

# Characterization of Single and Double Inactivation Strains Reveals New Physiological Roles for Group 2 $\sigma$ Factors in the Cyanobacterium *Synechocystis* sp. PCC 6803<sup>1[W]</sup>

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Cyanobacteria are eubacteria that perform oxygenic photosynthesis like plants. The initiation of transcription, mediated by the RNA polymerase holoenzyme, is the main determinant of gene regulation in eubacteria. The  $\sigma$  factor of the RNA polymerase holoenzyme is responsible for the recognition of a promoter sequence. In the cyanobacterium *Synechocystis* sp. PCC 6803, the primary  $\sigma$  factor, SigA, is essential for cell viability. The SigB, SigC, SigD, and SigE factors show significant sequence similarity with the SigA factor but are nonessential. In this study, we have used homology modeling to construct a three-dimensional model of *Synechocystis* RNA polymerase holoenzyme and all group 1 and 2  $\sigma$  factors. According to the models, the overall three-dimensional structures of group 1 and 2  $\sigma$  factors are similar, the SigB and SigD factors being the most similar ones. In addition, we have constructed a complete set of group 2  $\sigma$  factor double inactivation strains,  $\Delta$ sigBC,  $\Delta$ sigBD,  $\Delta$ sigBE,  $\Delta$ sigCD,  $\Delta$ sigCE, and  $\Delta$ sigDE. All double mutants grow well under standard conditions, but differences are observed in stress conditions. The transition from lag phase to exponential growth is slow in the  $\Delta$ sigBD strain, and all strains lacking the SigD factor were found to be sensitive to bright light. Furthermore, all group 2  $\sigma$  factors were found to be involved in acclimation to salt- or sorbitol-induced osmotic stresses.

Cyanobacteria are evolutionarily ancient eubacteria that perform oxygenic photosynthesis and are known as the ancestors of chloroplasts (Rodríguez-Ezpeleta et al., 2005). Present-day cyanobacteria are found in most natural habitats. Growth and survival under a range of different environmental stress conditions make cyanobacteria valuable model organisms in studies of molecular mechanisms underlying the acclimation processes of autotrophs. The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) has been used extensively in gene expression studies under a variety of different stress conditions (Hihara et al., 2001; Huang et al., 2002; Kanesaki et al., 2002; Shoumskaya et al., 2005; Foster et al., 2006; Singh et al., 2006; Summerfield and Sherman, 2007; Tuominen et al., 2008). These and other studies have demonstrated that acclimation to changing environmental conditions requires changes in gene expression over a wide range of different functions.

In eubacteria, the main determinant of gene regulation is the initiation of transcription mediated by the RNA polymerase holoenzyme. The eubacterial RNA polymerase holoenzyme is composed of a core enzyme (with the subunit composition  $\alpha_2$ ,  $\beta$ ,  $\beta'$ ,  $\omega$ ) and a  $\sigma$  factor. The core enzyme of the RNA polymerase exhibits the RNA polymerase activity, while the  $\sigma$  factor is responsible for the recognition of promoter sequences. Most bacteria synthesize several  $\sigma$  factors that compete for the same RNA polymerase core (Maeda et al., 2000), and it is believed that replacement of the  $\sigma$  factor with another  $\sigma$  factor is a major switch for changing the global transcription pattern in eubacteria.

Nine genes encode  $\sigma$  factors in *Synechocystis* (Kaneko et al., 1996). The *sigA* gene encodes the principal (group 1)  $\sigma$  factor that is essential for cell viability. The *sigB*, *sigC*, *sigD*, and *sigE* genes encode group 2  $\sigma$  factors (primary-like  $\sigma$  factors) that show extensive amino acid similarity with the SigA factor but are not essential for cell viability under optimal growth conditions (Imamura et al., 2003; Tuominen et al., 2003). Recent results have shown that group 2  $\sigma$  factors are important under suboptimal conditions (Osanai et al., 2005; Singh et al., 2006; Tuominen et al., 2006, 2008; Summerfield and Sherman 2007). A complicated regulatory network between the group 1 and group 2  $\sigma$  factors has been suggested to function in *Synechocystis* (Lemeille et al., 2005). The *sigF*, *sigG*, *sigH*, and *sigI* genes

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encode alternative  $\sigma$  factors that vary more considerably in amino acid sequence than those of group 1 and 2  $\sigma$  factors.

The structure of the bacterial RNA polymerase holoenzyme from the thermophilic bacteria *Thermus thermophilus* (Vassylyev et al., 2002, 2005) and *Thermus aquaticus* (Murakami et al., 2002a, 2002b) has been determined by x-ray crystallography. In this study, we took advantage of the high sequence identity between bacterial RNA polymerases and used the crystal structure of the RNA polymerase of *T. thermophilus* to construct a three-dimensional model of the RNA polymerase holoenzyme with SigA factor in *Synechocystis*. In addition, we constructed three-dimensional models of all group 2  $\sigma$  factors of *Synechocystis*. Based on the models, the overall three-dimensional structures of group 1 and 2  $\sigma$  factors resemble each other; the SigB and SigD factors being the most similar ones. In addition to homolog models, we have constructed a complete set of double inactivation strains of group 2  $\sigma$  factors, including  $\Delta$ sigBC,  $\Delta$ sigBD,  $\Delta$ sigBE,  $\Delta$ sigCD,  $\Delta$ sigCE, and  $\Delta$ sigDE. We show that although all double mutants grow well under standard conditions, the transfer from lag phase to exponential growth is slow in the  $\Delta$ sigBD strain. All strains lacking the SigD factor were found to be sensitive to bright light. Furthermore, all group 2  $\sigma$  factors were found to be involved in acclimation to osmotic stress.

## RESULTS

### Specific Features of the *Synechocystis* RNA Polymerase

We constructed the structural models of the *Synechocystis* RNA polymerase holoenzyme using the crystal structure of the RNA polymerase holoenzyme of *T. thermophilus* (Artsimovitch et al., 2005; Protein Data Bank [PDB] code 2A6E; chains A–F) as a template. The details of the different subunits of the RNA polymerase of *T. thermophilus*, including areas that are missing from the template structure, together with the RNA polymerase subunits of *Synechocystis*, are shown in Supplemental Table S1. The  $\omega$  subunit was excluded from the model because its sequence identity (20%) with the template was too low to ensure reliable modeling. The other subunits were 40% to 50% identical (Supplemental Table S1). Ramachandran plots of the models showed that approximately 90% of amino acid residues were in the most favorable regions and less than 1% were in disallowed regions (the corresponding values for the template were 83.9% and 0.1%), indicating good overall reliability for the models.

A specific feature of the cyanobacterial RNA polymerase is that the  $\beta'$  subunit has been split into two different polypeptides (Schneider and Hasekorn, 1988). The  $\gamma$  subunit of the RNA polymerase in cyanobacteria is homologous with the N-terminal part of the  $\beta'$  subunit of other eubacteria, and the cyanobacterial  $\beta'$  subunit is homologous with the C-terminal part of the

$\beta'$  subunit of other eubacteria. In *Synechocystis*, the  $\gamma$  subunit consists of 626 residues (light gray in Fig. 1) and the  $\beta'$  subunit consists of 1,317 residues (light blue in Fig. 1). According to the model, the splitting has very little effect on the overall structure of the RNA polymerase of *Synechocystis*, as the last amino acid residue of the  $\gamma$  subunit and the first amino acid of the  $\beta'$  subunit are located on the surface of RNA polymerase. Another special feature of the cyanobacterial  $\beta'$  subunit is that it includes a large insertion (Iyer et al., 2004) containing 635 amino acid residues in *Synechocystis*. The first and last amino acid residues of the insertion are shown in magenta in Figure 1A, but the insertion is not included in the model because of the lack of template for modeling. Although the three-dimensional structure of the cyanobacterial insertion remains to be solved, our model suggests that the insertion can be accommodated in the three-dimensional structure without introducing dramatic changes in the other parts of the RNA polymerase holoenzyme (Fig. 1A).

The three-dimensional models (Fig. 1B) and sequence alignment (Fig. 1C) are shown for the primary  $\sigma$  factor and for all group 2  $\sigma$  factors. The SigA factor is included in the holoenzyme model (Fig. 1A). The basic structure of all  $\sigma$  factors is similar, consisting essentially of  $\alpha$ -helices; the  $\alpha$ -helices are shown as colored barrels above the sequence alignment (Fig. 1C). Based on amino acid sequence homology, four homologous regions that are further divided into subregions have been identified in group 1 and 2  $\sigma$  factors (Lonetto et al., 1992). These subregions are indicated as boxes in Figure 1C. The 4.2 region (green) recognizes the  $-35$  promoter element, and the 2.4 region (blue) recognizes the  $-10$  promoter element. According to the three-dimensional models, these elements are similar in group 1 and group 2  $\sigma$  factors. The most variable part of the group 1 and group 2  $\sigma$  factors of *Synechocystis* is the nonconserved domain (NCD; red in Fig. 1) between the conserved domains 1.2 and 2.1. In primary  $\sigma$  factors of different bacteria, the length of the NCD region varies from two (*Bacillus subtilis*) to 315 (*Bradyrhizobium japonicum*) amino acids; in cyanobacteria, the variation is from 40 to 88 amino acids. In *Synechocystis*, the length of the NCD is 86 amino acids in SigA, 42 in SigB, 84 in SigC, 43 in SigD, and 44 in SigE. Based on secondary structure predictions (data not shown), the NCD is suggested to be helical in all group 1 and group 2  $\sigma$  factors of *Synechocystis*. However, sequence identities in the NCDs are low, and thus this area in the models is less reliable than the other regions. Although the length of the NCD is similar in SigB, SigD, and SigE factors, only the NCD sequences of the SigB and SigD factors are similar (47% identity). The long NCDs of SigA and SigC do not show high sequence identity.

### Single and Double Inactivation Strains of Group 2 $\sigma$ Factors in *Synechocystis*

In order to study the role of each group 2  $\sigma$  factor, we constructed single and double inactivation strains. The

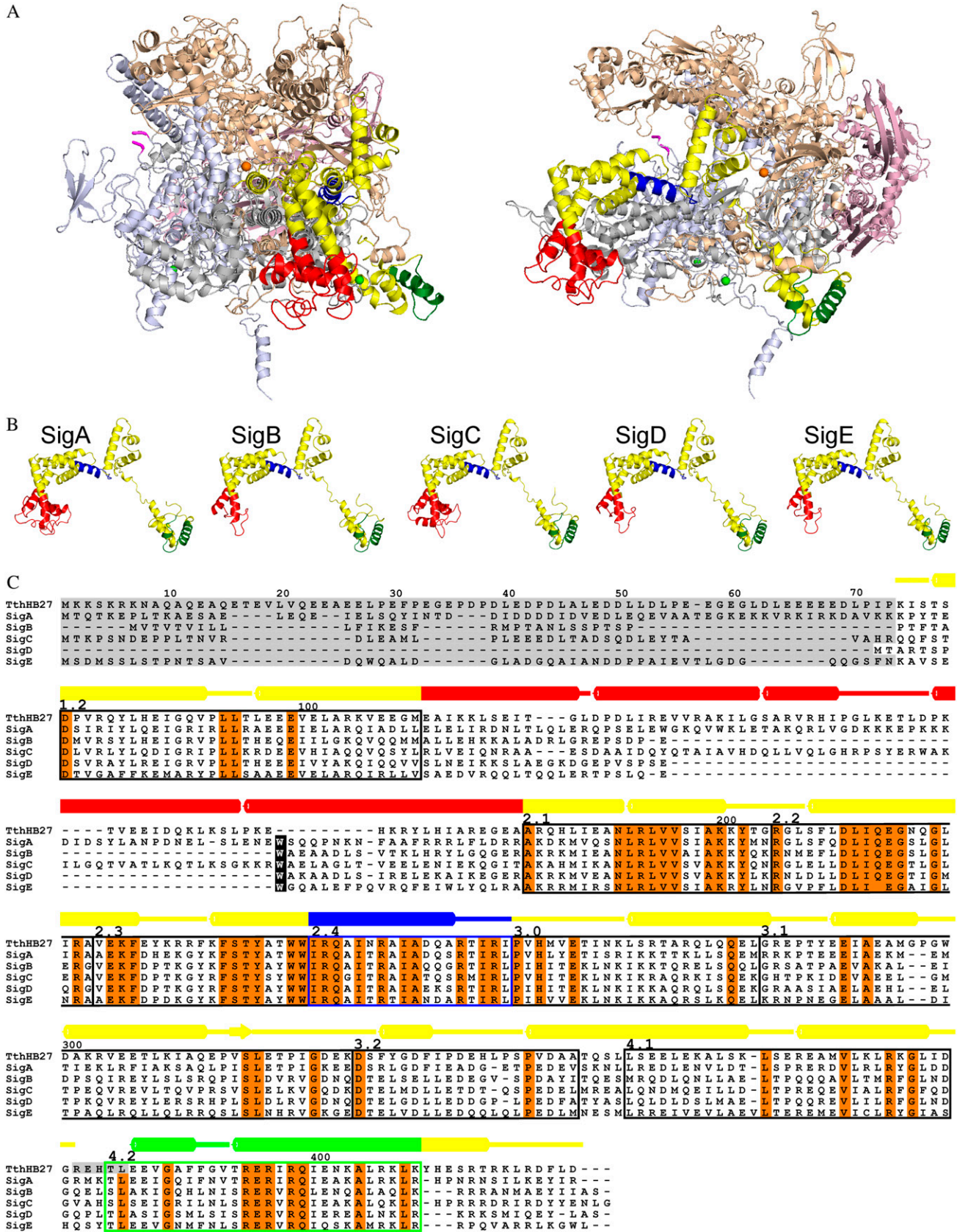


Figure 1. (Legend appears on following page.)

*sigB* (strain  $\Delta$ sigB), *sigC* (strain  $\Delta$ sigC), *sigD* (strain  $\Delta$ sigD), and *sigE* (strain  $\Delta$ sigE) genes were inactivated with a kanamycin (Kn) resistance cassette in *Synechocystis*. The constructs of all inactivation strains are shown in Supplemental Figure S1. The double inactivation strains were constructed by inactivating the second *sig* gene with a streptomycin/spectinomycin (Str/Spc) resistance cassette. The *sigC* gene was inactivated in the  $\Delta$ sigB strain (resulting in  $\Delta$ sigBC), the *sigD* gene was inactivated in the strains  $\Delta$ sigB (resulting in  $\Delta$ sigBD) and  $\Delta$ sigC (resulting in  $\Delta$ sigCD), and the *sigE* gene was inactivated in strains  $\Delta$ sigB (resulting in  $\Delta$ sigBE),  $\Delta$ sigC (resulting in  $\Delta$ sigCE), and  $\Delta$ sigD (resulting in  $\Delta$ sigDE). Two lines that were descendants of two independently raised colonies on the first selection plate were originally tested from each inactivation strain. Because the two lines behaved similarly, the results are shown only for one line. Testing of two independent lines minimizes the possibility that the results could be affected by secondary mutations. PCR verification confirmed that the strains were completely segregated (Supplemental Fig. S2).

First we measured the growth rates of all single and double inactivation strains under standard growth conditions (32°C, continuous light of 40  $\mu$ mol photons  $m^{-2} s^{-1}$ , ambient CO<sub>2</sub> concentration).  $A_{730}$  was set to 0.1, and the growth of a 50-mL cell culture in a 250-mL Erlenmeyer flask was followed for 14 d. All inactivation strains grew autotrophically with the same growth rate as the control strain (Fig. 2A). In our standard conditions, growth was exponential only at the very beginning of the growth experiment; thereafter, the growth was linear for a few days. Finally, growth of the cells slowed down, and by the 14th d the cells were hardly growing (Fig. 2A). As some researchers routinely grow *Synechocystis* cells under CO<sub>2</sub>-enriched atmosphere, we tested the growth of all inactivation strains under otherwise similar conditions as our standard conditions but supplemented the air of the growth chamber with 3% CO<sub>2</sub>. All strains grew faster under high-CO<sub>2</sub> conditions than under ambient CO<sub>2</sub> conditions, the doubling time being 8 h at high CO<sub>2</sub> and 14 h at air level CO<sub>2</sub> during the 1st d, but no differences were detected between the control and inactivation strains (Fig. 2B). The growth rate became slower throughout the whole experiment, both in 3% CO<sub>2</sub> and in ambient CO<sub>2</sub>.

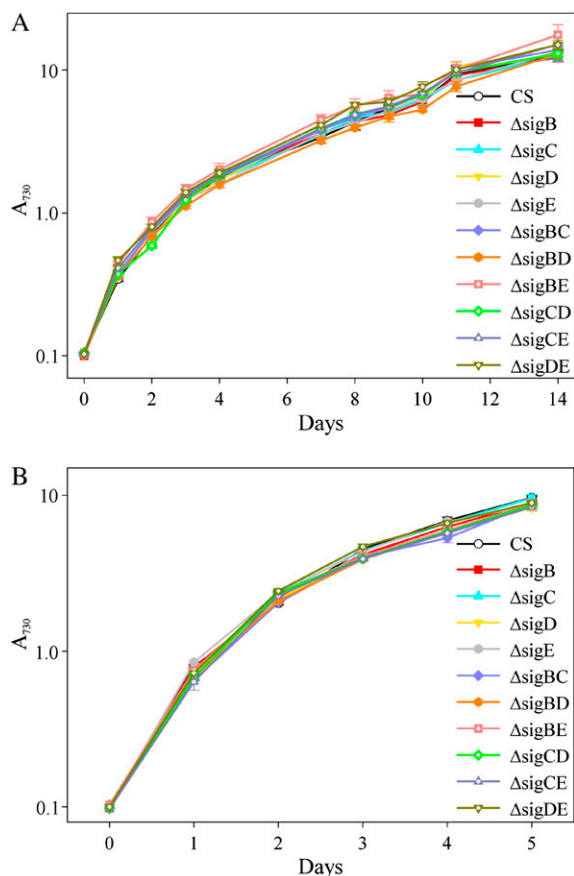
In accordance with similar growth rates, also photosynthetic activity, measured as light-saturated oxygen evolution, was found to be similar in all strains (Fig. 3A). Furthermore, similar PSII electron transport capacity was measured from the control and all inactivation strains (Fig. 3B). These findings indicate that two group 2  $\sigma$  factor genes can be inactivated simultaneously in any combination without affecting the growth rate or photosynthesis under standard growth conditions.

#### All Strains with an Inactivated *sigD* Gene Grow Slowly under Bright Light, and the Transition from Lag to Exponential Phase Is Slow in the $\Delta$ sigBD Strain

To follow the growth of the inactivation strains under different light conditions, dilute *Synechocystis* cultures ( $A_{730}$  was 0.1, corresponding to  $3.6 \times 10^6$  cells  $mL^{-1}$ ) were spotted on BG-11 plates and the plates were grown under constant irradiance of 20, 40, or 80  $\mu$ mol photons  $m^{-2} s^{-1}$  at 32°C. Under low light (20  $\mu$ mol photons  $m^{-2} s^{-1}$ ), the growth of the  $\Delta$ sigBD strain was delayed, and also the  $\Delta$ sigBC strain grew slightly more slowly than the control strain or the other inactivation strains (Fig. 4A).

Under standard growth conditions, the inactivation strains grew like the control strain, except that the  $\Delta$ sigBD strain grew slowly in the spot test (Fig. 4A). This was a surprise for us, as similar growth rates were measured for the control and  $\Delta$ sigBD strains in liquid cultures (Fig. 2) and we did not see any difference between the control and  $\Delta$ sigBD strains when we streaked a new plate using cells directly from the old plate (Fig. 4B). The difference in growth rates between the  $\Delta$ sigBD and control strains became larger when more dilute cultures were used as starting material in the spot experiments, but the difference disappeared completely if dense cultures (10-fold concentration after adjusting  $A_{730}$  to 1.0) were spotted on the plates (Fig. 4B). Because slow growth of the  $\Delta$ sigBD strain on plates was clearly dependent on cell density at the beginning of the experiment, we next tested the cell density dependence of growth in liquid culture. At initial  $A_{730}$  of 0.01, both control and  $\Delta$ sigBD strains grew rapidly. When  $A_{730}$  was 0.001, 100 times more dilute than was used in the beginning of our standard growth curve measurements, the doubling time of the

**Figure 1.** Model of *Synechocystis* RNA polymerase. A, Homology modeling of the RNA polymerase holoenzyme with the SigA factor was performed using the crystal structure of the RNA polymerase holoenzyme of *T. thermophilus* (Artsimovitch et al., 2005; PDB code 2A6E; chains A–F) as the template. Views are from the front toward the catalytic center (left) and from the side (right). The core enzyme consists of two  $\alpha$  subunits (light pink), the  $\beta$  subunit (light brown), the  $\beta'$  subunit (light blue), and the  $\gamma$  subunit (gray). The first and last residues of the cyanobacterial insertion in the  $\beta'$  subunit are indicated with magenta. In the otherwise yellow  $\sigma$  factor, the 4.2 region is green, the 2.4 region is blue, and the NCR connecting the conserved regions 1.2 and 2.1 is red. The catalytic magnesium ion is presented as an orange sphere, and two zinc ions are presented as green spheres. B, Models of principal (SigA) and group 2 (SigB–SigE)  $\sigma$  factors of *Synechocystis*. The coloring is as in A. C, Sequence alignment of *Synechocystis* and *T. thermophilus*  $\sigma$  factors. Absolutely conserved residues are shown in orange, and conserved regions from 1.2 to 4.2 are indicated with boxes. Residues missing from the structures are shaded gray. The numbering of amino acid residues and the secondary structure assignment, with barrels denoting  $\alpha$ -helices, are shown above the alignment for the template. The coloring of secondary structures is as in A. The cyanobacterial conserved Trp residue is indicated with a black background.



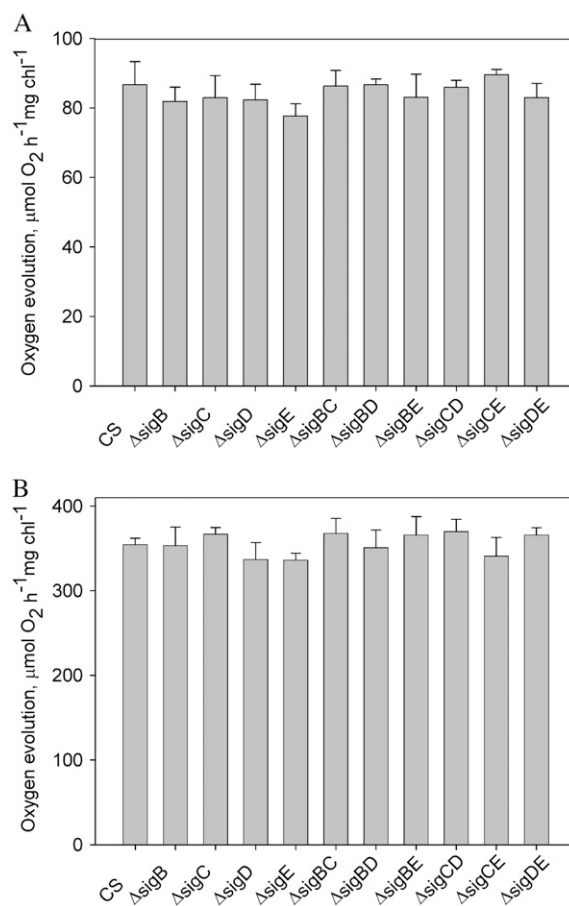
**Figure 2.** Growth of single and double inactivation strains of group 2  $\sigma$  factors of *Synechocystis*. The  $A_{730}$  of the cell culture was set to 0.1, and the cells were grown in BG-11 medium under the continuous illumination of  $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $32^\circ\text{C}$  under air levels of  $\text{CO}_2$  (A) or under 3%  $\text{CO}_2$  (B). Each growth curve represents the mean of five independent experiments, and the error bars denote SE.

control strain was only 6 h during the 1st d, indicating very fast growth. The  $\Delta\text{sigBD}$  strain, in turn, grew slowly during the 1st d, the doubling time being 17 h (Fig. 4C). During the next 2 d, the growth of the control strain slowed down as the density of the culture became higher, but the  $\Delta\text{sigBD}$  strain grew faster, and finally the growth difference between the strains almost disappeared. The finding that only the double mutant  $\Delta\text{sigBD}$ , not  $\Delta\text{sigB}$  or  $\Delta\text{sigD}$ , showed slow growth in dilute culture indicates that the presence of either the SigB or the SigD factor is sufficient for normal efficient transfer of the cells from the lag growth phase to the exponential growth phase. Partial redundancy of the functions of the SigB and SigD factors may be related to the fact that these are the two most homologous  $\sigma$  factors of *Synechocystis*.

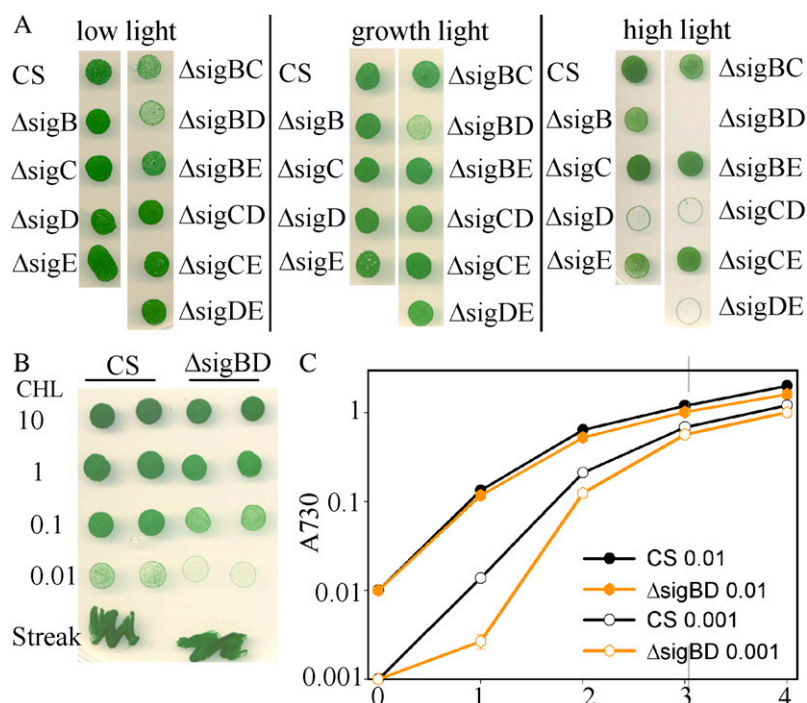
The dependence of the growth of the  $\Delta\text{sigBD}$  strain on cell density suggests that cell-to-cell communication might be important for growth. We tested the possibility that cells of the control and  $\Delta\text{sigBD}$  strains secrete different chemical signals to the growth me-

dium. The  $A_{730}$  was set to 0.001 and the control and  $\Delta\text{sigBD}$  strains were grown for 3 d. Thereafter, the cells were removed from the growth medium and new dilute batch cultures were started so that cells of the control strain grew in used  $\Delta\text{sigBD}$  strain-BG-11 medium and the new  $\Delta\text{sigBD}$  culture was started in used control strain medium. We compared the growth of control and  $\Delta\text{sigBD}$  strains in these and in fresh BG-11 media; at the beginning of the experiment, the  $A_{730}$  was set to 0.001. The  $\Delta\text{sigBD}$  strain always grew more slowly than the control strain, despite the used growth medium (data not shown), indicating that a secreted chemical signal is unlikely to explain the slow growth of the  $\Delta\text{sigBD}$  strain in dilute culture or that the chemical signal is short-lived.

In the spot test experiments, the clearest phenotypes were seen under high-light conditions. Strains with an inactivated *sigD* gene ( $\Delta\text{sigD}$ ,  $\Delta\text{sigCD}$ , and  $\Delta\text{sigDE}$ )



**Figure 3.** Light-saturated rates of photosynthesis (A) and PSII electron transport (B) in the  $\sigma$  factor inactivation strains. The rate of in vivo photosynthetic oxygen evolution was measured with an oxygen electrode in BG-11 medium supplemented with 10 mM  $\text{NaHCO}_3$  under saturating light ( $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $32^\circ\text{C}$ . The light-saturated rate of PSII electron transport was measured similarly as photosynthesis, except that 0.7 mM 2,6-dichlorobenzoquinone was used as an artificial electron acceptor. The measurements were repeated three times using independent liquid cultures each time. The error bars denote SE.



**Figure 4.** Growth of the inactivation strains under different light intensities. **A**, The  $A_{730}$  of each strain was set to 0.1, and 5  $\mu\text{L}$  of each cell culture was spotted on BG-11 plates. The plates were grown under continuous illumination of 20 (low light), 40 (growth light), or 80 (high light)  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 32°C for 7 d (growth and high light) or 8 d (low light). The data shown are representative of three independent experiments showing similar results. **B**, The  $A_{730}$  of the control (CS) and  $\Delta\text{sigBD}$  strains was set to 10, 1, 0.1, or 0.01, and 5  $\mu\text{L}$  of each dilution was spotted on BG-11 plates or cells were streaked directly from an old plate. The data shown are representative of three independent experiments showing similar results. **C**, Growth of the control and  $\Delta\text{sigBD}$  strains in liquid culture. The  $A_{730}$  of the cell culture was set to 0.01 (closed symbols) or 0.001 (open symbols), and the cells were grown in BG-11 medium under continuous illumination of 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 32°C for 4 d. Each growth curve represents the mean of three independent experiments, and the error bars denote SE.

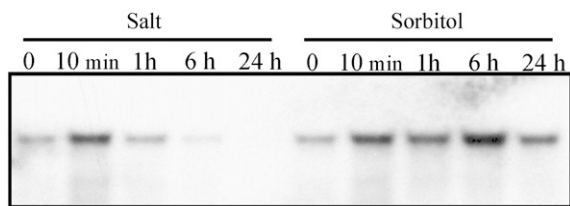
hardly grew at all at 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the double inactivation strain  $\Delta\text{sigBD}$  died (Fig. 4A). These results indicate that the SigD factor is extremely important for acclimation to bright light and further underline the redundancy of the functions of the SigB and SigD factors. The results also show that the antibiotic resistance cassette used to inactivate the gene does not interfere with the results, as the *sigD* gene was inactivated with a Kn cassette in strains  $\Delta\text{sigD}$  and  $\Delta\text{sigDE}$  and with a Str/Spc cassette in strains  $\Delta\text{sigBD}$  and  $\Delta\text{sigCD}$  (Supplemental Fig. S1).

#### All Group 2 $\sigma$ Factors Are Involved in Osmotic Acclimation of *Synechocystis*

Our earlier experiments indicated activation of the *sigB* gene by a short osmotic shock (Tuominen et al., 2003). We studied the expression of the *sigB* gene in more detail by following the amount of *sigB* transcripts under salt- and sorbitol-induced osmotic stress in the control strain. Addition of 0.7 M NaCl induced an 8-fold increase in the amount of *sigB* transcripts within 10 min, and after a 1-h treatment, the amount of *sigB* transcripts was still twice as high as that measured under the standard growth conditions (Fig. 5). Thereafter, the amount of *sigB* transcripts decreased below the amount measured under the standard growth conditions, and only traces of *sigB* transcripts were detected after 24 h of salt treatment (Fig. 5). Sorbitol-induced osmotic stress, in turn, caused more permanent up-regulation of *sigB* transcripts, and three times as high levels of *sigB* transcripts, compared with the amount measured under standard growth conditions, were measured even after 24 h of sorbitol-induced

osmotic stress (Fig. 5). In addition to the SigB factor, the SigD factor also has been suggested to be involved in signal transduction of short high-salt and high-sorbitol shock treatments (Shoumskaya et al., 2005). To get a more comprehensive picture of the importance of the different  $\sigma$  factors under osmotic stress, we grew cells of all inactivation strains for 5 d in BG-11 medium supplemented with either 0.7 M NaCl or 0.5 M sorbitol. These concentrations of salt and sorbitol were chosen because they slowed down the growth of the control strain by approximately 30% in our test experiments.

The growth of the  $\Delta\text{sigB}$  strain was seriously retarded in high-salt conditions and practically ceased after 2 d (Fig. 6A), while the  $\Delta\text{sigD}$  strain grew almost as well as the control strain, and only after a prolonged salt stress did the growth of the  $\Delta\text{sigD}$  strain decrease slightly compared with the control strain. Inactivation strains  $\Delta\text{sigC}$  and  $\Delta\text{sigE}$  grew slowly under salt stress throughout the experiment (Fig. 6A). From the double mutants, the  $\Delta\text{sigBC}$ ,  $\Delta\text{sigBD}$ , and  $\Delta\text{sigBE}$  strains grew as slowly as the  $\Delta\text{sigB}$  strain (Fig. 6B), indicating that inactivation of another group 2  $\sigma$  factor in addition to the SigB factor did not cause a more severe phenotype under high-salt stress. The other double mutant strains,  $\Delta\text{sigCD}$ ,  $\Delta\text{sigCE}$ , and  $\Delta\text{sigDE}$ , grew similarly as the single inactivation strains  $\Delta\text{sigC}$  and  $\Delta\text{sigE}$ . These results suggest that SigB is the most crucial  $\sigma$  factor for acclimation to high salt. In addition, the SigC and SigE factors are required for optimal acclimation to high-salt stress. The SigD factor, in turn, has only a minor role, if any, for salt acclimation. Furthermore, these results indicate that the redundancy of the SigD and SigB factors does not extend to all functions of these two  $\sigma$  factors.



**Figure 5.** Accumulation of *sigB* mRNA in the control strain under osmotic stress. The growth medium was supplemented with 0.7 M NaCl or 0.5 M sorbitol, as indicated, and samples were withdrawn before the addition (0) and after 10 min and 1, 6, and 24 h of incubation. Thereafter, total RNA was isolated and the amount of *sigB* mRNA was detected with the northern-blot technique. The data shown are representative of three independent northern-blot experiments showing similar results.

In accordance with the results of the high-salt experiments, all inactivation strains with inactivated *sigB* gene were susceptible to sorbitol-induced osmotic stress (Fig. 7, A and B). Contrary to salt-induced osmotic stress, inactivation of the SigE factor had only a minor effect on growth when osmotic stress was induced with sorbitol (Fig. 7A). The  $\Delta$ *sigC* strain showed reduced growth, but the effect of the SigC factor was milder under sorbitol-induced stress than under salt-induced stress. The SigD factor, in turn, was more important for acclimation to high sorbitol than for high-salt stress. Some double mutants showed peculiar behavior, as both  $\Delta$ *sigBE* and  $\Delta$ *sigBC* actually grew slightly better than the  $\Delta$ *sigB* strain. The reasons for this behavior remain to be solved.

## DISCUSSION

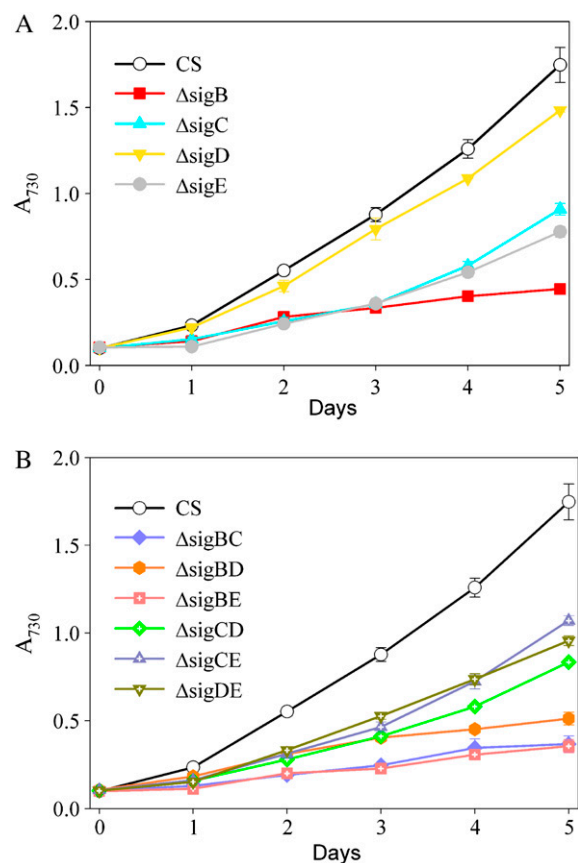
Cyanobacterial genomes typically code for several group 2  $\sigma$  factors. Studies of single inactivation strains of different group 2  $\sigma$  factors in *Synechocystis* (Asayama et al., 2004; Tuominen et al., 2006, 2008; Summerfield and Sherman, 2007), *Anabaena* sp. PCC 7120 (Khudyakov and Golden, 2001), *Synechococcus* sp. PCC 7002 (Caslake et al., 1997), and *Synechococcus* sp. PCC 7942 (Goto-Seki et al., 1999) as well as double inactivation strains in *Synechocystis* (Fig. 2) indicate that these  $\sigma$  factors have little or no effect on growth under optimal conditions. Evidence accumulated in recent years strongly suggests that group 2  $\sigma$  factors are required for acclimation under stress conditions (Table I).

The SigB factor can be considered as a general stress-responsive  $\sigma$  factor. Rapid transient increase of SigB transcripts has been detected in high-salt stress (Fig. 5), in heat stress (Imamura et al., 2003; Shoumskaya et al., 2005; Tuominen et al., 2006), and after a dark-to-light shift (Tuominen et al., 2003). Furthermore, up-regulation of the *sigB* gene has been found to occur under oxidative stress induced by hydrogen peroxide treatment (Kanesaki et al., 2007) and under nitrogen starvation (Imamura et al., 2006). SigB factor has also been implicated to be involved in the regulation of

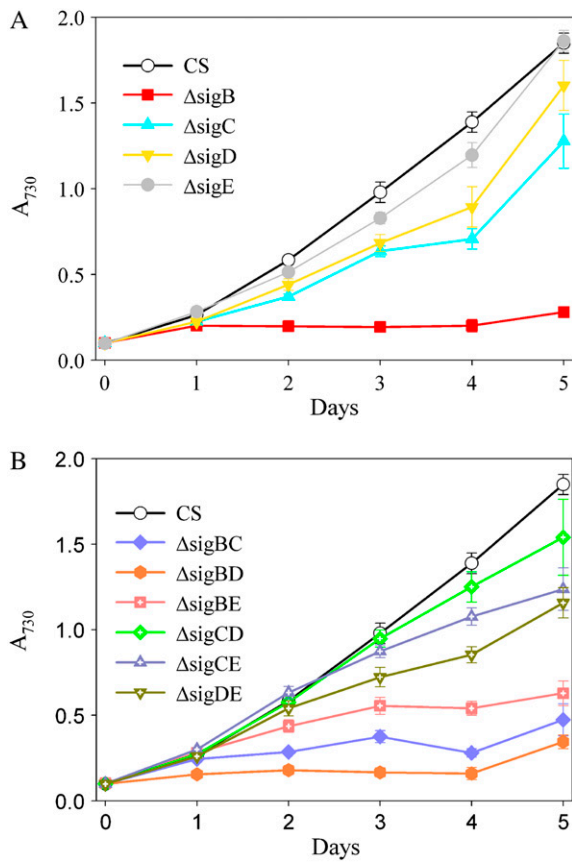
many genes in light-dark transitions (Summerfield and Sherman, 2007) and in exponential or linear growth phases (Foster et al., 2006), but in these studies the growth of the control and  $\Delta$ *sigB* strains was similar, suggesting that in these cases SigB can be complemented by other  $\sigma$  factors.

We show in this study that the SigB factor is rapidly up-regulated by osmotic stress induced by salt and by sorbitol and that cells do not grow without SigB under these conditions. On the other hand, similar rapid transient up-regulation of the *sigB* gene occurs at mild heat stress at 43°C, but the  $\Delta$ *sigB* strain grows almost as well as the control strain (Tuominen et al., 2006). These results indicate that there is no simple direct correlation between the increase in *sigB* transcripts and the importance of the SigB factor under a particular stress condition.

It is well documented that the SigB factor is involved in the up-regulation of heat shock genes, especially the *hspA* gene, at high temperatures (Imamura et al., 2003; Singh et al., 2006; Tuominen et al., 2006). Many heat shock proteins, particularly HspA, are important chaperones



**Figure 6.** Growth of single (A) and double (B) inactivation strains under high-salt stress. The  $A_{730}$  of the cell culture was set to 0.1, and the cells were grown in BG-11 medium supplemented with 0.7 M NaCl under continuous illumination of 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 32°C. Each growth curve represents the mean of three independent experiments, and the error bars denote SE.



**Figure 7.** Growth of single (A) and double (B) inactivation strains under high-sorbitol stress. The  $A_{730}$  of the cell culture was set to 0.1, and the cells were grown in BG-11 medium supplemented with 0.5 M sorbitol under continuous illumination of  $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $32^\circ\text{C}$ . Each growth curve represents the mean of at least five independent experiments, and the error bars denote SE.

in acclimation to osmotic stress (Asadulghani et al., 2004). We compared the expression of the *hspA* gene in the control and  $\Delta\text{sigB}$  strains in osmotic stresses. In salt stress, the amount of *hspA* mRNA remained lower in  $\Delta\text{sigB}$  than in the control strain (Supplemental Fig. S3), but the expression kinetics was similar in both strains. However, when osmotic stress was induced with sorbitol, the induction of the *hspA* gene was slower in the  $\Delta\text{sigB}$  strain than in the control strain, but after 1 h the amount of *hspA* mRNA in  $\Delta\text{sigB}$  exceeded that in the control strain (Supplemental Fig. S3). Interestingly, an *hspA* inactivation strain has been found to tolerate well mild (0.5 M NaCl) salt-induced osmotic stress (Asadulghani et al., 2004), and we have noticed that the  $\Delta\text{sigB}$  strain grows well in BG-11 medium supplemented with 0.5 M NaCl (data not shown). These results indicate that inactivation of the *sigB* gene affects the expression of the *hspA* gene not only in heat stress but also in salt- and sorbitol-induced stresses.

In this study, we show that the SigC factor is involved in acclimation to salt stress and also to a lesser

extent to sorbitol stress. Previously, SigC of *Synechocystis* and SigE, its closest homolog in *Synechococcus* sp. PCC 7002, were assigned roles in stationary phase-related gene expression and growth (Gruber and Bryant, 1998; Asayama et al., 2004; Imamura et al., 2006) and in *Synechocystis* also in acclimation to heat stress (Tuominen et al., 2008). We did not see differences in growth rate between the control and  $\Delta\text{sigC}$  strains during the studied 14 d in standard conditions (Fig. 2), but Asayama et al. (2004) noticed a slight delay of growth in a *sigC* inactivation strain after 3 weeks. A unique feature of the SigC factor is that the *sigC* gene is not specifically up-regulated at high temperature (Tuominen et al., 2008) or under osmotic stress (Tuominen et al., 2003, Shoumskaya et al., 2005) or stationary phase (Asayama et al., 2004), yet SigC factor is required for acclimation to those conditions. These results suggest that the *sigC* gene might be regulated posttranscriptionally. One possible explanation is that the RNA polymerase core has an increased tendency to recruit the SigC factor under these stress conditions. In *Escherichia coli*, the small signaling molecule ppGpp (for guanosine 5'-diphosphate 3'-diphosphate) directly binds to the RNA polymerase core (Artsimovitch et al., 2004), and active transcription from promoters that depend on  $\sigma^S$ , the only group 2  $\sigma$  factor in *E. coli*, requires ppGpp (Kvint et al., 2000). A similar system might explain why SigC is important in conditions in which the *sigC* gene is not up-regulated.

All strains with inactivated *sigD* gene were unable to grow under high light. DNA microarray analysis has shown that the expression of the *sigD* gene increases in response to high light in *Synechocystis* (Hihara et al., 2001; Huang et al., 2002). Furthermore, Imamura et al. (2003) reported an increase in the amount of SigD protein after high-light treatment. In another cyanobacterium, *Synechococcus elongatus* PCC 7942, one of the five group 2  $\sigma$  factors, RpoD3, was recognized as a high-light-responsive  $\sigma$  factor (Seki et al., 2007). The amino acid sequences of the RpoD3 factor of *Synechococcus elongatus* PCC 7942 and the SigD factor of *Synechocystis* suggest that these factors are closely related (Seki et al., 2007).

Enhancement of the expression of the *sigD* gene has been detected in both sorbitol- and salt-induced stresses (Shoumskaya et al., 2005). However, inactivation of the *sigD* gene only slightly slowed down the growth of *Synechocystis* under high-salt or high-sorbitol conditions (Figs. 6 and 7), and our earlier experiments did not show induction of *sigD* under salt stress (Tuominen et al., 2003). Shoumskaya et al. (2005) used more bright light in their experiments than we used in our experiments; thus, the amount of light during osmotic stress might explain the differences in the results. Interestingly, the SigD factor was suggested to be part of a signaling cascade in which signals are transduced by His kinase (Hik) 33 and the cognate response regulator (Rre) 31 during osmotic acclimation (Marin et al., 2003; Shoumskaya et al., 2005). We



**Table 1.** Growth of group 2  $\sigma$  factor inactivation strains under different environmental conditions

Growth is compared with the growth of the control strain under the same conditions. +++, Similar growth as in the control strain; ++(+), slightly slower growth; ++, slower growth; +, only very slow growth; +/-, slow growth for a short time; -, no growth; ND, not determined.

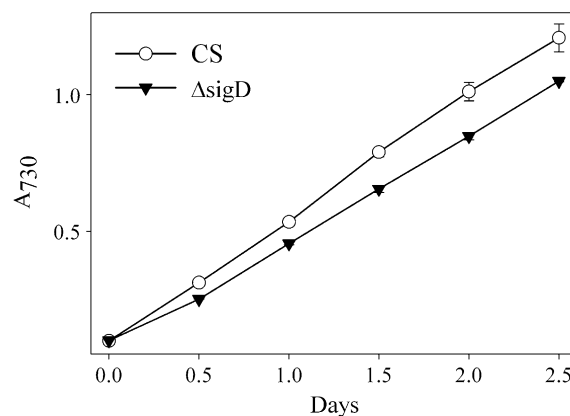
Growth Conditions	Inactivated <i>sig</i> Gene				Reference
	<i>sigB</i>	<i>sigC</i>	<i>sigD</i>	<i>sigE</i>	
Standard	+++	+++	+++	+++	This study
High CO <sub>2</sub>	+++	+++	+++	+++	This study
Heat	++(+)	+/-	++(+)	ND	Tuominen et al. (2006, 2008)
Salt stress	+/-	+	++(+)	+	This study
Sorbitol	+/-	++	++	++(+)	This study
High light	+++	+++	+	+++	This study
Light-activated heterotrophic growth	ND	ND	ND	-	Osanai et al. (2005)
Mixotrophic growth, 8 h of light/16 h of dark	+++	ND	+++	+	Summerfield and Sherman (2007)

noticed that genes (all 10 genes under salt stress and 11 of 12 genes under sorbitol stress) belonging to the Hik33-Rre31 cascade were among those genes that were up-regulated under UV light and high-light stress conditions (Huang et al., 2002). Furthermore, almost all of these same genes were less induced in the  $\Delta$ Hik33 strain than in the wild-type strain under oxidative stress caused by hydrogen peroxide treatment (Kanesaki et al., 2007). Because UV light and high-intensity visible light, as well as treatment with high salt, can induce the production of reactive oxygen species, it is possible that the SigD factor is required for acclimation to conditions that enhance the production of reactive oxygen species. We tested the effect of mild oxidative stress by growing the  $\Delta$ sigD and control strains in the presence of 0.1  $\mu$ M methyl viologen. Methyl viologen accepts an electron from PSI and reduces oxygen to O<sub>2</sub><sup>-</sup>, which is converted to hydrogen peroxide by superoxide dismutase. *Synechocystis* can grow in the presence of 0.5  $\mu$ M methyl viologen in moderate light (Tichy and Vermaas, 1999). Figure 8 shows that the  $\Delta$ sigD strain grew slightly more slowly than the control strain. This finding supports our idea that the SigD factor is involved in acclimation to oxidative stress.

The SigE factor was important for acclimation to salt stress, but growth of the  $\Delta$ sigE strain was only slightly affected under sorbitol stress (Figs. 6 and 7). Cyanobacteria accumulate the osmoprotective solute gluco-sylglycerol in response to salt stress, whereas sorbitol itself accumulates in cyanobacterial cells (Marin et al., 2006). Sorbitol was also shown to induce a more pronounced efflux of water from cells than salt (Kanesaki et al., 2002). DNA microarray analysis has revealed that although many genes are up-regulated or down-regulated in both salt-induced and sorbitol-induced osmotic stress, many differences are also found (Kanesaki et al., 2002; Shoumskaya et al., 2005). According to our results, SigE has an important role only in acclimation to salt stress. Earlier studies have also shown that

the SigE factor is involved in the regulation of sugar catabolic pathways (Osanai et al., 2005). In addition, inactivation of the *SigE* gene was found to affect the expression of photosynthetic genes in light-dark transition (Yoshimura et al., 2007), but in our continuous light experiments the  $\Delta$ sigE strain grew well in all tested light intensities.

The fact that the three-dimensional models of the SigB and SigD factors (Fig. 1) are the most similar among *Synechocystis*  $\sigma$  factors, and the finding that only the  $\Delta$ sigBD strain (not  $\Delta$ sigB or  $\Delta$ sigD) shows slow transition from lag phase to exponential growth (Fig. 4), suggest partial redundancy in the functions of these two  $\sigma$  factors. Furthermore, the  $\Delta$ sigBD strain is more sensitive to heat stress (Tuominen et al., 2006) and to high light (Fig. 4) than  $\Delta$ sigB or  $\Delta$ sigD. However, under osmotic stress, the  $\Delta$ sigBD strain was not



**Figure 8.** Growth of the  $\Delta$ sigD and control strains under mild oxidative stress. The A<sub>730</sub> of the cell culture was set to 0.1, 0.1  $\mu$ M methyl viologen was added, and the cells were grown under continuous irradiance of 40  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> at 32°C under air levels of CO<sub>2</sub>. Each growth curve represents the mean of three independent experiments, and the error bars denote SE.

more sensitive than the  $\Delta$ sigB strain. Of the other double mutants, the  $\Delta$ sigB $\Delta$ sigE strain was shown to grow badly under mixotrophic conditions in a 12-h-light/12-h-dark rhythm, although  $\Delta$ sigB and  $\Delta$ sigE strains grew well (Summerfield and Sherman, 2007). Both  $\Delta$ sigB and  $\Delta$ sigC strains are sensitive to heat stress (Tuominen et al., 2006, 2008). These two  $\sigma$  factors regulate completely different sets of genes at high temperature, which makes the double mutant  $\Delta$ sigBC extremely sensitive to heat stress (Tuominen et al., 2008).

In conclusion, the overall three-dimensional structures of all group 1 and 2  $\sigma$  factors in *Synechocystis* resemble each other, despite the NCD, which lies right next to the  $-10$  promoter region in the three-dimensional structure (Fig. 1A) and thus might play a regulatory role in promoter binding. The overall structural similarity suggests that the different  $\sigma$  factors may have overlapping functions. The physiological results obtained with the single and double inactivation strains support this suggestion. In particular, functional redundancy of group 2  $\sigma$  factors is confirmed by the finding that all possible combinations of group 2  $\sigma$  factor double inactivation mutants are viable in *Synechocystis*. Functional redundancy, found in transition from lag phase to exponential growth, is obvious between the SigB and SigD factors that show significant sequence identity even within the NCD connecting the conserved domains 1.2 and 2.1. However, treatments under different stress conditions also revealed that each group 2  $\sigma$  factor is important under a specific set of conditions. The SigD factor is involved in high-light responses in *Synechocystis*, and all group 2  $\sigma$  factors have a role in acclimation to salt- or sorbitol-induced osmotic stress, the SigB factor being the most important one.

## MATERIALS AND METHODS

### Structural Modeling of *Synechocystis* RNA Polymerase Holoenzyme

The amino acid sequences of the subunits of RNA polymerase of *Synechocystis* (Table I) were obtained from CyanoBase (www.kazusa.or.jp/cyanobase) and aligned with the sequences of their respective counterparts from the *Thermus thermophilus* RNA polymerase structure (Artsimovitch et al., 2005; PDB code 2A6E; chains A–F were used as the structural template) with the program MALIGN (Johnson and Overington, 1993) in the Bodil visualization and modeling package (Lehtonen et al., 2004). The secondary structure predictions for the sequences were made with PredictProtein (Rost and Liu, 2003). As the sequence identities between the subunits of *T. thermophilus* and *Synechocystis* RNA polymerases were reasonably high (Supplemental Table S1), alignment was fairly straightforward. The only exceptions were the  $\sigma$  factors, which were aligned as follows: First, SigA and SigC were aligned with the template, the alignment was fixed, and then SigB, SigD, and SigE were aligned with the previously fixed alignment. Finally, the alignment was modified so that the *Synechocystis*  $\sigma$  factors have a conserved Trp at the end of the NCD before region 2.1 (Fig. 1C). Structural modeling was done with MODELLER (Šali and Blundell, 1993) using the very thorough variable target function optimization method. Ten models were generated for each of the  $\sigma$  factors, and those with lowest objective function, as given by MODELLER, were chosen for further investigation. The stereochemical quality of the models was assessed with PROCHECK (Laskowski et al., 1993).

### Construction of Group 2 $\sigma$ Factor Inactivation Strains in *Synechocystis*

The Glc-tolerant strain of *Synechocystis* (Williams, 1988) was used as a control strain. The *sigB* (*sl10306*), *sigC* (*sl10184*), *sigD* (*sl12012*), and *sigE* (*sl11689*) genes were amplified by PCR with primers specific for *sigB* (5'-ATGGTAA-CAGTGACAGTTAT-3' and 5'-TAGCTCTGGCCATCGTTA-3'), *sigC* (5'-ATG-ACTAAACCAAGCAACGA-3' and 5'-AATCTAGCAAAATTCCTGC-3'), *sigD* (5'-ATGACTGCCAGAACCAGCCC-3' and 5'-GCCTCCTACAGTTG-GATCT-3'), and *sigE* (5'-ATGAGCGATATGCTTCCCT-3' and 5'-CTATAAC-CAACCTTGAGGC-3'). The PCR products were cloned into the pCR-Blunt II-TOPO vector (Invitrogen). The pCR-Blunt II-TOPO-*sigB* was digested with *KpnI* and *PstI*, and the *sigB* fragment was ligated into the *KpnI* and *PstI* double-digested pUC19. The pUC19-*sigB* was digested with *SmaI*, and *BamHI* polylinker (New England Biolabs) was added. The inactivation plasmid pUC19-*sigB*-Kn was constructed by ligating the *BamHI* fragment of pUC4K (Amersham Biosciences), carrying the Kn resistance cassette, into the *BamHI* site. The pCR-Blunt II-TOPO-*sigC* was digested with *SpeI* and *EcoRV*, and the *sigC* fragment was ligated into the *XbaI* and *SmaI* double-digested pUC19. To construct the inactivation plasmid pUC19-*sigC*-Kn, the pUC19-*sigC* was digested with *BglIII* and the *BamHI* fragment of pUC4K was then ligated into the *BglIII* restriction site. To construct the inactivation plasmid pUC19-*sigC*- $\Omega$ , the *BamHI* fragment (the  $\Omega$  fragment that confers resistance for *Spc* and *Str*) of pHP45 $\Omega$  (Prentki and Krisch, 1984) was ligated into *BglIII*-digested pUC19-*sigC*. The pCR-Blunt II-TOPO-*sigD* was digested with *SpeI* and *EcoRV*, and the *sigD* fragment was ligated into the *XbaI* and *SmaI* double-digested pUC19. The pUC19-*sigD*-Kn was constructed by ligating the *BamHI* fragment of pUC4K into *BamHI*-digested pUC19-*sigD*. To construct the inactivation plasmid pUC19-*sigD*- $\Omega$ , the *BamHI* fragment of pHP45 $\Omega$  was ligated into *BamHI*-digested pUC19-*sigD*. The pCR-Blunt II-TOPO-*sigE* was digested with *PstI* and *EcoRI*, and the *sigE* fragment was ligated into the *PstI* and *EcoRI* double-digested pUC19. The inactivation plasmids pUC19-*sigE*-Kn and pUC19-*sigE*- $\Omega$  were constructed by ligating the *BamHI* fragment of pUC4K and the *BamHI* fragment of pHP45 $\Omega$ , respectively, into *BamHI*-digested pUC19-*sigD*.

The control strain was transformed with the vector pUC19-*sigB*-Kn, pUC19-*sigC*-Kn, pUC19-*sigD*-Kn, or pUC19-*sigE*-Kn according to Williams (1988). Transformants were isolated on selective BG-11 agar plates containing Kn (50  $\mu$ g mL<sup>-1</sup>). For double mutants, the  $\Delta$ sigB and  $\Delta$ sigC inactivation strains were transformed with pUC19-*sigD*- $\Omega$ ; the  $\Delta$ sigB,  $\Delta$ sigC, and  $\Delta$ sigD inactivation strains were transformed with pUC19-*sigE*- $\Omega$ ; and the  $\Delta$ sigB strain was transformed with pUC19-*sigC*- $\Omega$ . Transformants were isolated on BG-11 agar plates containing Kn (25  $\mu$ g mL<sup>-1</sup>), *Spc* (20  $\mu$ g mL<sup>-1</sup>), and *Str* (10  $\mu$ g mL<sup>-1</sup>).

The complete replacement of the native gene with the inactivated gene was confirmed by PCR analysis of corresponding genomic DNA. Genomic DNA was isolated (Williams, 1988), and the *sigB*, *sigC*, *sigD*, or *sigE* gene, depending on the strain, was amplified by PCR using the same primers as in the cloning procedure.

### Growth Conditions and Measurements

*Synechocystis* was grown in BG-11 medium (Rippka et al., 1979) supplemented with 20 mM HEPES-NaOH, pH 7.5, under the continuous photosynthetic photon flux density (PPFD) of 40  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> and ambient CO<sub>2</sub> levels at 32°C. Liquid cultures were shaken at 90 rpm. These are referred to as standard growth conditions. For high-CO<sub>2</sub> conditions, air was supplemented with 3% CO<sub>2</sub>. The BG-11 agar plates for strains  $\Delta$ sigB,  $\Delta$ sigC,  $\Delta$ sigD, and  $\Delta$ sigE were supplemented with Kn (50  $\mu$ g mL<sup>-1</sup>), and plates for strains  $\Delta$ sigBD,  $\Delta$ sigBC,  $\Delta$ sigBE,  $\Delta$ sigCD,  $\Delta$ sigCE, and  $\Delta$ sigDE were supplemented with Kn (25  $\mu$ g mL<sup>-1</sup>), *Spc* (20  $\mu$ g mL<sup>-1</sup>), and *Str* (10  $\mu$ g mL<sup>-1</sup>). For the experiments, cells were grown without antibiotics in liquid BG-11 medium.

The  $A_{730}$  of liquid cultures was set to 0.1, and the growth of the cells (50 mL of cell culture in a 250-mL Erlenmeyer flask) was monitored under standard growth conditions (PPFD of 40  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, 32°C, shaking at 90 rpm) by measuring  $A_{730}$  for 14 d. Samples of dense cultures were diluted with BG-11 before the absorbance was measured, so that  $A_{730}$  did not exceed 0.4, and the dilutions were taken into account when the final results were calculated. In addition, growth of the control and  $\Delta$ sigBD strains was followed so that  $A_{730}$  was set to 0.01 and 0.001 at the beginning of the growth experiment. Osmotic stress was induced by supplementing BG-11 medium with either 0.7 M NaCl or 0.5 M sorbitol, as indicated. The  $A_{730}$  was set to 0.1, and the growth of the cells was monitored under standard growth conditions (PPFD of 40  $\mu$ mol m<sup>-2</sup>

$s^{-1}$ , 32°C) for 5 d. Oxidative stress was induced by supplementing BG-11 medium with 0.1  $\mu\text{M}$  methyl viologen. The  $A_{730}$  was set to 0.1, and the growth of the cells was monitored under standard growth conditions (PPFD of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 32°C) for 2.5 d.

The growth of the inactivation strains was screened on plates under various light conditions. The  $A_{730}$  of each strain was set to 0.1, and 5  $\mu\text{L}$  of each cell suspension was spotted onto a BG-11 plate. The plates were then kept under continuous light at the PPFD of 20, 40, or 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , as indicated, and photographed at the indicated times. The effect of dilution on growth on solid medium was tested in  $\Delta\text{sigBD}$  and control strains. The  $A_{730}$  of the cell suspension was first set to 10, 1, 0.1, 0.01, and 0.001, and 5  $\mu\text{L}$  of the cell suspensions was spotted onto BG-11 plates. The plates were kept in the standard growth conditions and photographed after 1 week.

## Determination of Photosynthetic and PSII Capacity

In vivo photosynthetic activity of the control and inactivation strains (1 mL of cell suspension containing 10  $\mu\text{g}$  chlorophyll  $\text{mL}^{-1}$ ) was measured in BG-11 medium supplemented with 10 mM  $\text{NaHCO}_3$  under saturating light (500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) with a Clark-type oxygen electrode (Hansatech) at 32°C. PSII capacity was measured similarly as photosynthetic capacity, except that 0.7 mM 2,6-dichloro-*p*-benzoquinone was used as an artificial electron acceptor and 0.7 mM ferricyanide was added to keep the quinone in an oxidized form.

## RNA Isolation and Northern Blotting

Total RNA was isolated as described by Tyystjärvi et al. (2001). The RNAs were separated on 1.2% agarose-glyoxal gels and subsequently transferred to Hybond- $\text{N}^+$  membranes (Amersham Biosciences) according to standard procedures (Sambrook and Russell, 2001). A 7- $\mu\text{g}$  aliquot of total RNA was loaded per lane. Equal loading of the gels was confirmed by methylene blue staining (Sambrook and Russell, 2001). The gene-specific probes were amplified by PCR with primers specific for *sigB* (5'-TGTAACAGTGACAGTTAT-3' and 5'-GCTTCAATCATTTCCTGTTT-3') and for *hspA/sll1514* (5'-GTCCTCATTCTTTACAATCC-3' and 5'-TTAGGAAAGCTGAACCTTTCAC-3'). The probes were labeled, hybridized, and detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the instruction manual of the kit.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** The  $\sigma$  factor inactivation strains of *Synechocystis*.

**Supplemental Figure S2.** PCR analysis of inactivation strains.

**Supplemental Figure S3.** The amount of *hspA* transcripts in the control and  $\Delta\text{sigB}$  strains under salt or sorbitol stress.

**Supplemental Table S1.** Similarity of RNA polymerase subunits in *Synechocystis* and *T. thermophilus*.

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