unable to grow under blue light (Wilde et al. 1997). Hik24 is the ortholog of CikA, one of the histidine kinases involved in the circadian clock of *Synechococcus* (Los et al. 2010). Recently, novel histidine kinases encoded by *pix* genes have emerged as photoreceptors in *Synechocystis*. PixA is involved in the regulation of phototaxis (Narikawa et al. 2011), while PixD is a blue light receptor (Tanaka et al. 2011). Transcription factor Sll1961 is associated with modulation of photosystem stoichiometry (PSI:PSII ratio) during acclimation to high light (Fujimori et al. 2005). The transcription factor PedR regulates several photosynthesis-related genes (Nakamura and Hihara 2006). The autorepressor PrqR is involved in the phototactic response (Kirik et al. 2008).

Hik34, Hik16, Hik41, and Hik33 regulate the expression of 26 genes inducible by H_2O_2, but the Rres associated with each Hik in this stress have not been identified (Kanesaki et al 2007); furthermore, the same Hiks are involved in other stresses as well. The transcription factor PerR regulates a set of H_2O_2 induced genes (Li et al. 2004). The *sigB* and especially *sigD* genes are strongly induced by H_2O_2, suggesting that these σ factors could play an important role in protection against peroxide (Li et al. 2004). Indeed, it was recently shown that when SigB is the only functional group 2 σ factor, *Synechocystis* is vulnerable to oxidative stress because it has higher amounts of ROS than the control strain (Hakkila et al. 2014).

1.3.3. Hyperosmotic stress

Although *Synechocystis* is a freshwater organism, it is regarded as moderately halotolerant *Synechocystis* can survive NaCl concentrations of up to 1.2 M, twice as high as the salinity of ocean water, because it accumulates glucosylglycerol (GG) as its main compatible solute (Reed and Stewart 1985, Reed et al. 1986a). The synthesis of GG starts only after the cells are exposed to NaCl, but non-ionic hyperosmotic shock does not activate synthesis of GG (Hagemann 2011). The response of cyanobacteria to a sudden increase in the salt content of the growth medium can be divided to five phases (Hagemann 2011). During the first milliseconds, the cells rapidly lose water and solutes. A similar reduction in cell volume is also observed when the hyperosmotic shock is induced by the non-ionic solute sorbitol (Shapiguzov et al 2005). The second phase of salt acclimation lasts for several minutes, and is characterised by the passive influx of ions including Na^+ and Cl^- and reuptake of water. The high ion concentration inhibits metabolic processes, particularly photosynthesis but also transcription and translation (Hagemann et al. 1994, Marin et al. 2004). In the third phase, toxic ions are exchanged for non-toxic ones, and synthesis of compatible solutes starts. In the fourth phase, hour(s) after the onset of salt stress, compatible solutes replace non-toxic ions and activation of transcription and translation finally help cells to acclimate to the new conditions. Intensive transcriptomics studies have revealed that 200-300 genes are upregulated and a similar amount of genes are downregulated after salt shock, but only a fraction of these are specific for salt stress (Kanesaki et al. 2002, Marin et al. 2003, 2004). The final phase, a fully acclimated steady state, is usually reached only day(s) after the initial exposure to salt.

In addition to accumulation of compatible solutes, unsaturation of membrane lipids has been shown to protect against salt stress (Sakamoto and Murata 2002). Protective carotenoids and

HSPs accumulate in salt stress (Asadulghani et al. 2004, Nikkinen et al. 2012). Furthermore, Na^+/H^+ antiporters, encoded by *nhaS* genes, have roles in salt stress. The *nhaS3* gene seems to be essential for *Synechocystis* (Elanskaya et al. 2002), and downregulation of *nhaS3* leads to high salt sensitivity in alkaline conditions (Wang et al. 2002). However, the NhaS3 protein is mainly localized to the thylakoid and not to the cytoplasmic membrane, and the protein level is regulated mainly by CO_2 and light rather than salt or hyperosmotic stress (Tsunekawa et al. 2009). *Synechocystis* cells grown in CO_2-enriched medium are much more tolerant to salt stress than cells grown without supplementary CO_2, implying an interaction between salt stress and inorganic carbon uptake (Wang et al. 2002).

The signal transduction mechanisms in salt stress are not known. Remarkably, the same five Hik-Rre systems (Hik33-Rre31, Hik10-Rre3, Hik16-Hik41-Rre17, Hik2-Rre1 and Hik34-Rre1) operate in the perception of both ionic and non-ionic hyperosmotic stress, but the individual target genes might be regulated differently in these stresses (Shoumskaya et al. 2005).

2. AIM OF THE STUDY

The aim of this study was to explore some specific structural features of cyanobacterial RNA polymerase and their roles in the regulation of gene expression in acclimation to environmental stress. I continued the work that others had started on group 2 σ factors of *Synechocystis,* measuring physiological responses to environmental stress. As a part of this, I constructed a homology model of cyanobacterial RNA polymerase holoenzyme and noticed that the tiny ω subunit was quite unique in cyanobacteria. Since virtually nothing was known previously about this subunit in cyanobacteria, I also decided to study its role further by combining reverse genetic methods with physiological characterization and transcriptional profiling.

3. METHODOLOGY

3.1. Strains and growth measurements

3.1.1. Control strain and standard conditions

A descendant of the glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used as the control strain (CS). The standard growth conditions referred to are liquid BG-11 medium (Rippka et al. 1979) supplemented with 20 mM Hepes, pH 7.5, a continuous photosynthetic photon flux density (PPFD) of 40 µmol m^{-2} s^{-1}, +32°C, and ambient CO_2. Liquid cultures were grown as 30-ml batches in 100-ml Erlenmeyer flasks with shaking at 90 rpm. Cultures were maintained for short time on BG-11 agar plates in standard conditions, and glycerol preps at -80 °C were used for long-time storage.

3.1.2. Construction of inactivation and complementation strains

The inactivation strains were constructed by interrupting the genes of interest with antibiotic resistance cassettes. The details for the single and double σ factor inactivation strains are described in Paper I and for the ΔrpoZ strain in Paper III. Briefly, the gene of interest was amplified with PCR and inserted into the cloning vector pUC19. Then the fragment containing the antibiotic resistance cassette was introduced to a suitable restriction site inside the gene to interrupt it. The construct was used to transform either CS cells to obtain single mutants, or single mutant strains, to obtain double mutants. The transformants were selected and the mutant cultures were maintained on BG-11 agar plates containing the corresponding antibiotic(s). *Synechocystis* has multiple copies of genome per cell, and therefore the selection procedure takes a few weeks. Complete segregation of strains was confirmed with PCR.

The complementation strain ΔrpoZ+rpoZ was constructed using the vector pAII (obtained from Dr. Marion Eisenhut), which has the up- and downstream regions of the *psbA2* gene for homologous recombination and the streptomycin/spectinomycin resistance cassette for selection. The *rpoZ* gene was amplified from *Synechocystis* genomic DNA and inserted between the NdeI and KpnI sites in the vector. This construct was then used to transform ΔrpoZ cells, which resulted in replacement of the *psbA2* coding region with the *rpoZ* coding region and the antibiotic cassette. The *psbA2* promoter is a strong promoter, and more ω subunit was detected in ΔrpoZ+rpoZ than in CS (Paper III).

For the ΔsigBCDE inactivation strain, the *sigC* gene (*sll0184*) was amplified from *Synechocystis* genomic DNA with PCR using primers 5'-AATTATGAGCTCCTAACCCAAATTTTCGTAAT-3' and 5'-TATAAACTGCAGATGACTAAACCAAGCAACGA-3'. The PCR product was digested with PstI and SacI and cloned into PstI and SacI digested pUC19. The pUC19-sigC construct was digested with AsiSI and a nourseothricin resistance cassette (obtained from Dr. Luis Lopez-Maury) was inserted to the digestion site. The construct was then used to transform ΔsigBDE cells (Pollari et al. 2011), and complete segregation was achieved within a few weeks (see Fig. 4A, p. 30).

3.1.3. Growth measurements

No antibiotics were supplemented in the liquid BG-11 medium when growth experiments were done. To measure changes in cell density, optical density of liquid cell culture at 730 nm (OD_{730}; A_{730} in Paper I) was measured. When OD_{730} is 0.1, the cell density is circa 3.6 x 10^6 ml^{-1}

(Tuominen et al. 2008). Growth was measured under standard conditions (Papers I and III), at PPFD 80 µmol m^{-2} s^{-1} (Paper I) and under high (Paper I) or low CO_2 atmosphere (Paper II). Growth was followed also at 43 °C, PPFD 35 µmol m^{-2} s^{-1} in air at pH 6.7, pH 7.5 and pH 8.3 (Paper II). In Paper IV, growth was measured also at 38 °C (otherwise standard conditions) and at 40 °C either at normal pH (7.5) or in alkaline conditions at pH 8.3.

Oxidative stress was induced by supplementing the growth medium with 0.1 µM methyl viologen (Paper I). Hyperosmotic stress was induced both ionically and non-ionically by supplementing the growth medium with 0.7 M NaCl or 0.5 M sorbitol, respectively (Paper I). In some experiments, 5 mM glucose, 0.1 mM cyclic adenosine 3',5'-monophosphate (cAMP), 20 mM 2-oxoglutarate, 20 mM pyruvate or 20 mM succinate was added to the cultures as indicated (Paper II).

3.2. Physiological characterization

3.2.1. Oxygen evolution

The light saturated rate of photosynthetic oxygen evolution was measured in vivo from cells grown under standard growth conditions (Papers I-IV), at 40 °C (Paper IV) or at 43 °C (Paper II). Oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech, UK) at 32 °C in the presence of 10 mM $NaHCO_3$.

The 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).

3.2.2. Analysis of pigments, α-tocopherol, lipids and cAMP

In vivo absorption spectra were measured from 400 nm to 800 nm. The carotenoid peak at 678 nm, the phycobilin peak at 625 nm, and the Chl *a* peak at 678 nm were used for pigment ratio analysis (Papers III and IV). The Chl *a* content of intact cells was measured after methanol extraction. Carotenoids and α-tocopherol were extracted with methanol and measured with HPLC (Paper III). Lipids were extracted from intact cells, and lipid classes were separated and quantified (Paper IV). cAMP was detected as described in Paper II.

3.2.3. Biophysical measurements

To estimate PSI:PSII ratio, 77 K fluorescence emission spectra were measured using blue light excitation and the spectra were normalized to the PSI emission peak value at 723 nm (Paper III).

Relaxation of Chl *a* fluorescence yield after a single-turnover flash was measured with and without DCMU to probe possible changes in PSII electron transfer reactions upon heat treatment (Paper II).

3.3. Protein analysis

3.3.1. Computational methods

The amino acid sequences of *Synechocystis* RNAP subunits were retrieved from CyanoBase (www.kazusa.or.jp/cyanobase) and aligned with the sequences of homologous subunits from *Thermus thermophilus* using the MALIGN algorithm in the Bodil package (Lehtonen et al. 2004) and manual adjusting. Secondary structure predictions were made with PredictProtein (Rost and Liu 2003) and three-dimensional models were constructed with MODELLER (Sali and Blundell 1993) using the 2.6 Å X-ray crystallography structure of *Thermus thermophilus* RNAP (Artsimovitch et al. 2005) as a template. The best model was chosen after visual examination and quality assessed with PROCHECK (Laskowski et al. 1993). For visualization of the models and sequence alignments, a variety of programs including Bodil, PyMol (DeLano Scientific, Inc.) and Jmol (www.jmol.org) have been used.

Further sequence analysis was undertaken with the tools available from NCBI. Versions of the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997) were used to find closely and more distantly related sequences of RNAP subunits. Settings were adjusted according to the query sequence, e.g. for the ω subunit, the BLOSUM-80 matrix was used instead of the default BLOSUM-65 matrix to better suit the short sequence. From the BLAST result alignments, distance trees were generated with default settings.

3.3.2. Immunoblotting

After the treatments described in the papers, cells were rapidly cooled and collected by centrifugation at 10 000 g for 5 min at 4 °C. Cells were broken in ice cold STNE buffer (0.4 M sucrose, 10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 20 mM Na-EDTA) by vortexing them with glass beads (150-212 mm, Sigma). Glass beads and unbroken cells were removed by centrifugation and the supernatant was used as a total-protein sample. Membranes were collected by additional centrifugation at 19500 g for 15 min at 4 °C, and the supernatant was collected and recentrifuged for 15 min at 19500 g at 4 °C to get the soluble protein fraction. To separate RNAP boud and free SigA, size fractionation of soluble proteins was performed as described in Paper III. The concentrations of proteins were measured with the Lowry protein assay. Proteins were solubilized for 10 min at 75°C and separated by using 10% NEXT GEL SDS-PAGE (Amresco). After electrophoresis, separated proteins were transferred to Immobilon-P membranes (Millipore). Specific proteins were detected with polyclonal antibodies received as generous gifts from professor Eva-Mari Aro (antibodies against Flv2, Flv3, NdhJ, NdhK and NdhD3), or purchased from Agrisera (custom antibodies against RNAP subunits α, β, ω, and SigA, and commercial antibodies against AtpE, RbcL, allophycocyanin, phycocyanin, PsaB, CP43 and HspA) or from Innovagen (custom SigC antibody). The goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Zymed) and the CDP Star chemiluminescence kit (New England Biolabs) were used for detection.

3.4. RNA analysis

3.4.1. Isolation of total RNA and Northern blotting

Total RNA was isolated from samples with hot acidic phenol extraction as described previously (Tyystjärvi et al. 2001). RNA concentration was measured with NanoDrop spectrophotometer.

For visualisation, RNAs were separated in 1.2 % agarose gel stained with ethidium bromide. For Northern blotting (Paper I), 10 μg of RNA was denaturated with glyoxal and separated on 1.2% agarose gels in phosphate buffer and subsequently transferred to Hybond-N membranes (Amersham Biosciences) according to standard procedures (Sambrook and Russell, 2001). The gene-specific probes were amplified by PCR from *Synechocystis* genomic DNA. The probes were labelled with digoxigenin-dUTP, hybridized, and detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the instruction manual of the kit.

3.4.2. Operon analysis

Putative terminators between the *rpoZ* and *sll1532* genes were searched from the WebGeSTer database (Mitra et al. 2011) (http://pallab.serc.iisc.ernet.in/gester), but none were found. To experimentally determine if the *rpoZ* and *sll1532* genes belong to the same operon, DNA-free RNA was extracted from CS cells (Paper III). Then, reverse transcription (RT) was performed with 900 ng of RNA using SuperScript III kit (Invitrogen) according to the manufacturer's instructions using gene-specific primers for *rpoZ* and *sll1532*. For control reactions, no reverse transcriptase was added. 2 μl of each RT reaction was used as a template in the PCR reactions, and 5 μl of the PCR reaction was loaded on 1% agarose gel stained with ethidium bromide.

3.4.3. Transcriptional profiling

For gene expression analysis (Papers III and IV), CS and ΔrpoZ cells were grown in standard conditions for three days (OD_{730} 0.8-1.0) after which half of the samples were collected and the other half was grown at 40 °C for another 24 h before collecting. Total RNA was extracted from the cells with the hot-phenol method and purified with the RNEasy Mini kit (Qiagen) including the DNase treatment steps. Samples were taken to Finnish Microarray and sequencing centre (FMSC; Turku, Finland), where the quality of RNA was checked. The platform chosen for expression analysis was Agilent 8 × 15 K custom DNA array for *Synechocystis* (Eisenhut et al. 2007). RNA labelling, hybridizations, and data collection were performed at the FMSC using the One-Color Microarray-Based Prokaryote Analysis method (Agilent), which uses the Stratagene Fairplay III Microarray Labeling Kit (Agilent) for RNA labeling. Images of the DNA microarray were acquired using a G2565CA Scanner (Agilent). Data collection was done with Agilent Feature Extraction Software (version 10.7.3) using protocol GE1_107_Sep09. The expression data were analysed with Chipster (Kallio et al. 2011). If not otherwise mentioned, the processed signal (treated for local and global background noise) was used for analysis. The values from different probes of the same gene were averaged before normalisation. The data were normalized with the quantile method. Differentially expressed genes were identified with the t-test. Annotation to functional categories was done with information available from CyanoBase. For visualisation of the microarray results, Venn diagrams were drawn with a web-based tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

4. RESULTS AND DISCUSSION

4.1. Structural features of cyanobacterial RNA polymerase

4.1.1. Comparative modelling reveals architectural and sequence conservation of RNAP

When a crystal structure cannot be obtained, comparative modelling can provide insight into the structure and function of the protein. In the case of bacterial RNAP, crystallization has been difficult because of the size and flexibility of the enzyme. However, crystal structures have been obtained from *Thermus aquaticus* at 3.3 Å (Zhang et al. 1999), *T. thermophilus* at 2.6 Å (Vassylyev et al. 2002), and recently even from *E. coli* at 3.7 Å (Murakami 2013). The resolved structures can be used as templates for homologous modelling if the amino acid sequence is sufficiently similar with the sequence of the protein to be modelled because similar sequences tend to fold into similar structures. Generally, if the sequences share less than 20% identical amino acids, the reliability of the model will be poor. Table 1 (Supplemental Table S1 from Paper I) shows a comparison of RNAP subunits from *T. thermophilus* and *Synechocystis*.

Table 1. Comparison of RNA polymerase subunits in *Synechocystis* and *Thermus thermophilus*.

subunit	chain ID 2A6E	gene name (locus tag) *Synechocystis*	number of residues / included residues		model/ template % identity
			Thermus thermophilus / 2A6E	*Synechocystis* / models	
α	A / B	*rpoA* (sll1818)	315 / 1-229	314 / 1-230	42.79
β	C	*rpoB* (sll1787)	1119 / 1-1119	1102 / 1-1082	50.18
β'	D	*rpoC2* (sll1789)	1524 / 2-251, 364-1505	1317 / 1-340, 976-1257	49.28
γ		*rpoC1* (slr1265)		626 / 1-626	
ω	E	*rpoZ* (ssl2982)	96 / 2-96	76 / -	19.74
σ	F	*sigA* (slr0653)	423 / 74-378, 384-423	425 / 66-425	48.41
		sigB (sll0306)		345 / 30-345	41.77
		sigC (sll0184)		404 / 44-404	39.71
		sigD (sll2012)		318 / 1-318	39.91
		sigE (sll1689)		369 / 52-369	39.62

The sequence identity for most subunits was high enough to allow reliable modelling, and thus a model of cyanobacterial RNAP (Fig. 1 in Paper I) could be constructed based on the *T. thermophilus* structure at 2.6 Å (Artsimovitch et al. 2005) as a template. Alignment of *Synechocystis* γ and β' subunits with *T. thermophilus* β' indicated that there are conserved amino acids on both sides of the cyanobacterial split, and only six additional amino acids that could not be found in *Synechocystis* were present in the template in this area. The split site is located on the surface of the complex quite near the predicted location of the ω subunit. Regions that are not present in the template structure cannot be modelled, as was the case for the large cyanobacterial insertion in the β' subunit. This insertion spans over 600 amino acids; for comparison, the σ factor SigA is 425 amino acids. From the model it can be seen that the insertion site is on the edge of the other claw of RNAP and can thus be easily accommodated (Paper I). The *E. coli* RNAP also harbours a large insertion of about 300 amino acids at the same site, but its electron density could not be fixed which suggests that it is highly flexible (Murakami 2013; see Fig. 1).

It was observed that all group 2 σ factors were very similar with each other and SigA (Paper I). A variable domain, σNCD, was not as conserved as other parts of the σ factor. The σNCD was either as long as in *T. thermophilus* (SigA and SigC) or shorter (SigB, SigD, and SigE), and the alignment was adjusted so that this domain could be included in the model (Paper I). It should be kept in mind that the folds of this domain can deviate quite a lot from the template. Situated near the σ2.4 domain which recognizes the -10 promoter element, σNCD might even have a role in binding some yet unknown transcription factors. SigB and SigD are the most similar in terms of amino acid sequence, also in the σNCD region (Paper I).

The cyanobacterial ω subunit is smaller than the ω subunit of *T. thermophilus* or *E. coli,* but has additional amino acids in the N terminus, whereas the C terminus is truncated (Fig. 2). Only the most conserved amino acids could be aligned, which was not enough to allow reliable modelling and the ω subunit was discarded from the final model of cyanobacterial RNAP. In a phylogenetic tree, the cyanobacterial ω polypeptides form a tight, monophyletic clade with ω subunits of chloroplast RNAP in red algae, glaucocystophyta and some green algae (Fig. 3‡, Supplemental figure S1 from Paper III). Interestingly, despite intensive searching, I have not been able to find close homologues to the ω subunit in higher plant genomes, although when search criteria are unspecific enough, the eukaryotic-type subunits appear in the results.

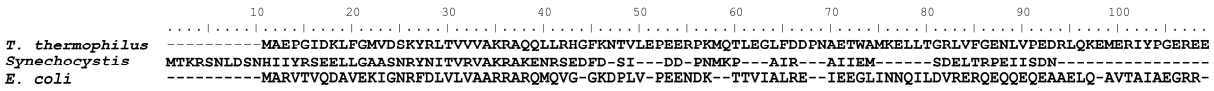

Figure 2. Amino acid sequence alignments of ω subunits from *T. thermophilus, Synechocystis* and *E. coli*.

‡ Figure 3 (next page). Phylogenetic tree of ω subunits. The NCBI database of non-redundant protein sequences was queried with the sequence of the ω subunit of *Synechocystis* sp. PCC 6803 (RefSeq ID: NP_441766.1) with DELTA-BLAST (Boratyn et al. 2012) using BLOSUM80 matrix. From the result list, only bacterial and plastid sequences annotated as the ω subunit with E-values better than the threshold 0.005 were selected and a distance tree was generated with default settings. Coloured triangles depict closely clustered sequences from different strains, the number of strains is indicated in brackets, and coloured spheres denote single strains.

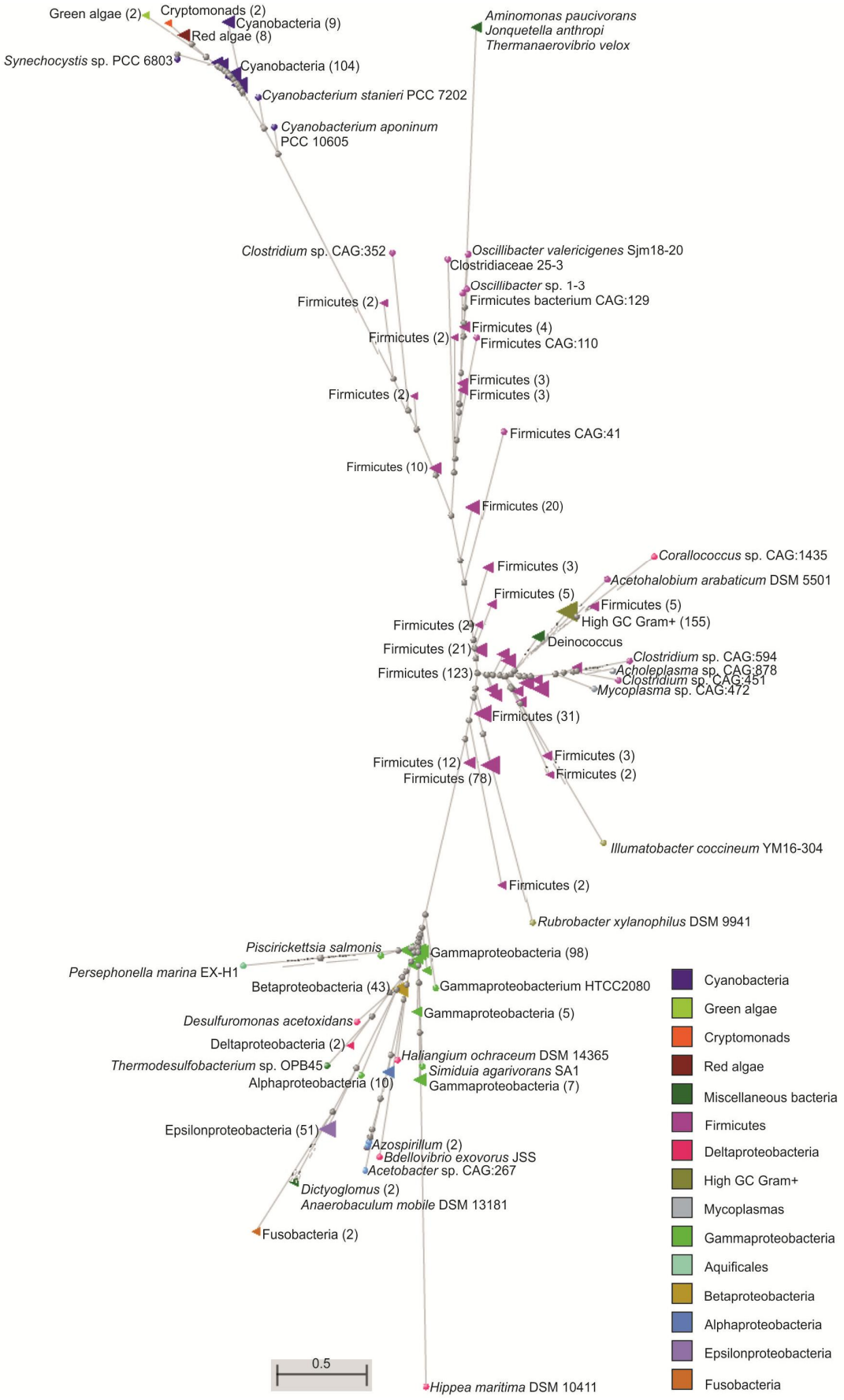

Figure 3. Phylogenetic tree of ω subunits. (Details on previous page.)

4.2. Group 2 sigma factors and environmental acclimation in cyanobacteria

4.2.1. Group 2 sigma factors are nonessential for *Synechocystis* in standard conditions

In Paper I, a complete series of *Synechocystis* group 2 σ factor single and double mutants was analysed. It was observed that in liquid cultures, all mutant strains grew like CS both in standard conditions and in 3% CO_2 (Paper I). Also photosynthetic activity and PSII electron transport capacity were similar in CS and all mutant strains (Paper I). In optimum conditions there seems to be no need for group 2 σ factors for *Synechocystis,* as several groups have succeeded in inactivating one (Muro-Pastor et al. 2001, Imamura et al. 2003a, Lemeille et al. 2005a), two (Summerfield and Sherman 2007), or three group 2 σ factors simultaneously (Summerfield and Sherman 2007). Furthermore, a complete set of triple inactivation strains was described recently (Pollari et al. 2011). Even the completely segregated quadruple mutant strain ΔsigBCDE, in which all group 2 σ factors have been simultaneously inactivated, has a growth rate similar to CS (Fig. 4). This confirms that SigA alone is sufficient to sustain normal growth.

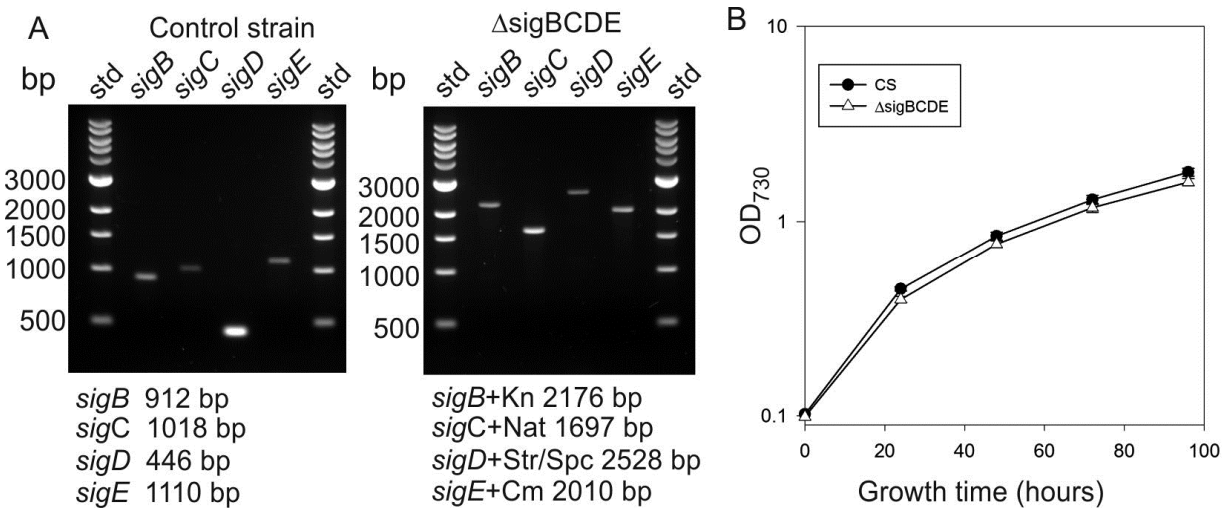

Figure 4. A. The *sig* genes were amplified with PCR from genomic DNA extracted from the control and ΔsigBCDE strains, and the products were separated on 1% agarose gel stained with ethidium bromide. The expected fragment sizes are given below each picture. Kn, kanamycin cassette; Nat, nourseothricin cassette; Str/Spc, streptomycin/spectinomycin cassette; Cm, chloramphenicol cassette. B. Growth of the control (CS, black circles) and ΔsigBCDE strains (white triangles) in standard conditions. The values are averages from six biological replicates and error bars, shown when larger than the symbol, denote standard error.

It is interesting to note that while the amount of the SigA protein remains relatively constant through different growth phases in normal conditions (Imamura et al. 2003b), the *sigA* transcripts diminished when cells were exposed to different stress conditions (Tuominen et al. 2003, Paper IV). It was reported that the absence of either SigB, SigD, or SigE reduced *sigA* transcripts by at least 50%, whereas without SigC, the amount of *sigA* transcripts rises to 150% of that detected in CS (Matsui et al. 2007). Furthermore, the concentration of the α subunit was found to be about 4.7 times higher than the concentration of SigA (Imamura et al. 2003b). This would mean that less than 50% of the RNAP core could bind SigA at any time; however, the

presence of the σ factor is obligatory only for transcription initiation. Inactivation of SigA seems impossible in *Synechocystis* (Imamura et al. 2003b).

4.2.2. SigB is involved in many stress responses

The amount of SigB protein remains constantly low during growth in normal conditions (Imamura et al. 2003b). The transcription of *sigB* was rapidly but only transiently upregulated in salt stress (Paper I, Kanesaki et al. 2002). In sorbitol-induced hyperosmotic shock *sigB* was rapidly induced, and the transcript level stayed high longer than in salt-induced stress (Paper I). Accordingly, the ΔsigB strain grew slowly in both salt- and sorbitol-induced stresses, and also all σ factor double mutants with an inactivated *sigB* gene grew slowly (Paper I), confirming the importance of SigB for acclimation to hyperosmotic stress. Recent results with the triple mutant strain ΔsigCDE show that when SigB is the only functional group 2 σ factor, the cells are slightly more tolerant against salt stress than CS cells (Nikkinen et al. 2012, Tyystjärvi et al. 2013). An apparent reason for poor growth of ΔsigB in high salt was underexpression of the *ggpS* gene, encoding glucosylglycerol phosphate synthase (Nikkinen et al. 2012). GgpS is a key enzyme in the synthesis of GG, and expression of *ggpS* is induced by salt shock (Marin et al. 2002). The expression of *ggpS* is increased in the ΔsigCDE strain, but impaired in the ΔsigB strain, compared to the control strain (Nikkinen et al. 2012, Tyystjärvi et al 2013). The growth of the ΔsigB strain in high salt could be restored by adding the compatible solute trehalose, which can be taken up by *Synechocystis* (Nikkinen et al. 2012). The group 3 σ factor SigF has been suggested to control the production of GG (Marin et al. 2002). However, the promoter of the *ggpS* gene does not contain a typical consensus sequence of SigF (Hagemann 2011) and it remains to be solved whether the effect of SigF is direct or indirect. In addition, *hspA* and protective carotenoids were downregulated in ΔsigB and upregulated in ΔsigCDE compared to the CS in high salt conditions (Nikkinen et al. 2012).

SigB has a role in light-dark shifts, although the response seems to be complicated. Tuominen et al. (2003) reported very rapid and short upregulation of *sigB* transcripts when cells were transferred from dark to light, and Imamura et al. (2003a) detected decrease of SigB protein 1 h after transfer from dark to light, whereas 1 h after transition from light to darkness, the SigB protein level was higher than in the light (Imamura et al. 2003a). Inactivation of *sigB* resulted in upregulation of many transcripts after cells were transferred to darkness (Summerfield and Sherman 2007).

The *sigB* gene has also been found to be inducible by increase in light intensity and by UV radiation (Huang et al. 2002), and it was observed that the ΔsigB mutant grew slightly more slowly on plates than CS in high light (Paper I). Interestingly, when SigB is the only functional group 2 σ factor, the cells were more resistant against the damaging reaction of photoinhibition than cells of CS (Hakkila et al. 2013) but otherwise suffered from oxidative stress (Hakkila et al. 2014).

Transcription of *sigB* is induced rapidly but transiently in heat shock (Imamura et al. 2003b, Tuominen et al. 2003, 2006, Singh et al. 2006). SigB positively regulates the transcription of the *hspA* gene, and survival of ΔsigB cells was lower than that of CS after a short lethal heat treatment (Tuominen et al. 2006). Furthermore, ΔsigB cells were not able to acquire thermal tolerance as efficiently as CS, although growth in mild heat stress was not impaired (Tuominen

et al. 2006). In addition to HspA, SigB may also control the expression of other heat responsive genes and chaperones (Singh et al. 2006).

In *Synechocystis,* the amount of SigB protein is doubled upon nitrogen deficiency and it was suggested to participate in the regulation of some NtcA-dependent genes in the logarithmic growth phase (Imamura et al. 2006). Nitrogen depletion upregulated SigB-type factors in other cyanobacteria as well; the SigB-type σ factor in *Synechococcus* sp. PCC 7002 (Caslake et al. 1997) and two out of four SigB-type σ factors in *Synechococcus* sp. PCC 7120 (Brahamsha and Haselkorn 1992, Khudyakov and Golden 2001) are upregulated in nitrogen deprivation.

4.2.3. SigC is required for sustained growth in high temperature

The expression of *sigC* is not induced by heat (Tuominen et al. 2003, Imamura et al. 2003b). However, the inactivation of *sigC* leads to a heat-sensitive phenotype, rendering the mutant unable to grow at 43 °C (Tuominen et al. 2008). Microarray results indicated that heat shock genes are similarly regulated in ΔsigC and CS, while many genes related to carbon concentrating systems were upregulated in the ΔsigC strain compared to CS after a 24 h treatment at 43 °C (Tuominen et al. 2008). When the CO_2 level in the growth chamber was raised to 3%, the growth of the ΔsigC mutant at 43 °C was partially restored (Tuominen et al. 2008). In higher temperatures, less inorganic carbon (C_i) is available in the growth medium, and in Paper II, this phenomenon was further investigated. It was observed that lowering the CO_2 level at normal growth temperature reduced the growth of ΔsigC more than the growth of CS. The form and total amount of available C_i can be changed by pH adjustment. Lowering the pH to 6.7 decreased the available C_i and reduced the growth of both ΔsigC and CS at 43 °C. On the contrary, when the pH of the medium was elevated to 8.3, the growth of the ΔsigC mutant at 43 °C was almost completely restored and CS grew faster than in the standard pH of 7.5 (Paper II). Interestingly, in alkaline conditions the amount of the SigC protein in CS stayed high throughout the growth measurement, whereas in acidic conditions the amount of SigC decayed faster than at the normal pH of 7.5 (Paper II).

Inactivating the *spkD* gene (*sll0776*) encoding a serine/threonine kinase renders *Synechocystis* unable to grow under normal air unless the growth medium is supplemented with HCO_3^- or with tricarboxylic acid cycle intermediates 2-oxoglutarate or succinate (Laurent et al., 2008). However, addition of organic carbon as glucose, pyruvate, succinate or 2-oxoglutarate did not restore growth of the ΔsigC strain at 43 °C (Paper II). The pH-dependent changes in photosynthetic activity of ΔsigC and CS were reasons for pH-dependent growth at 43 °C (Paper II). pH-dependent growth has been reported in some other *Synechocystis* mutants as well. Modification or inactivation of lumenal proteins of PSII (Eaton-Rye et al. 2003, Summerfield et al. 2005, 2013) or inactivation of the NdhB subunit of the NAD(P)H dehydrogenase complex (Zhang et al. 2004) renders the mutants unable to grow in neutral pH, and these strains need an alkaline environment to survive. Furthermore, ΔNdhD3/NdhD4, with an inactivated CO_2 uptake system, is able to grow at alkaline pH but not at neutral or acidic pH (Zhang et al. 2004).

The amount of cAMP was low in ΔsigC compared to CS in standard conditions, and stayed at the same level during the heat treatment, suggesting that the SigC factor is involved in the regulation of the cAMP content, but the growth of ΔsigC at 43 °C was only slightly improved when cAMP was added to the medium (Paper II). cAMP has been associated mainly with cell motility in *Synechocystis* (e.g. Bhaya et al. 2006); however, our control strain is non-motile by

default. Predicted targets for the cAMP binding transcription factor CRP include genes involved in photosynthesis, carbon metabolism, transport and transcription (Xu and Su 2009). It was also shown that bicarbonate regulates the activity of the adenyl cyclase which generates cAMP in *Synechocystis* (Masuda and Ono 2005); however, more experiments are needed to elucidate the role of this secondary messenger in full.

Cell viability of ΔsigC in stationary phase was reported to be lower than that of CS (Asayama et al. 2004), but growth curves of ΔsigC and CS were similar in our standard conditions (Paper I). A SigC-type σ factor has been suggested to control gene expression in stationary phase in *Synechococcus* sp. 7002 as well (Gruber and Bryant 1998). SigC has been suggested to regulate nitrogen metabolism during stationary phase in *Synechocystis* (Asayama et al. 2004, Imamura et al. 2006), and a SigC-type σ factor has been suggested to participate in the control of nitrogen metabolism also in *Anabaena* sp. 7120 (Aldea et al. 2007).

ΔsigC showed reduced growth both in salt- and sorbitol-induced hyperosmotic stress (Paper I). Interestingly, growth in high salt conditions was very slow also when SigC was the only functional group 2 σ factor; this was attributed to the decreased induction of *ggpS* and *hpsA* genes in the ΔsigBDE strain (Tyystjärvi et al. 2013). These results indicate that SigC is involved in acclimation to hyperosmotic stress at many levels, but more detailed studies are still needed.

4.2.4. SigD mediates responses to light and oxidative stress

The single and all double mutant strains with an inactivated *sigD* gene showed reduced growth on plates when growth light was doubled to PPFD 80 μmol m^{-2} s^{-1}, and ΔsigBD was virtually unable to grow at all (Paper I). Previous studies have shown that in *Synechocystis,* expression of the SigD factor is upregulated both at transcript (Hihara et al. 2001, Huang et al. 2002, Tuominen et al. 2003) and protein level (Imamura et al. 2003b) in high light, and the expression of the *sigD* gene is also induced by transferring cells from dark to light (Gill et al. 2002, Imamura et al. 2003a) or by treating cells with UV-B radiation (Huang et al. 2002). In *Synechococcus elongatus* PCC 7942 the SigD homolog, RpoD3, has been shown to be upregulated upon high light treatment, and the RpoD3 knockout strain was found to be sensitive to high light (Seki et al. 2007). The high-light induction of *rpoD3* was shown to be under the control of the transcriptional repressor protein RpaB (Hanaoka and Tanaka 2008).

The *sigD* gene is also highly induced by H_2O_2 (Li et al. 2004), indicating that SigD might be involved in high-light associated oxidative stress responses. Indeed, ΔsigD grew more slowly than CS in the presence of methyl viologen that mediates the production of superoxide (Paper I). Furthermore, when *Synechocystis* cells were treated with another oxidative stress causing agent, DCMU, the amounts of both *sigD* transcripts (Hihara et al. 2003) and SigD protein were increased (Imamura et al. 2003a).

The growth of the ΔsigD strain was only slightly impaired in salt- or sorbitol-induced hyperosmotic stress (Paper I), suggesting that SigD might have only a minor contribution in the acclimation to these stress conditions. This was unexpected, as the *sigD* gene is upregulated in both stress conditions (Shoumskaya et al. 2005). It should be noted that the triple mutant ΔsigBCE, where SigD is the only functional group 2 σ factor, grew surprisingly well in high salt conditions (Tyystjärvi et al. 2013). This might be due to the increased expression of *nhaS3,* encoding a Na+/H+ antiporter (Tyystjärvi et al. 2013).

SigB and SigD are the most similar group 2 σ factors in *Synechocystis,* both according to the structural model and sequence alignment (Paper I). The ΔsigBD double mutant was more sensitive to both high light and low light than the ΔsigB or ΔsigD single mutants (Paper I). It was later shown that while photosynthetic light reactions are fully functional, the adjustment of the light-harvesting system and PSII repair cycle are impaired in the ΔsigBD strain (Pollari et al. 2009). Previously, it had also been reported that ΔsigBD is also more sensitive to heat stress than the ΔsigB or ΔsigD single mutants (Tuominen et al. 2006). Clearly, there seems to be a certain level of redundancy in the functions of SigB and SigD, although they have distinct roles in certain stress conditions.

4.2.4. SigE has an additional role in initial acclimation to salt stress

SigE participates in acclimation to salt stress. The ΔsigE strain grew slowly during the first three days in salt-supplemented medium, but after that the growth rate increased (Paper I). Recent results suggest that full salt acclimation can be achieved, but is slowly initiated when only SigE is present (Tyystjärvi et al. 2013). However, sorbitol-induced hyperosmotic stress had only a minor effect on the growth of the ΔsigE strain (Paper I).

The promoter region of the *sigE* gene (originally named *rpoD-V*) of *Synechocystis* was found to contain a putative binding sequence for the nitrogen-deprivation activated transcription factor NtcA, and expression of *sigE* was shown to be induced by nitrogen limitation (Muro-Pastor et al. 2001). Cell viability during prolonged nitrogen starvation decreased without SigE (Muro-Pastor et al. 2001). A SigE-type σ factor was shown to be upregulated in nitrogen deficiency in *Synechococcus* sp. PCC 7002 as well (Caslake et al. 1997). It was also shown that in *Synechocystis,* SigE upregulates the expression of genes related to sugar catabolism (Osanai et al. 2005, 2006, 2011, Imamura et al. 2006). In light, ChlH (H subunit of Mg-chelatase) acts as an anti-sigma factor by binding SigE (Osanai et al. 2009).

4.3. The omega subunit and its role in gene expression

There was no information on the role of the cyanobacterial ω subunit previously. This prompted me to construct an inactivation strain and investigate how the absence of the ω subunit affects the basic functions of cyanobacterial cell.

4.3.1. Construction of the ΔrpoZ and ΔrpoZ+rpoZ strains

To produce the inactivation strain, the first step was to amplify the *rpoZ* gene by PCR. The *rpoZ* gene is only 231 bp, so some of the flanking sequences needed to be included in the PCR product to guarantee that homologous recombination would work efficiently in the transformation phase. The first attempts with PCR were unsuccessful, and eventually it was decided that this region would be sequenced. Sanger sequencing of the region revealed that our control strain has lost the transposase *sll1635* which is located only about 30 bp upstream of the start codon of *rpoZ* in the Kazusa strain (Supplemental figure S2, Paper III). Reports from other groups who had sequenced their control strains appeared in the same time (Kanesaki et al. 2012, Trautmann et al. 2012), indicating that the Kazusa reference genome is the only one in which the transposase is observed (Supplemental figure S3, Paper III). After the correct sequence was obtained, PCR and mutant construction proceeded without problems.

On the other side, 31 bp downstream from the *rpoZ* gene and on the same strand, lies *sll1532,* which encodes a hypothetical protein. RT-PCR confirmed that *rpoZ* and *sll1532* are transcribed as an operon (Paper III). Homologues to *sll1532* are only found in cyanobacteria, and they are almost always located immediately downstream of *rpoZ,* but nothing is known about the function of this protein. To confirm that any observed changes in the ΔrpoZ strain could be attributed to the absence of the ω subunit, a complementation strain, ΔrpoZ+rpoZ, was constructed (Paper III). In the complementation strain, the *rpoZ* gene was re-introduced into the genome of the ΔrpoZ strain by replacing the *psbA2* coding sequence with the *rpoZ* coding sequence followed by a selective antibiotic marker. The *psbA2* gene encodes the central D1 subunit of PSII and has a very strong promoter. The nearly identical *psbA3* gene, which normally contributes less than 10% of *psbA* transcripts, is highly up-regulated when *psbA2* is inactivated (Mohamed et al. 1993). Thus, the *rpoZ* coding sequence was combined with the strong promoter of *psbA2,* and Western blot analysis showed that the ΔrpoZ+rpoZ strain in fact overproduced the ω subunit compared to CS (Paper III).

4.3.2. Inactivation of *rpoZ* leads to a shift in transcription pattern

The ΔrpoZ inactivation strain grew well in our standard conditions (Paper III). To estimate changes in RNAP activity, the transcriptomes of CS and ΔrpoZ were monitored with DNA microarray. In standard conditions, 187 genes were at least two-fold up-regulated and 212 genes were down-regulated to half or less in the ΔrpoZ strain compared to CS (Paper III). Up-regulated genes included mainly genes encoding hypothetical or unknown proteins, while many photosynthesis and transport related genes were down-regulated (Paper III). Also the *rpoZ-sll1532* operon was down-regulated in the ΔrpoZ strain.

Most of the down-regulated genes in ΔrpoZ show high expression in CS, such as photosynthesis genes, whereas genes that were up-regulated in ΔrpoZ showed only low or moderate expression in CS (Paper III). Highly expressed genes can be assumed to be mainly transcribed by SigA, and indeed the expression of SigA was also down-regulated both at the RNA and protein levels in ΔrpoZ. Furthermore, it was observed that less SigA was bound to RNAP core in ΔrpoZ than in CS, and on the other hand, more free SigA was found in ΔrpoZ than in the CS while the amount of RNAP core remained unchanged (Paper III). This suggests that more RNAP core is available to bind group 2 and group 3 σ factors, and the finding that many genes that normally are not highly expressed are upregulated in ΔrpoZ supports this idea. For example, *hspA,* which is regulated by SigB, and *pil* genes, regulated by the group 3 σ factor SigF, were among the upregulated genes.

In *E. coli,* normally over 80% of the RNAP in a cell contains the ω subunit, whereas in *Bacillus subtilis,* the ω subunit is only found in about half of mature RNAP complexes (Doherty et al. 2010). RNAP can be constituted *in vitro* without ω (Heil and Zillig 1970), but this construction is unable to respond to the stringent response alarmone molecules guanosine tetra- and pentaphosphate, collectively referred to as (p)ppGpp (Vrentas et al. 2005). In *E. coli,* the *rpoZ* gene belongs to the same operon as *spoT,* which encodes a pyrophosphatase that controls the amount of (p)ppGpp, and inactivation of *rpoZ* has been shown to reduce expression of the downstream *spoT* gene, complicating the interpretation of mutant phenotypes (Igarashi et al. 1989, Gentry et al. 1991, Xiao et al. 1991). Binding of σ^S instead of σ^{70} to RNAP core is favoured in an ω-less strain (Geertz et al. 2011). Recently, it was shown that (p)ppGpp binds at the

interface of ω and β' and the N-terminal part of ω is involved in (p)ppGpp-mediated regulation of transcription in *E. coli* (Ross et al. 2013, Mechold et al. 2013, Zuo et al. 2013). Interestingly, the binding of alternative σ factors σ^S and σ^{32} to RNAP core was reduced in (p)ppGpp-depleted cells (Jishage et al. 2002). Also 6S RNA has been implicated as a regulator of (p)ppGpp levels, but the mechanisms remain elusive (Steuten et al. 2014).

In streptomycetes, (p)ppGpp stimulates the synthesis of some antibiotics (Bibb 2005). If (p)ppGpp binds to RNAP via the ω subunit like in *E. coli,* this might explain the impaired antibiotic production in the ω-less strains of *Streptomyces kasugaensis* and *S. coelicolor* (Kojima et al. 2002, Santos-Beneit et al. 2011). However, the structure of RNAP in streptomycetes has not been studied. Furthermore, (p)ppGpp might not inhibit RNAP activity directly in all bacteria (Kasai et al. 2006, Krásný and Gourse 2004). In cyanobacteria, (p)ppGpp-mediated regulation of gene expression has not been investigated in detail. A recent study suggested a role for (p)ppGpp in heterocyst development in *Anabaena* sp. PCC 7120 (Zhang et al. 2013). It was also suggested that in plant plastids, (p)ppGpp binds to the β' subunit of the cyanobacterial-type RNAP (Sato et al. 2009). For (p)ppGpp regulation in *Synechocystis,* only one study written in Chinese is currently available, and the abstract reported that *sll1325,* encoding a RelA/SpoT homologue, was found essential for *Synechocystis* (Miao et al. 2011).

Promoter analysis from differentially regulated genes in ΔrpoZ (Paper III) showed that there was a conserved -10 element, similar to the -10 element in *E. coli* promoters (Lisser and Margalit 1993), in both up- and down-regulated genes. Furthermore, an extended -10 element typical for highly expressed genes in *E. coli* (Barne et al. 1997) was more common in genes that were down-regulated in ΔrpoZ than in up-regulated genes. However, the -35 regions of up- and down-regulated genes in ΔrpoZ were different (Paper III), and neither of them shared resemblance with the -35 element of *E. coli* (Lisser and Margalit 1993). The differences in the promoter elements of up- and down-regulated genes further support the idea that transcriptional differences between CS and ΔrpoZ are caused by differences in σ factor recruitment.

4.3.3. The ΔrpoZ strain shows altered physiology in standard conditions

The ΔrpoZ strain grew well in standard conditions, but there was a visual yellowing of the culture after a few days, which was further investigated with pigment analysis. The Chl *a* and phycobilin contents of ΔrpoZ were similar to those measured in CS but carotenoids accumulated in high levels in the mutant cells (Paper III). Gene expression analysis showed that most genes of the carotenoid synthesis pathway were down-regulated in the ΔrpoZ strain (Paper III), suggesting that the key enzymes of carotenoid synthesis are under posttranscriptional control. Moreover, the high carotenoid content did not alter the lipid composition of membranes significantly. The ΔrpoZ strain contained less Rubisco than the CS, and in accordance, light-saturated photosynthetic activity was lower in the ΔrpoZ strain (Paper III). However, amounts of PSI and PSII, as well as amounts of the antenna proteins phycocyanin and allophycocyanin, were similar in ΔrpoZ and CS.

Gene expression analysis also showed that in addition to *rbcS* and *rbcL,* encoding Rubisco subunits, many genes encoding photosynthetic complexes, including ATP synthase, NDH-1 complex, and carbon concentrating mechanisms, were downregulated, and some of these changes were also verified at the protein level. An imbalance between photosynthetic light

reactions and carbon fixation might lead to accumulation of reactive oxygen species. The expression of the protective flavodiiron proteins Flv2, Flv3 and Flv4 was lower in the ΔrpoZ strain than in CS; however, the extra carotenoids provide protection against singlet oxygen (Hakkila et al. 2013).

Inactivation of the ω subunit does not seem to affect the survival of diverse bacteria dramatically in standard conditions, although slow growth and pleiotropic phenotypes have been reported. Antibiotic production was reduced in *S. coelicolor* and *S. kasugaensis,* and altered cell surface properties and colony morphology were reported in the streptomycetes and *M. smegmatis* (Mathew et al. 2006, Kojima et al. 2002, Santos-Beneit et al. 2011). The *Synechocystis* ΔrpoZ colonies were visually similar to CS colonies, but ΔrpoZ cells adhered more firmly to BG-11 plates and to walls of centrifuge tubes, suggesting that inactivation of *rpoZ* causes changes in cell surface properties also in cyanobacteria (Paper III).

4.3.4. The ω subunit is involved in thermal acclimation response

Inactivation of the *rpoZ* gene leads to temperature-sensitive phenotypes in other bacteria (Gentry et al. 1991, Santos-Beneit et al. 2011), which prompted us to monitor the growth of the ΔrpoZ strain in different temperatures. At 38 °C, the control strain grew essentially similarly as in standard conditions, whereas the ΔrpoZ strain had a slightly reduced initial growth rate but cells were able to acclimate after the first 24 h (Paper IV). At 40 °C the inactivation strain has major difficulties already during the first 24 h and the culture bleached completely during the next 24 h (Paper IV). The control strain grew at 40 °C with no visual signs of stress, and the ΔrpoZ+rpoZ complementation strain behaved like CS at 40 °C.

Since expression of many genes encoding the components of carbon concentrating mechanisms were downregulated in ΔrpoZ in standard conditions (Paper III) and temperature rise decreases the amount of available C_i (Paper II), we tested if the growth of ΔrpoZ at 40 °C could be improved using a more alkaline medium, which would improve the availability of C_i. However, alkaline pH did not rescue the growth of ΔrpoZ at 40 °C (Paper IV).

At 32 °C, the growth of CS was enhanced in high CO_2 conditions (Fig. 5), but the growth rate of ΔrpoZ cells was only slightly enhanced during the first 24 h compared to standard conditions. The medium in which ΔrpoZ was grown became cyanotic after 48 hours, indicating that cells were dying. The ΔrpoZ+rpoZ strain grew almost like the control strain in high CO_2 conditions. The slightly slower initial growth rate might be due to the deletion of the *psbA2* gene.

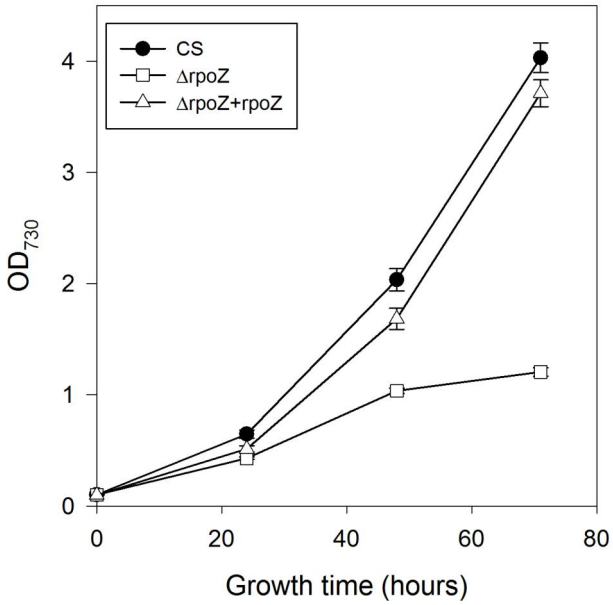

Figure 5. Growth of the control (CS, black circles), ΔrpoZ (white squares) and ΔrpoZ+rpoZ (white triangles) strains in air supplemented with 3% CO_2. The values are averages from at least three biological replicates and the error bars, shown when larger than the symbol, denote standard error.

Because the ΔrpoZ strain was unable to grow at 40 °C, additional measurements were done with cells that were first grown in standard conditions to OD_{730} ~1 and then transferred to 40 °C for 24 h. Both strains grew during the treatment, suggesting that cell density might protect ΔrpoZ cells from heat. The total RNA content of cells decreased compared to standard conditions in both strains, and the decrease was more pronounced in CS than in ΔrpoZ (Paper IV). In accordance with this, the amounts of the α and β subunits of RNAP decreased in both strains. Photosynthetic activity decreased significantly in ΔrpoZ upon the temperature shift, while CS and the complementation strain ΔrpoZ+rpoZ showed only minor changes (Paper IV). This could be related to the observation that the amount of Rubisco was low in the ΔrpoZ strain.

Transcriptomes of ΔrpoZ and CS were determined after the 24 h treatment at 40 °C (Paper IV) and the results were compared with those determined in standard conditions (Paper III). In the CS, mild heat treatment upregulated 71 transcripts and downregulated 91 transcripts, while the ΔrpoZ strain showed more extensive changes with 200 genes up-regulated and 194 down-regulated. The majority of heat-responsive genes in both strains encode proteins with unknown functions. Two other highly represented gene categories of CyanoBase were transport and binding proteins and photosynthesis genes. Many genes for biosynthesis of amino acids and cofactors, prosthetic groups and carriers were up-regulated upon heat stress in ΔrpoZ strain but only few in CS. 62 heat-responsive genes were similarly regulated in both strains, and the most striking feature was the upregulation of transport and binding related transcripts in both strains. Seven genes showed opposite changes in ΔrpoZ and CS upon mild heat stress. Of the five genes that were downregulated in ΔrpoZ but upregulated in CS, only *trmD* has an assigned function, encoding tRNA (guanine-N1-)-methyltransferase. The two genes that were upregulated in ΔrpoZ but downregulated in CS were *gcvP,* encoding glycine dehydrogenase, and *ccmR,* which encodes a repressor protein regulating many genes involved in carbon concentrating mechanisms (Wang et al. 2004).

The heat treatment was mild and long, and the expression of heat-regulated genes was not particularly prominent in CS. The *hspA* gene was upregulated, while *groES* and *htpG* were downregulated. Interestingly, also *hik34* was downregulated, although it has been shown that the Hik34 protein negatively regulates the expression of *htpG* (Suzuki et al. 2005).

Many genes that were upregulated in the ΔrpoZ strain upon the mild heat stress were downregulated in the ΔrpoZ strain compared to CS in standard conditions; the most prominent examples come from genes related to photosynthesis. The opposite was also observed: many genes that were downregulated in the ΔrpoZ strain following the heat stress were upregulated in ΔrpoZ in standard conditions (Paper IV).

Comparison of all transcriptomics data showed that 21 genes were up-regulated and 17 genes down-regulated in ΔrpoZ compared to CS in both conditions. Most of these genes encoded for proteins with unknown or hypothetical functions, but interestingly, one of the up-regulated genes is *sigH,* a group 3 σ factor which has been shown to be induced in inorganic carbon limitation (Wang et al. 2004). Other upregulated annotated genes were *hypA1* (putative hydrogenase formation protein HypA1), *nifJ* (pyruvate flavodoxin oxidoreductase), and *gltB* (NADH-dependent glutamate synthase large subunit). Annotated down-regulated genes were *aqpZ* (the water channel protein aquaporin), *atpA, atpC, atpD* and *atpG* (subunits of ATP synthase), *gpmB* (phosphoglycerate mutase), *hemL* (glutamate-1-semialdehyde aminomutase), and *pgk* (phosphoglycerate kinase).

5. CONCLUSIONS AND FUTURE ASPECTS

Papers I and II give a more detailed picture of the specific roles of group 2 σ factors, as well as giving a first glimpse on the structure of cyanobacterial RNAP holoenzyme by comparative modelling. Group 2 σ factors are not essential for *Synechocystis* in standard laboratory conditions, as the completely segregated ΔsigBCDE strain without any functional group 2 σ factors was able to grow as well as the CS. However, group 2 σ factors are involved in acclimation to new conditions. The roles of group 2 σ factors had been studied previously, but the studies presented here helped to elucidate further specific conditions in which each of the group 2 σ factor is involved:

SigB
- is essential for growth in both ionic and non-ionic hyperosmotic stress
- is involved in light acclimation, together with SigD

SigC
- is required for sustained growth under heat stress
- has a role in the regulation of carbon metabolism in heat stress
- is involved in the acclimation to salt- and sorbitol-induced hyperosmotic stress

SigD
- is crucial for acclimation to varying light conditions
- has a minor role in acclimation to salt- and sorbitol-induced hyperosmotic stress

SigE
- has a role in initial acclimation to salt stress

Papers III and IV show that although the tiny ω subunit is not essential for growth in standard conditions, inactivating the *rpoZ* gene leads to considerable physiological changes. There was less Rubisco in ΔrpoZ cells than in the control strain, which led to lower light-saturated photosynthetic activity. The amounts of photosystems were normal, and protective carotenoids and α-tocopherol accumulated in ΔrpoZ indicating that the production of reactive oxygen species was high. Transcriptional profiling showed that highly expressed genes, including many genes related to photosynthesis and carbon acquisition, were downregulated, while many genes with low expression level were upregulated in the inactivation strain. The primary σ factor SigA was downregulated both at transcript and protein level, and less SigA was bound to RNAP core in the ΔrpoZ strain. This might suggest that the ω subunit is involved in the binding of SigA to the RNAP core. Furthermore, the ω subunit is essential for thermal acclimation of *Synechocystis,* as ΔrpoZ was unable to grow in a very mild heat stress that did not affect the growth of the control strain.

The results presented in this thesis have paved the way for further analysis of the function of the cyanobacterial RNAP holoenzyme. Interesting questions that remain unanswered include the lineage-specific insertion in the β' subunit, as well as the splitting of the *rpoC* gene. Intriguingly, in *Synechocystis,* the *rpoC1* gene has been relocated in the genome, leaving only *rpoB* and *rpoC2* in an operon. Also the *rpoZ* operon needs further studies to decipher the role of the hypothetical protein Sll1532. *In vitro* studies could shed light on how σ factors actually bind to the RNAP core in the presence or absence of the ω subunit. Analysis of the quadruple mutant ΔsigBCDE should help clarifying the role of the primary σ factor SigA.

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“*Äiti! Missä mun kumisaappaat on? Miten televisio toimii? Miks pitää olla ihmisiä?*”

”Mama! Where are my boots? How does television work? Why do humans have to exist?”

So long, and thanks for all the fish!

Liisa

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