Stress responses of *Synechocystis* sp. strain PCC 6803 mutants impaired in genes encoding putative alternative sigma factors

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In the complete genome sequence of the cyanobacterium *Synechocystis* sp. strain PCC 6803 [Kaneko *et al.* (1996). *DNA Res* 3, 109–136] genes were identified encoding putative group 3 σ -factors SigH (Slr-0856), SigG (Slr-1545) and SigF (Slr-1564) and the regulatory protein RsbU (Slr-2031). Mutations in these genes were generated by interposon mutagenesis to study their importance in stress acclimation. For the genes *sigH*, *sigF* and *rsbU*, the loci segregated completely. However, attempts to mutagenize the *sigG* locus resulted in merodiploids. Under standard growth conditions only minor differences were detected between the mutants and wild-type. However, cells of the RsbU mutant showed a clear defect in regenerating growth after a nitrogen- and sulphur-starvation-induced stationary phase. After applying salt, heat and high-light shocks, stress protein synthesis was analysed by means of one- and two-dimensional electrophoresis. Cells of the SigF mutant showed a severe defect in the induction of salt stress proteins. Although the acclimation to moderate salt stress up to 684 mM NaCl was not significantly changed in this mutant, its ability to acclimate to higher concentrations of NaCl was reduced. Northern blot experiments showed a constitutive expression of the *rsbU* and *sigF* genes. The expression of the *sigH* gene was found to be stress-stimulated, particularly in heat-shocked cells, whilst that of *sigG* was transiently decreased under stress conditions. Possible functions of these regulatory proteins in stress acclimation of *Synechocystis* cells are discussed.

Keywords: cyanobacteria, environmental stress, sigma factors, stationary phase, stress proteins

INTRODUCTION

Processes of acclimation to environmental stresses in micro-organisms are mainly regulated at the level of transcriptional activation or repression. These alterations have been investigated by comparing the protein synthesis patterns of control and stressed cells. Differences between these patterns reflect changes in the actual gene expression programme (Hecker *et al.*, 1996). Besides mechanisms that act on single genes, groups of genes are regulated in stressed cells via the activity of alternative sigma factors, which replace the primary sigma factor under unfavourable growth conditions.

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; Km, kanamycin; PSII, photosystem II; WT, wild-type.

The eubacterial σ -factors can be principally divided into two evolutionarily distinct families, the σ^{70} - and σ^{54} -related factors. The σ^{70} family has been divided into several groups based upon the structural and functional properties of its members (Wösten, 1998). Group 1 comprises only the primary σ -factor, which is responsible for the transcription of most genes during exponential-growth phase and is essential for cell viability. Group 2 includes all nonessential primary-like σ -factors, which are structurally very close to the principal σ -factor. Group 3 contains alternative σ -factors, which are structurally different from proteins of group 1 and 2 and are involved in the transcription of special regulons necessary for flagella synthesis, heat shock response, sporulation or response to extra-cytoplasmic signals, etc.

The cyanobacterial σ^{70} -like group 1 and the group 2 factors form evolutionarily distinct groups as deduced by comparing their sequences to homologous proteins from other eubacteria (Gruber & Bryant, 1997). In the strains *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) and *Synechococcus* sp. PCC 7002 (Gruber & Bryant, 1997), four group 2 σ -factors have been identified in addition to the principal σ -factor. Additional group 2 σ -factors were also found in the strains *Anabaena* sp. PCC 7120 (Brahamsha & Haselkorn, 1992), *Microcystis aeruginosa* K-81 (Asayama *et al.*, 1997), *Synechococcus* sp. PCC 7942 (Goto-Seki *et al.*, 1999; Tsinomeras *et al.*, 1996) and *Nostoc punctiforme* (Cambell *et al.*, 1998). *In vitro* analysis has shown that the group 1 and some group 2 σ -factors of *Synechococcus* sp. strain PCC 7942 exhibited the same promoter specificity (Goto-Seki *et al.*, 1999). Several σ -factor-encoding genes were mutated to study their function in the cyanobacterial cell. One group 2 σ -factor of *Synechococcus* sp. strain PCC 7002, called SigE, was found to be involved in the transcription of genes specifically expressed in post-exponential phase (Gruber & Bryant, 1998), whilst the SigB and SigC proteins seem to be involved in responses to changes in carbon and nitrogen supply (Caslake *et al.*, 1997). Cyanobacterial group 2 σ -factors also play a role in the establishment of symbioses with plants and in circadian-regulated gene expression (Cambell *et al.*, 1998; Tsinomeras *et al.*, 1996). According to extensive sequence comparisons, three genes of the complete genome sequence of *Synechocystis* sp. strain PCC 6803 putatively encode alternative σ -factors of group 3 (Table 1). They were initially named *rpoE* (*sll-0856* and *slr-1545*) and *rpoF* (*slr-1564*) according to the closest eubacterial homologues (Kaneko *et al.*, 1996). In most of the recent studies on cyanobacterial σ -factors, instead of *rpo* these genes have been named using the *sig* nomenclature (Caslake *et al.*, 1997; Gruber & Bryant, 1997, 1998). To facilitate comparison with these studies on the strain *Synechococcus* sp. PCC 7002, the genes for putative group 3 σ -factors of *Synechocystis* sp. strain PCC 6803 were in this study designated according to their closest homologues in *Synechococcus* sp. strain PCC 7002: *sigF* (*rpoF*, *slr-1564*), *sigG* (*rpoE*, *slr-1545*) and *sigH* (*rpoE*, *sll-0856*). Recently, a SigF mutant of *Synechocystis* sp. strain PCC 6803 was constructed and it was found that this mutant has a defect in phototactic movement (Bhaya *et al.*, 1999).

Cyanobacterial acclimation to extreme environmental conditions such as high temperatures, changing salinities, high light intensities, etc., is accompanied by the expression of a special set of stress proteins (e.g. Fulda *et al.*, 1999; Hagemann *et al.*, 1991; Webb & Sherman, 1994). These stress proteins can be divided into two groups, special stress proteins, which are only induced by a defined stress, and general stress proteins, which are induced by several stresses (Hagemann *et al.*, 1991). In cyanobacteria, the molecular mechanisms involved in the activation of gene expression during acclimation processes are mostly unknown. As in other bacteria, cyanobacteria may use alternative σ -factors to regulate

the expression of stress proteins. Group 3 σ -factors, which include the extracytoplasmic-function-(ECF)- σ -factors, are also often involved in environmental acclimation processes of Gram-negative bacteria (Missiakas & Raina, 1998). The induction of general stress proteins, such as osmotically and stationary-phase-regulated proteins, in *Escherichia coli* and other Gram-negative bacteria is driven by RpoS (Hengge-Aronis, 1996). RpoS homologues form a distinct group among σ^{70} -like-factors, to which none of the cyanobacterial group 2 factors bear homology (Gruber & Bryant, 1997). In Gram-positive bacteria such as *Bacillus subtilis*, general stress proteins form the SigB-dependent operon (Hecker *et al.*, 1996). This group 3 σ -factor is activated by internal and external stimuli. These signals are transmitted via several proteins of the *rsb* operon, which act as anti- σ -factors, protein kinases or phosphatases. In particular, the protein phosphatase RsbU plays a central role in SigB activation under environmental stress (Hecker *et al.*, 1996). A close homologue to RsbU has been identified in the genome of *Synechocystis* sp. strain PCC 6803 (Table 1), which is encoded by the ORF *slr-2031* (Kaneko *et al.*, 1996).

To define the role of alternative sigma factors in cyanobacterial acclimation to environmental stresses, genes encoding putative group 3 σ -factors (SigF, SigG and SigH homologues) and the RsbU homologue (Kaneko *et al.*, 1996) in the strain *Synechocystis* sp. PCC 6803 were selected to generate mutants defective in these genes. The acclimation of these mutants to environmental stresses was analysed. Stress protein synthesis and expression of the *sig* and *rsbU* genes were characterized. Furthermore, the survival time under lethal stress treatments was compared.

METHODS

Strains and culture conditions. A derivative of the *Synechocystis* sp. strain PCC 6803 (henceforth referred to as *Synechocystis*) with enhanced transforming capacity was used in all experiments and was obtained from S. Shestakov (Moscow State University, Russia). *Synechocystis* belongs to the group of moderately halotolerant cyanobacteria resisting up to 1.2 M NaCl by accumulating the compatible solute glucosylglycerol (Reed & Stewart, 1985). Axenic cells were cultivated in batch cultures at 30 °C with CO₂-enriched air (5%, v/v) and constant illumination (170 $\mu\text{mol m}^{-2} \text{s}^{-1}$) using a potassium-nitrate-containing standard medium with 2 mM NaCl (Allen & Arnon, 1955). Salt treatment experiments were performed in batch cultures of mutant and wild-type (WT) cells after addition of NaCl (usually up to 684 mM) to the standard medium. For heat and high-light shock experiments, cultures were transferred to 43 °C and to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively, for the times indicated in the figures and tables. To test recovery from the stationary phase, mutant and WT cells were pre-cultured in BG11 medium (Rippka *et al.*, 1979) in shaking Erlenmeyer flasks at 30 °C and 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light. To induce stationary phase, cells were harvested by centrifugation (10 min, 5000 g), washed twice with N-free BG11 and cultured in N-free BG11 medium for two weeks. Thereafter, cells were harvested by centrifugation and transferred into N-containing BG11 medium to observe recovery

Table 1. Sequence comparisons of the *Synechocystis* proteins (Kaneko *et al.*, 1996) that were analysed in this study, indicating that they are putative group 3 σ -factors and a regulatory protein

Only those similar proteins which have been functionally characterized are shown.

<i>Synechocystis</i> protein	Identity/ similarity (%)	Bacterial homologue (protein, organism)	Regulatory functions of homologue	Reference
Slr-1564/SigF	31/53	SigB, <i>Bacillus subtilis</i> *	General stress response	Hecker <i>et al.</i> (1996)
	31/49	SigF, <i>Streptomyces setonii</i>	Carotenoid biosynthesis	Kato <i>et al.</i> (1995)
			Morphological differentiation	Potuckova <i>et al.</i> (1995)
Slr-1545/SigG	32/48	AlgU, <i>Pseudomonas aeruginosa</i>	Alginate biosynthesis and stress tolerance	Martin <i>et al.</i> (1993)
	33/48	RpoE, <i>Escherichia coli</i> *	Response to strong heat shocks	Gross (1996)
	28/45	FecI, <i>Escherichia coli</i>	Iron transport and acquisition	Van Hove <i>et al.</i> (1990)
Sll-0856/SigH	32/50	AlgU, <i>Pseudomonas aeruginosa</i>	Alginate biosynthesis and stress tolerance	Martin <i>et al.</i> (1993)
	32/52	RpoE, <i>Escherichia coli</i> *	Response to strong heat shocks	Gross (1996)
	27/46	RsbU, <i>Bacillus subtilis</i> *	Protein phosphatase activating SigB	Völker <i>et al.</i> (1995)

* Comparable similarities were found to many functionally related proteins from other Gram-positive and Gram-negative bacteria.

Table 2. Primers used to amplify DNA fragments containing genes of *Synechocystis* sp. strain PCC 6803 (Kaneko *et al.*, 1996)

Restriction sites are shown in bold letters.

Designation	Primer	Enzyme
SIGF 5*	5'-CTTTAGTCCATGGGGATCTG-3'	<i>NcoI</i>
SIGF 3*	5'-GCGATCGCCATGGCCTAGAC-3'	<i>NcoI</i>
SIGH 5*	5'-GACTGAGTTTAAACGGGTTC-3'	<i>DraI</i>
SIGH 3*	5'-ACGGGAATTTAAACGGCGTT-3'	<i>DraI</i>
SIGG 5*	5'-ATTACTGGCCACGGCATTGT-3'	<i>BalI</i>
SIGG 3*	5'-TCTTCATCCATGGCTACGAT-3'	<i>NcoI</i>
RSBU 5*	5'-CAGGTTCAAGCTTTGCAAAA-3'	<i>HindIII</i>
RSBU 3*	5'-AGCGAGGATATCAATGGAT-3'	<i>EcoRV</i>
SIGFP 5†	5'-ATGACCAATGCCACCAGCGA-3'	—
SIGFP 3†	5'-TCCGTATTTAAGGCTTTTTT-3'	—
SIGHP 5†	5'-GAGGCCTTTCGTCAACTTTA-3'	—
SIGHP 3†	5'-TTAAAAGACATCTTGAAGTC-3'	—
SIGGP 5†	5'-GTGTTGGGTAAATCCATCAA-3'	—
SIGGP 3†	5'-CCAAATAGGGCTGTAAAAA-3'	—
RSBUP5†	5'-TCAAGGAGTTGGTGGCTAAG-3'	—
RSBUP3†	5'-TCACTCCGTCGATTTTACCC-3'	—
groEL-fw†	5'-CATGGACATTCTGGCGGAAG-3'	—
groEL-rev†	5'-GGTCAATACCATGCCAGCGA-3'	—
16SrDNA27†	5'-AGAGTTTGATCMTGGCTCAG-3‡	—
16SrDNA1525†	5'-AAGGAGGTGWTCCARCC-3‡	—

* Primers used to construct mutants (see Fig. 1).

† Primers used to generate gene-specific probes for Northern blot experiments.

‡ Primer sequences according to Lane (1991).

Fig. 1. Schematic drawing showing the genetic organization, restriction map and protein-encoding region of the chromosomal sites affected in the *Synechocystis* sp. strain PCC 6803 mutants SigG (a), SigH (b), SigF (c) and RsbU (d). The sequences of the protein-encoding regions were taken from the complete genome sequence of this strain (Kaneko *et al.*, 1996). The sites of insertion of the *aphII* gene cartridges into the different genes so as to obtain directed mutants of different ORFs are shown above each region. Black triangles, primer binding sites used to generate mutants; open triangles, primer-binding sites used to generate gene-specific probes; black arrows, affected genes; open arrows, genes neighbouring affected genes.

from the starvation-induced stationary phase. The survival of lethal stresses was tested by incubation of cyanobacterial cells in test tubes under stress conditions close to the maximal resistance levels (45 °C, 4 °C, 855 mM NaCl, 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, complete lack of single nutrients). After different incubation times (hours to weeks depending on the particular stress, see text), 1 ml samples were taken from the test tubes. The cells were collected by centrifugation (10 min, 5000 g) and resuspended in BG11 medium. Defined aliquots were dropped onto Petri dishes containing medium C (Kratz & Myers, 1955) supplemented with 0.8% agar. After incubation of the plates at 29 °C and continuous light of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for one week, surviving cells were evaluated and documented by photography. The *E. coli* strain TG1 (Sambrook *et al.*, 1989) was used for routine DNA manipulations. *E. coli* was cultivated in Luria broth medium (Sambrook *et al.*, 1989) at 37 °C.

DNA manipulations. Total DNA from *Synechocystis* was isolated according to Hagemann *et al.* (1996). Chromosomal DNA for PCR analyses was obtained after treatment of 200 μl cyanobacterial solution with hot phenol and chloroform. All other techniques, such as plasmid isolation, transformation of *E. coli*, ligation and restriction analysis (restriction enzymes were obtained from New England Biolabs) were standard methods (Sambrook *et al.*, 1989). DNA probes were labelled with digoxigenin for Southern hybridization using the PCR DIG Probe Synthesis kit (Boehringer Mannheim). DNA and protein sequences were analysed using DNASIS/PROSIS, CLUSTAL X and BLAST (Altschul *et al.*, 1997) software packages. For the PCR reactions, PCR-supermix or Elongase (Life Technologies) and the following temperature cycle was applied ($\times 30$): 15 s at 94 °C, 30 s at 52 °C, 2 min at 72 °C.

Generation of insertion mutants. Mutants impaired in selected genes were generated by reverse genetics. The coding sequences and neighbouring sequences were amplified by PCR. The approximately 2 kb PCR products were cloned into pUCBM20/21 (Boehringer Mannheim). The primers for amplification were designed using the complete genome sequence of *Synechocystis* (Kaneko *et al.*, 1996). Sequences were selected which contained appropriate restriction sites to improve cloning of the fragments (Table 2). The *aphII* gene [aminoglycoside phosphotransferase II conferring kanamycin (Km) resistance] isolated from plasmid pUC4K (Pharmacia) was inserted into unique restriction sites of the encoding sequences. Transformation of *Synechocystis* has been described previously (Hagemann & Zuther, 1992). Transformants were initially selected on medium C (Kratz & Myers, 1955) containing 10 $\mu\text{g Km ml}^{-1}$ (Sigma), whilst the segregation of clones was performed by restreaking of primary clones on plates supplemented with 50 $\mu\text{g Km ml}^{-1}$ several times (at least three transfers). During cultivation of mutants, 50 $\mu\text{g Km ml}^{-1}$ was added to the liquid media.

Protein labelling and electrophoretic separation. For labelling of proteins, 5 ml cells was incubated with 2.8 MBq L-[^{35}S]methionine (specific activity $> 29.6 \text{ TBq mmol}^{-1}$; Amersham Buchler) for 30 min under the culture or shock conditions described above, but without bubbling with CO_2 -enriched air. Proteins were extracted in 10 mM HEPES/NaOH (pH 7.3) containing 1 mM PMSF by means of an ice-cooled cell mill (E. Buhler) with glass beads (diameter 0.1–0.2 mm) twice for 1 min. The homogenate was centrifuged (48000 g, 4 °C; Sorvall) and the supernatant was stored at -20 °C. After estimation of [^{35}S]methionine incorporation, aliquots of the samples were concentrated by lyophilization. Equal amounts of radioactivity (10^5 and 10^6 c.p.m. for one- and two-dimensional separations, respectively) were applied to each lane or gel. One-dimensional (1D) SDS-PAGE was performed as described previously (Fulda *et al.*, 1999). The molecular masses of protein bands were determined in comparison to labelled rainbow protein standards (Amersham Buchler) by videodensitometry with the Bioprofil 1D software (Vilbert Lourmat). For two-dimensional (2D) PAGE separations (SDS used in the second dimension), the 2D Investigator system (Millipore) was applied, following exactly the instructions of the manufacturer regarding the preparation and running of IEF and PAGE gels.

RNA isolation and Northern blot experiments. RNA was isolated from cells of 10 ml culture, which were harvested by centrifugation (4000 g, 10 min, 2 °C), immediately frozen and stored at -80 °C. RNA was extracted using the High Pure RNA Isolation kit (Boehringer Mannheim). Methods used for the separation of RNA, blotting and hybridization were

Table 3. Plasmids and *Synechocystis* sp. strain PCC 6803 mutants used and constructed in this study (see also Fig. 1)

Plasmid or mutant	Description
Plasmids	
pNsigF	pUCBM20 containing <i>sigF</i> (<i>slr-1564</i>) as a 2.1 kb <i>NcoI</i> fragment obtained by PCR with SIGF 5 and SIGF 3 primers; final size, 4.87 kb
pNFSK	pNsigF containing an inactivated <i>sigF</i> gene (<i>aphII</i> gene is inserted at the unique <i>StuI</i> site in opposite direction to <i>sigF</i>); final size, 6.12 kb
pSNG	pUCBM21 containing <i>sigG</i> (<i>slr-1545</i>) as a 1.9 kb <i>BalI</i> / <i>NcoI</i> fragment obtained by PCR with SIGG 5 and SIGG 3 primers; final size, 4.58 kb
pSNGHK	pSNG containing an inactivated <i>sigG</i> gene (<i>aphII</i> gene is inserted at the unique <i>HpaI</i> site in the same direction as <i>sigG</i>); final size, 5.83 kb
pSsigH	pUCBM21 containing <i>sigH</i> (<i>sll-0856</i>) as a 2.3 kb <i>DraI</i> fragment obtained by PCR with SIGH 5 and SIGH 3 primers; final size, 5.04 kb
pSsigHSK	pSsigH containing an inactivated <i>sigH</i> gene (<i>aphII</i> gene is inserted at the unique <i>StuI</i> site in opposite direction to <i>sigH</i>); final size, 6.29 kb
pHSU	pUCBM20 containing <i>rsbU</i> (<i>slr-2031</i>) as a 2.32 kb <i>HindIII</i> / <i>EcoRV</i> fragment obtained by PCR with RSBU 5 and RSBU 3 primers; final size, 4.96 kb
pHSUHK	pHSU containing an inactivated <i>rsbU</i> gene (<i>aphII</i> gene is inserted at the unique <i>HpaI</i> site in opposite direction to <i>rsbU</i>); final size, 6.21 kb
Mutants	
SigF mutant	<i>Synechocystis</i> mutant impaired in <i>sigF</i> (<i>slr-1564</i>) obtained after transformation of the WT with pNFSK
SigH mutant	<i>Synechocystis</i> mutant impaired in <i>sigH</i> (<i>sll-0856</i>) obtained after transformation of the WT with pSsigHSK
SigG mutant	<i>Synechocystis</i> mutant impaired in <i>sigG</i> (<i>slr-1545</i>) obtained after transformation of the WT with pSNGHK
RsbU mutant	<i>Synechocystis</i> mutant impaired in <i>rsbU</i> (<i>slr-2031</i>) obtained after transformation of the WT with pHSUHK

described in detail previously (Hagemann *et al.*, 1997). Gene-specific DNA probes for the Northern blot experiments were obtained after PCR amplification of the coding sequences of the corresponding genes using primers (see Table 2) binding to their 5' and 3' ends. The DNA was labelled with [α - 32 P]dATP (Amersham Buchler) using a random prime labelling kit (MBI Fermentas). Hybridization signals were recorded and quantified by means of a phosphorimager (BAS1000; Fuji). To quantify the data and correct errors in gel loading, all calculations were made on the basis of hybridization signals obtained after applying a radiolabelled 16S rDNA probe (for primers see Table 2) to the same filters.

Physiological characterization. The content of low-molecular-mass stress metabolites was analysed by HPLC (Hagemann *et al.*, 1997). Photosynthetic oxygen evolution in the light and respiratory oxygen consumption in the dark were measured using a Clark-type electrode. Growth and cell density were monitored by reading the optical density of diluted cyanobacterial suspensions at 750 nm using a spectrophotometer (U2000; Hitachi). Pigment concentrations were estimated using *in vivo* absorption measurements (U2000; Hitachi) and the formulae for corrections of peak interferences (Sigalat & de Kouchkovsky, 1975). Degradation of pigments in nitrogen-starved cells was followed by reading whole-cell absorbance spectra, which were corrected for cell scattering (Sauer *et al.*, 1999). Photosystem II (PSII) fluorescence with and without 10 μ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was recorded using a microplate fluorimeter (Fluoroscan II;

Labsystems) after excitation at 570 nm and emission at 685 nm.

All experiments were repeated at least three times using independent cultures. In the tables and figures, means and standard deviations are given or the results of one typical experiment are shown.

RESULTS

Generation of mutants

To study the importance of group 3 σ -factors for stress acclimation of *Synechocystis*, mutants affected in three genes encoding these σ -factors and in the regulatory protein RsbU were generated by interposon mutagenesis. After PCR amplification and cloning of coding sequences together with flanking sequences, the *aphII* resistance gene cassette was introduced into unique restriction sites. The plasmid DNA of Km-resistant *E. coli* clones was analysed by PCR and restriction analyses. For further experiments, constructs in which the *aphII* gene was inserted in a transcription direction opposite to that of *sigH*, *sigF* and *rsbU* were selected, except for *sigG* for which plasmids were only obtained with the *aphII* gene integrated collinearly (Fig. 1, Table 3). *Synechocystis* WT cells were transformed with

Fig. 2. PCR analyses using chromosomal DNA of the WT (lanes 1, 3, 5 and 7) and of mutants (lane 2, SigH; lane 4, SigG; lane 6, SigF; lane 8, RsbU; see Fig. 1, Table 3) of *Synechocystis* sp. strain PCC 6803 as a template and primers specific for selected genes (lanes 1 and 2, SIGH 5 and SIGH 3; lanes 3 and 4, SIGG 5 and SIGG 3; lanes 5 and 6, SIGF 5 and SIGF 3; lanes 7 and 8, RSBU 5 and RSBU 3; see Table 2) in order to verify the lesions in the chromosomal DNA of the mutants. Lane M, *Hind*III-cut DNA used as fragment size marker.

plasmids harbouring inactivated genes putatively encoding σ -factors and RsbU- and Km-resistant clones were selected.

Segregation was performed by restreaking of primary colonies on plates supplemented with $50 \mu\text{g Km ml}^{-1}$ for at least three weeks. The DNA of mutant clones was analysed by PCR using the same primers that were used

to amplify the DNA fragments from WT DNA. In all cases, using mutant DNA the PCR reactions produced enlarged fragments compared to the fragments obtained with WT DNA (Fig. 2). The size differences exactly corresponded to the increases expected following insertion of an *aphII* gene cassette (1.2 kb). Furthermore, when DNA of the SigH, SigF and RsbU mutants was used, the WT fragments were completely absent (Fig. 2). Only in DNA of the SigG mutant did a dominant fragment corresponding to the size of the WT DNA-fragment remain visible, indicating incomplete segregation of the genome of this mutant. Additionally, a fragment of intermediate size was generated, which probably resulted from an intramolecular template exchange by the elongase enzyme mix. All attempts to obtain a completely segregated SigG mutant or to minimize the proportion of the WT-size fragment using cultivation at enhanced Km concentrations or different salt and temperature conditions failed, indicating that this gene is essential for *Synechocystis* under all growth conditions tested in this study. The results of the PCR analyses were confirmed by Southern hybridization experiments (not shown).

Physiological characterization

Initially, growth and pigmentation of the mutants were compared to the WT under standard growth conditions (Table 4). Surprisingly, only the SigG mutant showed a decreased growth rate in spite of its incomplete segregation. No significant differences in pigment contents could be detected between the WT and *sig* mutants. However, cells of the RsbU mutant exhibited an increased pigmentation under our standard growth conditions. Photosynthetic and respiratory activities

Table 4. Comparison of physiological parameters of WT and mutant cells of *Synechocystis* sp. strain PCC 6803 after cultivation at a low salt concentration of 2 mM NaCl

Values are means $\pm$ SD.

Parameter*	WT	SigH	SigG	SigF	RsbU
Growth rate (h^{-1})	0.0399 ± 0.0063	0.0367 ± 0.004	0.0285 ± 0.0049	0.0400 ± 0.006	0.386 ± 0.0051
Photosynthetic oxygen evolution ($\text{mg O}_2^{-1} \text{ h}^{-1} \mu\text{mol Chl } a^{-1}$)	2.83 ± 0.83	2.84 ± 0.44	3.24 ± 0.87	3.05 ± 1.07	2.44 ± 0.75
PSII fluorescence ratio (F_v/F_{max})	0.401 ± 0.016	0.416 ± 0.012	0.389 ± 0.020	0.416 ± 0.014	0.446 ± 0.009
Respiratory oxygen consumption ($\text{mg O}_2^{-1} \text{ h}^{-1} \mu\text{mol Chl } a^{-1}$)	0.40 ± 0.13	0.37 ± 0.15	0.40 ± 0.15	0.43 ± 0.21	0.32 ± 0.27
Chl <i>a</i> content ($\mu\text{mol Chl } a \text{ l}^{-1}$ $\text{OD}_{750} \text{ unit}^{-1}$)	1.736 ± 0.095	1.650 ± 0.096	1.441 ± 0.268	1.598 ± 0.094	2.179 ± 0.176
Car content ($\mu\text{mol Car l}^{-1} \text{OD}_{750}$ unit^{-1})	2.458 ± 0.524	2.701 ± 0.752	1.938 ± 0.698	2.88 ± 0.504	4.727 ± 0.829
PC content ($\mu\text{mol PC l}^{-1} \text{OD}_{750}$ unit^{-1})	0.508 ± 0.086	0.525 ± 0.125	0.391 ± 0.116	0.551 ± 0.075	0.834 ± 0.114
GG content ($\mu\text{g GG ml}^{-1} \text{OD}_{750}$ unit^{-1})†	36.87 ± 0.55	41.38 ± 0.51	43.10 ± 1.15	40.15 ± 0.99	38.04 ± 1.00

* Car, carotenoid; Chl *a*, chlorophyll *a*; GG, glucosylglycerol; PC, phycocyanin.

† After acclimation to a high salt concentration of 684 mM NaCl.

Fig. 3. (a) Restoration of growth after N-starvation-induced stationary phase in cells of the WT and mutants SigH, SigG, SigF and RsbU of *Synechocystis* sp. strain PCC 6803. Cells were pre-cultured in complete BG11 medium and then grown in N-free BG11 for about 2 weeks. The arrow indicates the time point at which all cultures were transferred back into complete BG11 medium. Growth of the wild-type (WT 6803, $\diamond$) and mutants SigH ($\square$), SigG ($\triangle$), SigF ($\circ$) and RsbU ($\times$) was recorded as increase in optical density at 750 nm (OD₇₅₀). (b, c) Degradation of pigments was followed at different time points in cells of the wild-type (WT 6803, b) and the RsbU mutant (c) during cultivation under N-free conditions.

were almost identical in cells of the WT and the mutants. The growth of the mutant at different NaCl concentrations was compared to that of WT cells in the range 2–684 mM NaCl, corresponding to 0–4% NaCl. No significant differences were recorded under these low and moderate salt stress conditions. Also, the content of the osmolyte glucosylglycerol did not change in any of the mutants compared to salt-shocked WT cells (Table 4). However, very strong salt shocks were found to be lethal for the SigF mutant (see Fig. 8). Interestingly, cells of the SigF mutant showed impaired mobility on agar plates used to segregate the mutant clones, whilst all other mutants were able to move on agar surfaces like WT cells. This motility defect was recently described in an independent study (Bhaya *et al.*, 1999). However, under the cultivation conditions used in this work, our SigF mutant strain did not secrete a yellow-brown and UV-absorbing pigment as reported for the SigF mutant of Bhaya *et al.* (1999).

Mutants and WT were compared with respect to their ability to recover from starvation-induced stationary phase. After cultivation for two weeks in a N-deficient

Fig. 4. Stress-induced alterations of protein synthesis pattern in cells of the WT and mutants SigH and SigG (a) and SigF and RsbU (b) of *Synechocystis* sp. strain PCC 6803 analysed by pulse-labelling with [³⁵S]methionine and separation by 1D-PAGE. Cells were salt-shocked with 684 mM NaCl for 0.5 h (lanes S), heat-shocked at 43 °C for 0.5 h (lanes H), or light-shocked at 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 h (lanes L). Lanes M, molecular mass marker; lanes C, unshocked control. Asterisks indicate stress proteins absent or reduced in mutant cells.

growth medium, cells were retransferred into complete BG11 medium (Fig. 3). After a short lag phase, cells of the WT and the three Sig mutants were able to resume growth. In contrast, cells of the RsbU mutant were not able to regenerate and were completely lysed after a few days in N-replete medium. Since the stationary phase was induced by transfer of cells into N-free medium, cells underwent a chlorotic degradation of pigments. In cells of the RsbU mutant the chlorosis occurred faster. After one week the phycobilisome peak at 630 nm was already absent and chlorophyll content was significantly decreased. After about 10 d the chlorophyll was completely degraded (Fig. 3c). At this time point, cells of the WT (Fig. 3b) and the Sig mutants (not shown) still contained residual phycobilisomes and considerable amounts of chlorophyll *a*.

Protein synthesis pattern

Alterations of gene expression were analysed in salt-, heat- and high-light-treated cells by *in vivo* labelling of

Fig. 5. Salt-stress (ss)-induced alterations of protein synthesis pattern in cells of the WT and mutants SigH and SigF of *Synechocystis* sp. strain 6803 analysed by pulse-labelling with [35 S]methionine and separation by 2D-PAGE. Cells were salt-shocked with 684 mM NaCl for 0.5 h. Arrows indicate positions of salt-stress proteins and dotted spots mark positions of protein spots which were absent or reduced in labelled protein extracts of mutant cells. Control, protein labelling pattern obtained in WT cells under control conditions.

proteins with [35 S]methionine and subsequent electrophoretic separation of soluble proteins from cell lysates. Results from 1D-PAGE indicated that the protein synthesis pattern was changed in salt-stressed cells of the SigF mutant (Fig. 4). Compared to cells of the WT, which exhibited a set of stress proteins expressed within 30 min after a salt shock of 684 mM NaCl, the SigF mutant synthesized, though at a reduced rate, only three stress proteins, while the others were hardly detectable.

The response to heat and high-light shocks remained almost unchanged in cells of the SigF mutant. Only an about 16 kDa protein, which was also induced in salt-shocked cells, was clearly less labelled in a light-shocked SigF mutant (Fig. 4). The expression of a similar-sized protein was also decreased in the SigH mutant (Fig. 4). Cells of the nonsegregated mutant SigG showed no differences in stress protein labelling after 1D-separation. In SigF, SigH and RsbU mutants, expression of

Fig. 6. Northern blot experiments to detect heat-stress-induced alterations of the steady-state mRNA levels of *sigG* and *sigH* genes in comparison to the expression of the typical heat-shock genes *groEL*. For hybridization, gene-specific probes were generated by PCR (see Table 2) and RNA from heat-shocked WT cells of *Synechocystis* sp. strain PCC 6803 (43 °C, times as indicated) was used as the target. The filters were rehybridized with a 16S-rRNA-specific probe as control for RNA loading.

the high-molecular-mass heat-shock proteins seemed to be enhanced compared to the WT (Fig. 4).

A more detailed view of stress protein synthesis patterns after salt shock was obtained by separation of soluble proteins by 2D-PAGE (Fig. 5). After cultivation under control conditions, no significant alterations were observed in the protein labelling pattern of WT and mutant cells (not shown). As expected from the 1D-gels, salt shock had a most dramatic effect on the SigF mutant (Fig. 5). Only three of the salt-stress protein spots found in WT cells remained visible. Additionally, several constitutive proteins were diminished in salt-shocked SigF mutant cells. 2D-PAGE also showed the absence of some salt-shock proteins in the SigH mutant, which were also missing in the SigF mutant (Fig. 5). Cells of mutants SigG and RsbU (not shown) showed almost the same protein synthesis pattern after salt shock as WT cells. Heat shock repressed synthesis of many proteins

Fig. 7. Alterations of PSII fluorescence (a, ratio of F_t/F_{max} , relative values are shown, level of control cells was normalized to 100%, see Table 4) and glucosylglycerol (GG) content (b) in cells of the WT (solid lines) and SigF mutant (broken lines) of *Synechocystis* sp. strain PCC 6803 shocked for different times with 684 mM NaCl.

seen in control cultures. Only a few constitutive and a set of heat-shock proteins remained labelled in heat-shocked WT cells. Synthesis of these heat-shock proteins was not altered in the mutants analysed in this study (not shown).

Northern blot experiments

Analysis of the expression of genes encoding regulatory proteins under different growth conditions may give additional hints towards identification of processes regulated by these proteins. Therefore, the relative mRNA amounts of the σ -factor- and the RsbU-encoding genes were estimated in Northern blot experiments using total RNA from WT cells exposed to different environmental stresses. The amount of *sigF*- and *rsbU*-specific mRNAs did not change significantly after applying salt, heat or high-light stress to WT cells (not shown). The sizes of the main transcripts for the *sigF* and *rsbU* genes were estimated to be about 1.5 and 1.7 knt, respectively. Transcript sizes exceeded the length of probed ORFs, indicating that the genes were located on polycistronic mRNAs containing adjacent genes (compare Fig. 1). Clear responses to salt- and heat-stress treatments, but not towards light stress, were found in the transcript levels of the *sigG* and *sigH* genes. In control cells, *sigG* mRNA was clearly detectable and that of *sigH* nearly absent, whilst in heat- and salt-stressed cells the *sigH* mRNA amount exceeded that of

Fig. 8. Survival of extreme stress treatments by cells of the WT and SigF, SigG, SigH and RsbU mutants of *Synechocystis* sp. strain PC 6803. Cells were incubated for different times under stress conditions and thereafter aliquots were dropped onto control medium to evaluate survival of cells. (a) Cells were incubated for 2 d at different NaCl concentrations as indicated (mM); (b) cells were incubated for 3 weeks in S-, P- and Fe-free BG11 medium; (c) cells were exposed for different times as indicated (h) to a high light intensity of $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

sigG (Fig. 6). Maximal expression of *sigH* was detected only 7 h after the application of a heat shock of 43°C . At this time, the transiently activated expression of the heat-shock genes *groEL* was already reduced nearly to the control level (Fig. 6). Quantitative analyses revealed that the amount of *sigH*-specific mRNA increased about 30-fold in heat-shocked cells and 2.5-fold in salt-shocked cells, whilst the *sigG*-specific mRNA level was reduced to about 20 and 50%, respectively. The signal obtained for the mRNAs in Northern blot experiments was smeared, indicating low stability of these transcripts. In the case of *sigH*, the estimated full-length transcript of about 0.7 knt corresponded well to the length of its coding sequence, whilst a full-length transcript of at least 1.0 knt was observed for *sigG*, which clearly exceeds the size of its coding region. The nearly complete

disappearance of *sigG*-specific mRNA in salt- and heat-stressed cells led to the assumption that under these growth conditions this σ -factor could be dispensable. However, in both salt-treated and high-temperature treated cells, again no complete segregation of the SigG mutant was achieved.

Salt tolerance of the SigF mutant

Although salt-treated cells of the SigF mutant exhibited a strongly decreased level of stress protein synthesis, cells were still able to grow at a salt concentration of 684 mM NaCl in CO_2 -gassed cultures. Since most salt-stress proteins of *Synechocystis* are only transiently detectable (Hagemann *et al.*, 1991), it may be concluded that those proteins are only important during the early acclimation phase. Therefore, investigations of the early salt acclimation process were performed using WT and SigF mutant cells (Fig. 7). Photosynthesis measured as the ratio of variable and maximal photosystem II fluorescence and glucosylglycerol accumulation were chosen as parameters to evaluate a successful salt acclimation. In both cases, cells of the SigF mutant showed no sign of an enhanced lag phase or any delay in the acclimation process. In both the WT and SigF strains, photosynthesis was immediately diminished in cells shocked by 684 mM NaCl. A decreased fluorescence ratio was observed during the first 10 h after salt shock, whilst it recovered to the control level parallel to glucosylglycerol accumulation during further acclimation (Fig. 7). Additionally, the resistance to very high salt concentrations of the SigF mutant was compared to WT cells and that of the other mutants in BG11 medium. In these experiments, a decrease in the maximal salt tolerance level of the SigF mutant became obvious (Fig. 8). After 7 d incubation at 855 mM (5%) NaCl, cells of the SigF mutant were completely lysed and could not recover under optimal growth conditions, whilst all other mutants and the WT survived such a stress condition. Exposure of cells to 1 M NaCl was found to be lethal for the WT as well as the mutants (not shown).

Survival after extreme stress treatments

Under control conditions and moderate stress we did not observe any significant alterations in the phenotype of the mutants in comparison to WT cells. This may not be the case when these cells are exposed to harsh stresses, as was found for extreme high-salt stress. Therefore, mutants were deprived for up to 5 weeks of different nutrients as described in Methods. As after N-starvation (see Fig. 3), the RsbU mutant was again not able to recover after long term S-starvation, whilst the Sig mutants and the WT strains survived this treatment (Fig. 8). Long-term P- and Fe-starvations were tolerated by all strains. In further experiments, the cells were exposed to high and low temperatures. A heat shock of 45°C for 3–4 h was found to be lethal for all strains (not shown). In a few experiments, cells of the SigH mutant showed a tendency to be damaged earlier than the other

cells. The incubation of mutant and WT at low temperatures (4 °C) for several days was tolerated by all cultures (not shown). Exposure of cells to about 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 2–3 h at 29 °C was found to be lethal for cells of the SigF mutant and, surprisingly, also for the nonsegregated mutant SigG (Fig. 8). Cells of the WT as well as the SigH and RsbU mutant tolerated this stress treatment for at least 5 h.

DISCUSSION

To study the role of alternative σ -factors in the acclimation of *Synechocystis* to environmental stresses, genes encoding putative group 3 σ -factors and one regulatory protein were disrupted using interposon mutagenesis. Only the σ -factor SigG (Slr-1545) was found to be essential for *Synechocystis* cells. A similar result has been demonstrated for its close homologue RpoE from *E. coli*, indicating that this σ -factor is involved in the expression of essential genes under ambient temperature (Missiakas & Raina, 1998). In accordance with a possible involvement of SigG in transcription under standard growth conditions, the highest mRNA level of this gene was found in cells grown under these conditions. This putative σ -factor of *Synechocystis* also showed high similarities to the second ECF- σ -factor FecI of *E. coli* (Van Hove *et al.*, 1990), which is involved in the regulation of iron uptake. However, the SigG mutant survived a long-term iron starvation without any remarkable phenotypic changes. Because of its merodiploid status, it was not surprising that almost no phenotypical alterations were detected in the SigG mutant compared to WT cells. Only under high-light conditions was a clearly reduced survival time of cells of the SigG mutant found. Possibly, the reduced gene dosage of WT *sigG* in the mutant led to a reduced expression of protein(s) required to prevent lesions caused by high light. In photosynthetic cells, high light intensities induce oxidative stress and it is intriguing to see that RpoE-like σ -factors of *E. coli* are also involved in response to oxidative stress (Missiakas & Raina, 1998).

SigH (Slr-0856), the second RpoE homologue of *Synechocystis*, was found to be dispensable. The fully segregated mutant was able to tolerate all growth and stress conditions used in this study. The two *rpoE* gene homologues of *Synechocystis* seem to be complementarily regulated. The *sigH* transcript increased when the cells were subjected to heat-shock conditions, whilst the transcript of *sigG* decreased under these conditions. However, compared to the induction of the typical heat-shock genes *groEL*, the induction of *sigH* occurred rather late making it unlikely that the alternative σ -factor SigH is responsible for the regulation of these heat-shock genes. Furthermore, since the *sigG* gene could not be completely deleted, it seems that the SigH cannot functionally replace the essential SigG.

The completely segregated SigF mutant of *Synechocystis* exhibited a pronounced defect in salt-stress-induced

gene expression. Most of the stress proteins were absent in salt-shocked cells of this mutant, whilst levels of proteins induced by heat and high-light shock were almost unchanged. Despite the reduction in the synthesis of salt stress proteins, during the first hours after a salt shock no differences were observed regarding glucosylglycerol accumulation and recovery of photosynthesis. However, long term application of high-salt stress clearly diminished the survival rate of the SigF mutant in comparison to WT cells. These results indicate that the salt-shock proteins are most important for the long-term salt acclimation and tolerance of salt concentrations near the resistance level. But they are apparently not required for glucosylglycerol synthesis and ion export, two processes shown to be mainly regulated by post-translational activation of pre-existing enzymes (Hagemann *et al.*, 1996). Until now most of the proteins induced by salt stress were unknown. Many of them were also found after other stress treatments, therefore they can be regarded as general stress proteins (Fulda *et al.*, 1999; Hagemann *et al.*, 1991). The specific effect of salt, demonstrated in the phenotype of the SigF mutant, provides evidence that SigF represents a terminal element of a signal-transducing pathway sensing salt. This pathway apparently targets unknown stress proteins and proteins involved in the synthesis of pili, required for light-induced mobility of *Synechocystis* (Bhaya *et al.*, 1999).

The induction of salt-stress proteins seems to be primarily regulated by modulation of the activity of the SigF protein, since *sigF* transcription remained almost constant in stressed cells. Post-translational regulation is characteristic of the structurally similar SigB in *B. subtilis* (Hecker *et al.*, 1996) and the functionally similar RpoS in *E. coli* (Hengge-Aronis, 1996), two σ -factors that are required for the increased expression of several genes after a salt or osmotic shock. However, in contrast to SigB of *B. subtilis*, the SigF of *Synechocystis* seems not to be involved in transition to stationary phase since our SigF mutant did not show any phenotype regarding its ability to resume growth after starvation-induced stationary phase. Remarkably, a deletion of *rsbU*, encoding a close homologue of a regulatory protein involved in the activation cascade of SigB in *B. subtilis* (Völker *et al.*, 1995; Hecker *et al.*, 1996), showed a defect in recovering from stationary phase but no defect in inducing salt stress proteins and survival of high-salt and high-light stress treatments. Besides N-starvation-induced stationary phase, the RsbU mutant could not survive long-term S-starvation. Thus, in *Synechocystis* RsbU is not involved in the activation of SigF to drive salt-induced gene expression, but may participate in the response to nutrient deficiency, which includes a controlled degradation of phycobiliproteins followed by chlorophyll *a* (Sauer *et al.*, 1999). In the cyanobacterial strain *Synechococcus* sp. strain PCC 7002, a group 2 σ -factor called SigE was identified and found to be responsible for stationary-phase-induced gene activation (Gruber & Bryant, 1998). However, further studies will be required to show that RsbU eventually functions

in regulation of group 2 σ -factors in *Synechocystis* and other cyanobacteria.

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