Effects of Deficiency and Overdose of Group 2 Sigma Factors in Triple Inactivation Strains of *Synechocystis* sp. Strain PCC 6803^{∇†}

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Acclimation of cyanobacteria to environmental changes includes major changes in the gene expression patterns partly orchestrated by the replacement of a particular σ subunit with another in the RNA polymerase holoenzyme. The cyanobacterium *Synechocystis* sp. strain PCC 6803 encodes nine σ factors, all belonging to the σ^{70} family. Cyanobacteria typically encode many group 2 σ factors that closely resemble the principal σ factor. We inactivated three out of the four group 2 σ factors of *Synechocystis* simultaneously in all possible combinations and found that all triple inactivation strains grow well under standard conditions. Unlike the other strains, the $\Delta sigBCD$ strain, which contains SigE as the only functional group 2 σ factor, did not grow faster under mixotrophic than under autotrophic conditions. The SigB and SigD factors were important in low-temperature acclimation, especially under diurnal light rhythm. The $\Delta sigBCD$, $\Delta sigBCE$, and $\Delta sigBDE$ strains were sensitive to high-light-induced photoinhibition, indicating a central role of the SigB factor in high-light tolerance. Furthermore, the $\Delta sigBCE$ strain (SigD is the only functional group 2 σ factor) appeared to be locked in the high-fluorescence state (state 1) and grew slowly in blue but not in orange or white light. Our results suggest that features of the triple inactivation strains can be categorized as (i) direct consequences of the inactivation of a particular σ factor(s) and (ii) effects resulting from the higher probability that the remaining group 2 σ factors associate with the RNA polymerase core.

Cyanobacteria are eubacteria capable of oxygen-producing photosynthesis, and the chloroplasts of plants and algae evolved from cyanobacteria (26). *Synechocystis* sp. strain PCC 6803 (here *Synechocystis*) is a unicellular cyanobacterium commonly used as a model organism (11). The glucose-tolerant strain of *Synechocystis* is especially popular, as genetic manipulation of this naturally competent strain is easy, and the cells can be grown under autotrophic, mixotrophic, photoheterotrophic, or light-activated heterotrophic growth conditions (1, 38).

Light is among the key environmental factors, and cyanobacteria are able to respond to both light quality and light quantity. Cyanobacteria acclimate to light intensity changes by adjusting the expression of many genes, including those coding for components of the photosynthetic apparatus (8–10). In addition, the orange carotenoid protein (OCP) (39, 40), the IsiA pigment protein complex (7, 41), high-light-induced proteins (6, 36), and the efficient photosystem II (PSII) repair cycle (33, 34) keep the photosynthetic machinery functional under high-light conditions. The most dramatic light quality effect is seen in those cyanobacteria that perform complementary chromatic adaptation (15, 17). They change color from blue-green to brick red when the cells change phycocyanin to

phycoerythrin in phycobilisome antennae upon transfer from red light to green light (15, 17). *Synechocystis* is not able to perform complementary chromatic adaptation, but it can balance energy distribution between photosystem I (PSI) and PSII according to the light quality (14, 35). Phycobilisomes, which function as major light-harvesting antennae of PSII, efficiently collect orange light, while the chlorophyll (Chl) *a* antennae of PSI harvest mainly blue and red light. State transitions balance energy distribution between the photosystems according to the light quality: illumination with orange PSII light leads to state 2, in which energy is transferred more efficiently to PSI, and treatment with PSI light (blue light) leads to compensatory energy flow to PSII (state 1) (14, 35).

Acclimation to different environmental conditions is based on adjustments of gene expression. The promoter-recognizing σ subunit of the RNA polymerase (RNAP) holoenzyme has a central role in this process. Different σ factors compete for a limited number of catalytically active RNA polymerase cores, and switching between different σ factors is a major determinant of the overall gene expression pattern in eubacteria (19, 21). In cyanobacteria, all σ factors belong to the σ^{70} family, which is divided into three subgroups (18, 22). The group 1 σ factor is essential for cell viability, group 2 σ factors are very similar to the group 1 σ factor but are nonessential, and group 3 σ factors differ considerably from groups 1 and 2.

Cyanobacteria typically code for several group 2 σ factors, and recent studies have revealed that group 2 σ factors play crucial roles in acclimation to suboptimal conditions (for a review, see reference 22). *Synechocystis* encodes four group 2 σ factors. Studies with single inactivation strains have revealed that the SigB (13, 28, 30, 31) and SigC (5, 32) factors are involved in acclimation to high-temperature stress. The SigD

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TABLE 1. Primers used for amplification of *sig* genes

Primer	Sequence
<i>sigB</i> forward.....	5'-ATGGTAACAGTGACAGTTAT-3'
<i>sigB</i> reverse.....	5'-TAGCTCTTGGCCATCGTTA-3'
<i>sigC</i> forward.....	5'-ATGACTAAACCAAGCAACGA-3'
<i>sigC</i> reverse.....	5'-AATCTAGCAAAATTTCTGC-3'
<i>sigD</i> forward.....	5'-ATGACTGCCAGAACCCAGCCC-3'
<i>sigD</i> reverse.....	5'-AGATCCAACGTAGGGAGGC-3'
<i>sigE</i> forward.....	5'-ATGAGCGATATGTCTTCCCT-3'
<i>sigE</i> reverse.....	5'-CTATAACCAACCTTTGAGGC-3'

factor, together with the SigB and SigE factors, is involved in light regulation both in light-dark transitions and upon light intensity changes (12, 13, 24, 25, 29, 42). The SigE factor has a role in sugar metabolism and is required for light-activated heterotrophic growth (23). All group 2 σ factors affect the acclimation of *Synechocystis* cells to osmotic stress conditions (25).

In the present study, we constructed strains in which three group 2 σ factor genes were interrupted simultaneously in all possible combinations, leaving only one group 2 σ factor gene active in each strain. Our results point to two distinct features of the inactivation strains: (i) direct consequences of the inac-

tivation of a particular σ factor(s) and (ii) effects resulting from an overdose of the remaining group 2 σ factor.

MATERIALS AND METHODS

Construction of triple inactivation strains of group 2 σ factors in *Synechocystis*. The glucose-tolerant strain *Synechocystis* sp. PCC 6803 (38) was used as a control strain (CS). To construct the triple inactivation strains, the *sigC* and *sigE* genes were amplified by PCR using the primers shown in Table 1. The PCR products were cloned into a pCR Blunt II-TOPO vector (Invitrogen). pCR Blunt II-TOPO-*sigC* was digested with SpeI and EcoRV, and the *sigC* fragment was ligated into an XbaI and SmaI double-digested pUC19. pCR Blunt II-TOPO-*sigE* was digested with PstI and EcoRI, and the *sigE* fragment was ligated into PstI and EcoRI double-digested pUC19. The fragment conferring chloramphenicol (Cm) resistance was obtained from pKRP10 (CVector) by BamHI digestion and subsequently ligated into BamHI-digested pUC19-*sigC* or pUC19-*sigE*. The resulting inactivation plasmid, pUC19-*sigC*-Cm^r, was used to transform the $\Delta sigBD$, $\Delta sigBE$, and $\Delta sigDE$ strains (25) to obtain the $\Delta sigBCD$, $\Delta sigBCE$, and $\Delta sigCDE$ strains, respectively, and to obtain the $\Delta sigBDE$ strain, the $\Delta sigBD$ strain was transformed with pUC19-*sigE*-Cm^r (see Fig. S1 in the supplemental material for constructs of the triple inactivation strains). Selective agar plates contained kanamycin (Kn) (50 μ g/ml), streptomycin (Str) (20 μ g/ml), spectinomycin (Spc) (10 μ g/ml), and Cm (10 μ g/ml).

PCR analysis was used to verify the complete replacement of the native gene with the inactivated gene (Fig. 1A). Genomic DNA was isolated according to the method of Williams (38), and the *sigB*, *sigC*, *sigD*, and *sigE* genes were amplified using gene-specific primers (Table 1).

Growth of *Synechocystis*. Standard growth conditions for *Synechocystis* were BG-11 medium (25) supplemented with 20 mM HEPES-NaOH, pH 7.5; a con-

FIG. 1. Triple inactivation strains of group 2 σ factors in *Synechocystis*. (A) PCR analysis of the $\Delta sigBCD$, $\Delta sigBCE$, $\Delta sigBDE$, and $\Delta sigCDE$ inactivation strains. Genomic DNA was isolated from the inactivation strains, and each *sig* gene was amplified by PCR. The expected size of each PCR product is shown under each gel. std, standard. (B) Growth of strains. The A_{730} of the cultures was set to 0.1, and the cells were grown in BG-11 medium, pH 7.5, at a PPFD of 40 μ mol $m^{-2} s^{-1}$ in continuous light (solid lines) or in a 12-h light/12-h dark rhythm (dashed lines) at 32°C. Each data point represents the mean of at least three biological replicates with independent liquid cultures, and the error bars indicate standard errors (SE). CS, control strain. (C) Doubling times of the strains in the presence and absence of 5 mM glucose at a PPFD of 40 μ mol $m^{-2} s^{-1}$. The doubling times were calculated on the basis of growth during the first 24 h. Each data point represents the mean of at least four biological replicates with independent liquid cultures, and the error bars indicate SE. The asterisks denote significant differences between the growth rate in the absence and in the presence of glucose ($P < 0.01$; Student's *t* test).

tinuous photosynthetic photon flux density (PPFD) of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; 32°C ; and ambient CO_2 . The BG-11 agar plates of triple mutants were supplemented with Kn ($50 \mu\text{g/ml}$), Str ($20 \mu\text{g/ml}$), Spc ($10 \mu\text{g/ml}$), and Cm ($10 \mu\text{g/ml}$) and those of single mutants with Kn ($50 \mu\text{g/ml}$). For the measurements, cells were grown in liquid media without addition of antibiotics.

For growth measurements, the A_{730} of liquid cultures was set to 0.1 (30 ml cell culture in a 100-ml Erlenmeyer flask), and the A_{730} was measured every 24 h. Samples of dense cultures were diluted so that the A_{730} did not exceed 0.3, and the dilutions were taken into account when the final results were calculated. Similar relationships between the A_{730} and cell numbers can be expected in the control strain and in all inactivation strains because cells harvested on an A_{730} basis contain similar amounts of chlorophyll, RNA, and proteins and have the same photosynthetic activity in all strains. Furthermore, the whole-cell absorption profiles of all strains are virtually identical.

Cells were grown under standard conditions under continuous illumination or in a 12-h light/12-h dark rhythm. In addition, growth was measured at 22°C under continuous illumination (PPFD, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$) or in a 12-h light/12-h dark rhythm. For light rhythm measurements, the experiments were started in the middle of the light period. Cells were also grown under continuous blue or orange light. Blue light was obtained by illuminating the cultures through a filter with a transmission peak at 425 nm (HT 119 Dark Blue; Lee Filters) and orange light by using filters 105 Orange (Lee Filters) and Rosco number 89; this combination has a transmission peak at 568 nm. Mixotrophic growth conditions were obtained by adding 5 mM glucose to the growth medium under otherwise standard conditions. The growth during the first day was used for the doubling-time calculations.

Determination of photosynthetic and PSII capacities. Light-saturated photosynthetic activity *in vivo* was measured from 1 ml of cell suspension ($10 \mu\text{g Chl/ml}$) in BG-11 medium supplemented with 10 mM 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 . For PSII capacity measurements, 0.7 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) was used as an artificial electron acceptor, and 0.7 mM ferricyanide was added to keep the quinone in an oxidized form.

Photoinhibition treatments *in vivo*. In the photoinhibition experiments, cell cultures containing $10 \mu\text{g Chl/ml}$ were illuminated at a PPFD of $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a slide projector at 32°C . One-milliliter samples were drawn for PSII measurements from untreated cultures and after 15, 30, and 45 min of illumination.

Chl *a* fluorescence measurements. For nonphotochemical quenching (NPQ) measurements, cells were grown under standard growth conditions and concentrated to an A_{730} of 2.5. Then, the cultures were further incubated for 1 h under standard growth conditions or illuminated with blue light (HT 119 Dark Blue filter) at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The cell suspension (1 ml) was placed in a temperature-controlled cuvette, and chlorophyll *a* fluorescence was measured with a PAM-2000 fluorometer (Heinz Walz GmbH) at 32°C . The suspension was first incubated in the dark for 3 min, the initial fluorescence level (F_0) was measured, and then the maximum fluorescence level (F_M) was measured using a saturating flash of 0.4 s and a PPFD of $5,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Illumination with blue-green light (PPFD, $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) was initiated, and the maximum fluorescence level in the light (F_M') was measured at 40-s intervals by firing a saturating flash. For state transition measurements at room temperature, the cells were taken from standard growth conditions and incubated in the dark for 3 min. Thereafter, the cell suspension was illuminated for 135 s with blue-green light (PPFD, $80 \mu\text{mol m}^{-2} \text{s}^{-1}$) obtained by filtering the output from a slide projector through 400-nm Corion long-pass and 500-nm Corion short-pass filters and thereafter for 195 s with orange light (PPFD, $20 \mu\text{mol m}^{-2} \text{s}^{-1}$), obtained through 600-nm Corion long-pass and 650-nm Corion short-pass filters. A saturating flash was fired every 30 s throughout the procedure to measure F_M' .

77 K emission spectroscopy. For state transition measurements with 77 K emission spectroscopy, cells ($50 \mu\text{g Chl/ml}$; $50 \mu\text{l}$ samples) were rapidly frozen with liquid nitrogen directly from growth conditions or cells were successively illuminated with blue light (450-nm Corion low-pass filter) at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 min, kept in the dark for 5 min, and illuminated again with blue light for 5 min and then frozen. Fluorescence emission spectra were measured at 77 K with an S2000 spectrometer (Ocean Optics) by exciting the sample with orange light obtained by filtering output from a slide projector through a 580-nm narrow-band filter (Corion). The spectra were corrected by subtracting a low background signal, smoothed with a moving median using a 2-nm window and normalized by dividing by the peak value of PSI emission at 723 nm.

***In vivo* absorption spectra.** *In vivo* absorption spectra were measured with a UV-3000 spectrophotometer (Shimadzu, Japan) from 350 nm to 800 nm.

Western blotting. The cells (25-ml cell culture; $A_{730} = 1$) were harvested directly from growth light conditions after 1-h treatments at high light (PPFD,

TABLE 2. Photosynthetic and PSII activities of triple inactivation strains of group 2 σ factors in *Synechocystis* sp. strain PCC 6803

Strain	Light-saturated photosynthetic activity ($\mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$)	Light-saturated PSII activity ($\mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$)
Control	125.8 ± 7.8	387.4 ± 7.6
ΔsigBCD	125.3 ± 3.0	413.1 ± 3.0
ΔsigBCE	124.6 ± 2.8	392.1 ± 9.6
ΔsigBDE	126.6 ± 1.4	387.0 ± 20.1
ΔsigCDE	132.2 ± 5.3	427.5 ± 13.2

$1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) or blue light (HT 119 Dark Blue filter; $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) by centrifugation at $10,000 \times g$ for 5 min at 4°C . The cell pellets were washed with ice-cold STNE buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 20 mM Na-EDTA) and resuspended in 150 μl of STNE buffer. One-third volume of acid-washed glass beads (Sigma) was added, and the cells were broken by vortexing them three times for 2 min each time at 4°C ; 40 μl of STNE buffer was added, and the glass beads and unbroken cells were removed by centrifugation at $4,000 \times g$ for 4 min at 4°C . The centrifugation was repeated, and the collected supernatant was used as a total-protein sample. For thylakoid isolation, the supernatant was further centrifuged at $18,000 \times g$ for 15 min, and the thylakoid pellet was resuspended in STNE buffer. Chlorophyll concentrations were measured in 80% acetone. Polypeptides were solubilized with Next Gel sample loading buffer (Amresco) for 5 min at 75°C , and samples containing 0.5 μg of Chl *a* were loaded and separated by 10% Next Gel SDS-PAGE according to the manufacturer's instructions (Amresco). After electrophoresis, the separated proteins were transferred to Immobilon-P membranes (Millipore). The membranes were stained with Ponceau S solution (Sigma) to verify equal loading and even transfer of the samples. The OCP was detected from total-protein samples using a 1:1,500 dilution of an OCP-specific antibody, a generous gift from Diana Kirilovsky. Commercial antibodies (Agrisera) were used to detect the PSII reaction center protein D1 (AS06 124A) and the PSI reaction center protein PsaA (AS06 172) from thylakoid samples. Both antibodies were used at 1:1,000 dilution. The goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Zymed) and the CDP star chemiluminescence kit (New England Biolabs) were used for detection in Western blotting. The immunoblots were quantified with a FluorChem image analyzer (Alpha Innotech Corp.).

RESULTS

Triple inactivation strains of group 2 σ factors in *Synechocystis*. We constructed all possible triple inactivation strains of group 2 σ factors in *Synechocystis*. The *sigC* and *sigE* genes were inactivated with a chloramphenicol resistance cassette, and transformation of the ΔsigBD , ΔsigBE , and ΔsigDE double inactivation strains (25) resulted in the ΔsigBCD , ΔsigBCE , ΔsigBDE , and ΔsigCDE triple inactivation strains. The complete segregation of the inactivated gene from the native gene was confirmed by PCR analysis (Fig. 1A). Two lines that were descendants of independently raised colonies on the first selection plate were used in measurements to minimize the possibility that secondary mutations would affect the results. However, the two lines behaved similarly, and the results are shown for one line only.

The growth curves of all triple inactivation strains (Fig. 1B) were similar to that of the control strain under standard growth conditions (BG-11 medium, pH 7.5; continuous light; PPFD of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; 32°C ; and ambient CO_2). Light-saturated photosynthetic and PSII activities were similar in all strains, as well (Table 2). These results, together with the complete segregation of the inactivation strains, show that three out of the four *Synechocystis* group 2 σ factors can be inactivated simultaneously in any combina-

FIG. 2. Growth of single and triple inactivation strains at low temperature. The cell cultures were grown at 22°C in continuous light (A and B) or in a 12-h light/12-h dark rhythm (C and D) at a PPFD of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each data point represents the mean of at least three biological replicates with independent liquid cultures, and the error bars denote SE.

tion without affecting the viability and growth of the cells under standard conditions. All triple inactivation strains grew well in a 12-h light/12-h dark rhythm (Fig. 1B).

In laboratory batch cultures of *Synechocystis*, a short exponential growth phase is followed by a phase of linear growth and finally a stationary phase (4, 25). The doubling times during the first day of growth were measured for autotrophically and mixotrophically growing cells under continuous illumination (PPFD, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 32°C. The control and the ΔsigBCE , ΔsigBDE , and ΔsigCDE strains grew faster under mixotrophic than under autotrophic growth conditions (Fig. 1C), while the ΔsigBCD strain grew similarly under autotrophic and mixotrophic conditions.

SigB and SigD factors are involved in acclimation to low-temperature stress. The roles of σ factors in acclimation to low-temperature stress were not tested previously in cyanobacteria. We used 22°C; at this temperature, cells of the control strain grow, but slowly, with a doubling time of circa 19 h. We first tested the single inactivation strains and found that none of them grew as well as the control strain at 22°C, and especially, the ΔsigB and ΔsigD strains grew slowly (Fig. 2A). In accordance with the results obtained with single inactivation strains, the triple inactivation strains containing only SigC (ΔsigBDE) or only SigE (ΔsigBCD) grew poorly at 22°C, whereas those strains containing only SigD (ΔsigBCE) or only SigB (ΔsigCDE) grew like the control strain (Fig. 2B). These results point to the importance of the SigB and SigD factors for cold acclimation.

Next, low-temperature stress was combined with a light/dark rhythm. All single mutants grew more slowly at low tempera-

ture in a light rhythm than the control strain; the ΔsigB strain was the most sensitive (Fig. 2C). The importance of the SigB and SigD factors was obvious under light rhythm at 22°C, as strains that contained only SigC (ΔsigBDE) or SigE (ΔsigBCD) hardly grew at all (Fig. 2D), while strains with only SigB (ΔsigCDE) or only SigD (ΔsigBCE) grew only somewhat more slowly than the control strain, at a rate similar to that of the ΔsigB strain. The SigB and SigD factors showed the highest mutual similarity among all group 2 σ factors (25), and these results suggest that they have functional redundancy under low-temperature conditions. We have also noticed redundancy in their functions under high light, under high-temperature stress, and when very dilute cell cultures are grown (25).

High-light acclimation is disturbed in triple inactivation strains. The sensitivities of the strains to high light were studied by subjecting the cells to $1,500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Photoinhibition was determined as loss of PSII capacity compared to untreated samples. The ΔsigBCD , ΔsigBCE , and ΔsigBDE strains lost their PSII activities 20% faster than the control strain, but the ΔsigCDE strain behaved like the control strain (Fig. 3). These results indicate that the SigB factor is important in bright-light responses in *Synechocystis*.

The ΔsigBCE strain has defects in light quality acclimation. The light quality acclimation capacities of the triple inactivation strains were tested by growing the cells under continuous orange or blue light. The PPFD of blue and orange light was adjusted to $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, the same as the PPFD of the white light of standard growth conditions. The doubling times of the control strain were 12 h, 15 h, and 19 h under white, orange, and blue light, respectively. These growth rates

FIG. 3. Photoinhibition of the triple inactivation strains. Cell cultures were illuminated at a PPFD of $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$, and light-saturated PSII activity was measured after 0, 15, 30, and 45 min with a Clark-type oxygen electrode at 32°C using 0.7 mM 2,6-dichlorobenzquinone as an artificial electron acceptor. PSII activity is expressed as a percentage of the activity measured from untreated control samples. Each data point represents an average of five independent experiments, and the error bars denote SE. After 45 min of illumination, the difference between the control strain and the ΔsigBCD , ΔsigBCE , and ΔsigBDE strains was statistically significant ($P < 0.05$; Student's t test).

are in agreement with higher *in vivo* photosynthetic activity of *Synechocystis* cells under orange than under blue light (34). The ΔsigBCE strain grew more slowly than the control or the other triple inactivation strains in blue light, with a doubling time of 47 h (Fig. 4A). In orange light, however, all triple mutants grew like the control strain (Fig. 4B).

In cyanobacteria, the OCP is essential for phycobilisome-associated NPQ, a safety mechanism that reduces energy trans-

FIG. 4. Growth of the control strain and the triple inactivation strains in blue light, preferentially absorbed by PSI (A), or in orange light, preferentially absorbed by PSII (B). The PPFD was $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Each data point represents the mean of three independent cultures, and the error bars denote SE.

FIG. 5. Orange carotenoid protein content and nonphotochemical quenching in the control and ΔsigBCE strains. (A) Western blot showing the amounts of the orange carotenoid protein under standard growth conditions (standard), after 1 h of illumination at a PPFD of $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (high light), and after 1 h of illumination with blue light at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (blue light). (B and C) Chlorophyll *a* fluorescence yield measured with the PAM fluorometer from dark-adapted cells of the control strain (B) or the ΔsigBCE strain (C) during illumination with blue-green light (PPFD, $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$). Each peak corresponds to a saturating flash. rel, relative.

fer from the phycobilisomes to the photosystems (39, 40). As NPQ in cyanobacteria is induced by blue-green light (3) and slow growth of the ΔsigBCE strain is a blue-light-specific phenomenon, we compared the OCP content and NPQ in the control and ΔsigBCE strains. Western blot analysis revealed that the amounts of the OCP were similar in the control and ΔsigBCE strains under standard growth conditions but that after a 1-h treatment in high ($1,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or blue ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) light, the OCP contents of the ΔsigBCE strain were 108% and 107%, respectively, of that measured for the control strain under the same conditions (Fig. 5A). For NPQ measurements, cells were first illuminated for 1 h with blue light ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), F_0 and F_M were measured with a PAM-2000 fluorometer after 3 min of incubation in the dark, and then the cells were exposed to bright blue-green light ($1,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) under the probe of the fluorometer. Saturating flashes were fired every 40 s to measure F_M' . No differences were detected between cells of the different strains in the blue-green-light-induced nonphotochemical fluorescence quenching (Fig. 5B and C). The control and ΔsigBCE strains also behaved similarly

FIG. 6. State transitions and PSII/PSI ratios in the control and $\Delta sigBCE$ strains. Emission spectra were measured at 77 K from control (A) and $\Delta sigBCE$ (B) cells taken directly from growth light conditions or from cells illuminated first for 5 min with blue light (blue 1), then incubated for 5 min in the dark, and finally illuminated for 5 min with blue light (blue 2). Light at 590 nm was used for excitation, and the data were normalized by dividing by the height of the photosystem I emission peak at 723 nm. Each spectrum represents an average of four independent experiments. (C) Absorption spectra of control and $\Delta sigBCE$ cells grown under standard conditions. The spectra were normalized to the Chl *a* absorption peak at 440 nm. (D) Amounts of PSII reaction center protein D1 and PSI reaction center protein PsaA. Proteins of isolated thylakoids (0.5 μ g of Chl *a*) were separated by SDS-PAGE, and the amounts of the reaction center proteins were determined by Western blotting using D1 and PsaA protein-specific antibodies. (E and F) Fluorescence yields from dark-adapted control (E) and $\Delta sigBCE$ (F) cells were measured with a PAM-2000 fluorometer. The illumination protocol consisted of darkness, blue-green light (PPFD, 80 μ mol $m^{-2} s^{-1}$), orange light (PPFD, 20 μ mol $m^{-2} s^{-1}$), and finally darkness, as indicated. A saturating pulse was fired every 30 s to measure F_M .

when NPQ was measured for cells grown under standard conditions (data not shown).

As no differences in NPQ between the $\Delta sigBCE$ and control strains were detected, we continued by studying the state transitions that function to balance energy distribution between PSI and PSII. To follow state transitions, fluorescence emission spectra at 77 K were measured from the control and $\Delta sigBCE$ strains. For the measurements, cells taken from standard growth conditions or cells that had passed a treatment consisting of 5 min in blue light, 5 min in the dark, and 5 min in blue light again were rapidly frozen in liquid nitrogen. In the dark, cells of the control strain showed a low PSII fluorescence state (state 2), and treatment of the cells with blue light in-

duced a typical high PSII fluorescence state (state 1) (Fig. 6A). In contrast, the blue-dark-blue treatment did not induce clear state transitions in the $\Delta sigBCE$ strain (Fig. 6B). In all measured spectra, the PSII fluorescence peaks at 685 and 695 nm were higher in the $\Delta sigBCE$ strain than in the control strain (Fig. 6A and B). Different PSI/PSII ratios in the mutant and control strains could explain this observation. However, *in vivo* whole-cell absorption spectra were identical in the control and $\Delta sigBCE$ strains, indicating similar relative absorption of chlorophyll *a* and phycobilisomes in both strains (Fig. 6C). Furthermore, the levels of the PSII reaction center protein D1 and the PSI reaction center protein PsaA (Fig. 6D) were virtually identical in the control and $\Delta sigBCE$ cells, suggesting similar

Strain	Functional group 2 σ factors	Continuous light slow growth at 22 °C	Diurnal light rhythm slow growth at 22 °C	Diurnal light rhythm slow growth at 32 °C	Sensitive to photoinhibition	Blue light slow growth
CS		—	—	—	—	—
$\Delta sigCDE$		—	+	—	—	—
$\Delta sigBDE$		+	++	—	+	—
$\Delta sigBCE$		—	+	—	+	+
$\Delta sigBCD$		+	++	—	+	—
$\Delta sigBD$		ns	ns	—	+	—
$\Delta sigD$		+	+/-	—	—	—
$\Delta sigB$		+	+	—	—	+/-

FIG. 7. Summary of light and low-temperature acclimation properties of different inactivation strains. The results concerning photoinhibition of the $\Delta sigBD$, $\Delta sigD$, and $\Delta sigB$ strains were published previously (24); other data are from this study. Symbols indicate no phenotype (—) or a slight (+/-), clear (+), or prominent (++) phenotype. ns, not studied.

photosystem stoichiometries in the control and $\Delta sigBCE$ strains under standard conditions. Thus, the photosynthetic machinery of the $\Delta sigBCE$ strain appears to be locked in state 1.

The state transitions were further studied at room temperature, using a PAM fluorometer. In these measurements, the control strain showed a clear increase of F_M' in blue-green light and a decrease of F_M' in orange light (Fig. 6E). The blue-light-induced increase in F_M' did not occur in the $\Delta sigBCE$ strain, and only a very small decrease of F_M' occurred during illumination with orange light (Fig. 6F), confirming that the $\Delta sigBCE$ strain is not able to perform normal state transitions.

DISCUSSION

To our knowledge, this is the first time that a complete series of inactivation strains with only one group 2 σ factor left active has been constructed and examined in a cyanobacterium. Summerfield and Sherman (29) constructed a $\Delta sigBDE$ triple inactivation strain in *Synechocystis* and, similar to our results, found no differences in growth under standard conditions between the control and inactivation strains. Double inactivation strains of *Synechocystis* (25, 29), *Anabaena* sp. strain PCC 7120 (16), and *Synechococcus elongatus* PCC 7942 (20) have also been shown to grow well under standard conditions.

Previous studies with the *sigE* gene inactivation strain revealed that the SigE factor is important for light-activated heterotrophic growth, and SigE has been shown to activate some genes involved in sugar catabolic pathways (23). The $\Delta sigBCD$ strain, containing SigE as the only functional group 2 σ factor, was not able to utilize glucose in the light like the other strains (Fig. 1). As all σ factors compete for binding to the same RNA polymerase core, inactivation of three group 2

σ factors could actually promote the binding of the remaining group 2 σ factor. Thus, our finding may suggest that inactivation of the other three group 2 σ factors leads to an overdose of the SigE factor in the RNA polymerase holoenzyme, and this overdose disturbs the utilization of glucose under continuous light. The data strengthen the idea that the SigE factor is an important regulator of sugar metabolism.

According to the data presented here, group 2 σ factors, particularly SigB and SigD, are important for acclimation to cold and light stresses in *Synechocystis*. The results are summarized in Fig. 7. Maximal growth of the triple inactivation strains containing either SigB or SigD as the only group 2 σ factor and the slightly retarded growth of the $\Delta sigB$ and $\Delta sigD$ strains suggest that either SigB or SigD is sufficient to ensure optimal growth at low temperatures if the σ factor does not need to compete with other group 2 σ factors for a limited pool of RNAP core enzymes. In *Escherichia coli* K-12, the only group 2 σ factor, RpoS, is involved in acclimation to low temperatures. Recent microarray analysis revealed that 7% of the genes in *E. coli* K-12 are upregulated under cold stress conditions, and 40% of those genes have been shown to be controlled by RpoS under at least one stress condition or during the stationary growth phase (37).

Cyanobacteria are unique among eubacteria in having endogenous circadian rhythms (2). This phenomenon has been investigated in detail in the cyanobacterium *S. elongatus* PCC 7942. Inactivation of any of the four group 2 σ factor genes individually or in pairs or overproduction of the group 2 σ factors altered the circadian expression of different reporter genes in *S. elongatus* PCC 7942, indicating that group 2 σ factors are involved in circadian regulation but that none of them alone is responsible (20). In *Synechocystis*, all triple inactivation strains grow well under a diurnal light rhythm at optimal temperature, but they are more sensitive to low tem-

peratures under a light rhythm than under continuous light, suggesting that group 2 sigma factors might also be involved in circadian regulation in *Synechocystis*, although none of them alone is crucial (Fig. 7).

For light quantity acclimation, the SigB and SigD factors seem to be the most important. The *sigB* and *sigD* genes are upregulated upon transfer of *Synechocystis* cells to high-light conditions (9, 10). A close homolog of SigD, RpoD3 of *S. elongatus* PCC 7942, is upregulated in high light, and inactivation of the *rpoD3* gene results in a light-sensitive phenotype (27). In *Synechocystis*, the $\Delta sigBD$ and $\Delta sigD$ strains were not able to enhance growth like the control strain upon doubling of the growth light intensity (24).

Under high-light conditions (PPFD, 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$), the half-life of the D1 protein is only 20 min (33) and the survival of the cells is crucially dependent on efficient repair of photoinhibited PSII. We have previously shown that the double inactivation strain $\Delta sigBD$ is sensitive to high light (24). The PSII repair cycle of the $\Delta sigBD$ strain does not function efficiently, because transcription of the *psbA* genes encoding the PSII reaction center protein D1 is not upregulated normally (24). The results of this study show that the SigB factor alone is sufficient to provide normal tolerance for high-light-induced photoinhibition. However, the $\Delta sigB$ strain can tolerate high-light treatment as well as the control strain (24), suggesting that, together with the SigC and SigE factors, the SigD factor is able to substitute for the SigB factor under high-light stress.

Another aspect of light acclimation is acclimation to different qualities of light. The slow growth of the $\Delta sigBCE$ strain in blue light (with a doubling time 2.5 times longer than in the control strain) might be due to an increased probability of having the SigD factor in the RNA polymerase holoenzyme because the other group 2 σ factors are missing. For a more comprehensive picture of the blue-light phenomenon, growth of the $\Delta sigBD$, $\Delta sigB$, and $\Delta sigD$ strains was also measured in blue light. The growth of the $\Delta sigB$ strain was slightly reduced (doubling time, 1.14 times longer than in the control strain). In the $\Delta sigBCE$ strain, SigD is the only group 2 σ factor, and in the $\Delta sigB$ strain, the absence of the SigB factor might promote binding of the other group 2 σ factors, including the SigD factor, to the RNA polymerase core (Fig. 7). In *S. elongatus* PCC 7942, overproduction of group 2 σ factors under an IPTG (isopropyl- β -D-thiogalactopyranoside)-induced promoter has been shown to affect circadian regulation of reporter genes (20).

The $\Delta sigBCE$ strain lacks the capacity for normal state transitions induced by blue light (Fig. 6). Obviously, the absence of state transitions leads to an inability to balance the functions of the two photosystems in blue light, although the $\Delta sigBCE$ strain appears to be locked in state 1, which is the state normally induced by blue light. On the other hand, another light-balancing system, the OCP-related NPQ, appears to function normally in the $\Delta sigBCE$ strain (Fig. 5). Thus, our results with the $\Delta sigBCE$ strain fully agree with the conclusion, drawn from experiments with the ΔOCP strain and phycobilisome and PSII mutants (39, 40), that state transitions and NPQ are separate phenomena in cyanobacteria.

In conclusion, three out of four group 2 σ factors can be inactivated simultaneously in any combination without affecting growth or photosynthetic activity under our standard

growth conditions. The presence of either the SigB or the SigD factor is important for low-temperature acclimation, particularly under a diurnal light rhythm. The results obtained with the full series of single, double, and triple inactivation strains suggest that competition of group 2 σ factors for binding to the RNA polymerase core might have an important role. The lack of the other three group 2 σ factors in the $\Delta sigBCD$ strain might promote association of the SigE factor with the RNA polymerase core and lead to abnormalities in glucose utilization. Similarly, the competition advantage of the SigD factor in the $\Delta sigBCE$ strain leads to slow growth in blue light, apparently because state transitions do not occur normally in the $\Delta sigBCE$ strain.

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