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ORIGINAL RESEARCH

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Engineering RNA polymerase to construct biotechnological host strains of cyanobacteria

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Abstract

Application of cyanobacteria for bioproduction, bioremediation and biotransformation is being increasingly explored. Photoautotrophs are carbon-negative by default, offering a direct pathway to reducing emissions in production systems. More robust and versatile host strains are needed for constructing production strains that would function as efficient and carbon-neutral cyanofactories. We have tested if the engineering of sigma factors, regulatory units of the bacterial RNA polymerase, could be used to generate better host strains of the model cyanobacterium *Synechocystis* sp. PCC 6803. Overexpressing the stress-responsive *sigB* gene under the strong *psbA2* promoter (SigB-oe) led to improved tolerance against heat, oxidative stress and toxic end-products. By targeting transcription initiation in the SigB-oe strain, we could simultaneously activate a wide spectrum of cellular protective mechanisms, including carotenoids, the HspA heat shock protein, and highly activated non-photochemical quenching. Yellow fluorescent protein was used to test the capacity of the SigB-oe strain to produce heterologous proteins. In standard conditions, the SigB-oe strain reached a similar production as the control strain, but when cultures were challenged with oxidative stress, the production capacity of SigB-oe surpassed the control strain. We also tested the production of growth-rate-controlled host strains via manipulation of RNA polymerase, but post-transcriptional regulation prevented excessive overexpression of the primary sigma factor SigA, and overproduction of the growth-restricting SigC factor was lethal. Thus, more research is needed before cyanobacteria growth can be manipulated by engineering RNA polymerase.

1 | INTRODUCTION

Cyanobacteria offer a future solution for carbon-neutral production of fuels and valuable compounds. In the most optimal scenario, cyanobacteria produce high-value products and simultaneously collect macronutrients from wastewater and use CO_2 emissions for photosynthesis. Removing phosphate and nitrate, ammonium or urea from wastewater and CO_2 from industrial emissions to supplement cyanobacterial production will help mitigate climate change and natural waters' eutrophication. Currently, the utilization of cyanobacteria in biotechnological applications is limited by low productivity and high production costs (Farrokh et al. 2019; Agarwal et al. 2022). Further research is required to construct production vectors with strong and adjustable expression levels and robust strains showing tolerance against harmful end products and varying environmental conditions. Furthermore, for efficient photosynthetic cultivation, methods have to be developed to overcome the dilemma of high enough cell density and sufficient illumination (Jodlbauer et al. 2021).

Acclimation of cyanobacteria to suboptimal environmental conditions is largely dependent on reprogrammed gene expression. The major controllers of gene expression are the σ subunits of RNA polymerase (RNAP). Together, the RNAP core and a σ subunit form a holoenzyme capable of transcription initiation. The binding of different σ subunits to the RNAP core changes the transcription pattern. The model cyanobacterium *Synechocystis* sp. PCC 6803 (abbreviated *Synechocystis*) encodes nine different σ subunits (Imamura et al. 2003). The primary σ factor SigA is essential and mainly responsible for gene expression during exponential growth. SigB, SigC, SigD and SigE belong to group 2 σ factors (Imamura et al. 2003) that structurally resemble the primary SigA factor. However, group 2 σ factors are non-essential in favorable growth conditions; strains missing one or multiple group 2 σ factors, including the ΔsigBCDE strain, retain a similar growth rate as the control strain in standard growth conditions (Pollari et al. 2008, 2011; Koskinen et al. 2016). Instead, group 2 σ factors play major roles in acclimation to suboptimal conditions (for a recent review, see Srivastava et al. 2020).

We have suggested that manipulation of the σ factor content may be used for controlling cyanobacteria growth or stress resistance, which could be beneficial for different biotechnological applications (Stensjö et al. 2018). The primary SigA factor is responsible for the expression of household genes and, therefore, overexpression of the SigA protein might lead to an increased growth rate. In contrast, the SigC factor is linked to the stationary growth phase (Asayama et al. 2004), and the high accumulation of RNAP-SigC holoenzyme efficiently prohibits growth (Antal et al. 2016; Heilmann et al. 2017). For group 2 σ factors, the SigB factor has been connected to multiple stress acclimation responses. In standard growth conditions, cells produce only low amounts of the *sigB* transcript and the SigB protein (Tuominen et al. 2003; Koskinen et al. 2016), and the transcriptome of the ΔsigB strain closely resembles that of the control strain (Turunen et al. 2022). The *sigB* gene is transiently induced upon heat treatment (Singh et al. 2006; Tuominen et al. 2006). Studies with the ΔsigB strain have shown that SigB is important for survival in extreme heat stress at 48°C, and also for the formation of acquired heat tolerance (Singh et al., 2006; Tuominen et al., 2006), although the ΔsigB strain grows well in mild heat stress at 40–42°C (Turunen et al., 2022). Without the SigB factor, *Synechocystis* cells do not acclimate well to high salt stress or to osmotic stress, whereas the ΔsigCDE strain containing SigB as its only group 2 σ factor shows improved tolerance to salinity, in comparison to the control strain (Pollari et al. 2008; Tyystjärvi et al. 2013). SigB also plays a role in high light and oxidative stresses by activating carotenoid synthesis and reactive oxygen species (ROS) scavenging mechanisms (Pollari et al. 2009; Hakkila et al. 2013, 2014, 2019; Turunen et al. 2022).

In the present manuscript, we have explored if engineering RNAP could be used to control the growth rate of *Synechocystis* cells in optimal conditions or to increase stress tolerance of the cells in suboptimal conditions. Overproduction of the primary SigA factor might accelerate growth in favorable conditions as transcription of household genes should be enhanced. On the contrary, overproduction of the growth-restricting SigC factor should retard growth. And finally, overproduction of the stress-responsive SigB factor might increase the stress tolerance of the cells. Overexpression strains were constructed by inserting an extra copy of the *sigA*, *sigB* or *sigC* gene under the strong *psbA2* promoter into the genome of *Synechocystis*, and properties of the mutant strains were studied.

2 | MATERIALS AND METHODS

2.1 | Strains and standard growth conditions

The *Synechocystis* sp. PCC 6803 substrain GT-T (Koskinen et al. 2023) was used as a control and host strain. To overexpress SigA, SigB or SigC protein, an extra copy of the gene under the strong *psbA2* promoter was inserted into *Synechocystis* genome in the middle of the *psbA1* gene (*sigB*) or the *psbA2* (*sigA* and *sigC*) gene (Figure S1).

The *sigA*, *sigC* and *sigB* genes with compatible restriction sites at both ends were ordered as synthetic DNA fragments (GeneScript). The *sigA* and *sigC* coding regions were cut with NdeI and KpnI restriction enzymes and ligated to pAII plasmid (a generous gift from Dr. Marion Eisenhut) backbone containing homologous regions 500 bp up- and down-stream of the *psbA2* gene to produce pAII-sigA and pAII-sigC plasmids (Figure S1A-B). The *sigB* coding region was cut with NheI and EcoRI restriction enzymes and ligated to a pUC57 plasmid backbone with homologous regions 500 bp up- and down-stream of the *psbA1* gene (Turunen et al. 2022) to produce the pUC57-sigB plasmid (Figure S1C). *Synechocystis* GT-T substrain was transformed with pUC57-sigB, pAII-sigA and pAII-sigC plasmids by incubating a 0.5 mL cell culture ($OD_{730} \approx 0.5$) for 5 h in our standard growth conditions (see below). Plasmids were linearized with EcoRI and added to final concentrations of 30 μg/mL. Cells were spread on BG-11 selection plates containing 10 μg/mL nourseothricin for *sigB* overexpression strain (SigB-oe) and 20 μg/mL spectinomycin and 10 μg/mL streptomycin for SigA-oe and SigC-oe strains. After two weeks, mutant colonies were transferred to new selection plates and selection was continued for six to eight weeks. Then, segregation of the mutant strains was verified with PCR (Figure S2) using the *psbA1_ forward* and *psbA1_ reverse* primers for SigB-oe, and *psbA2_forward* and *psbA2_reverse* primers for SigA-oe (Table S1). Finally, the modified areas of the chromosome were sequenced to verify that the strains were as planned.

To add a His-tag to the C-terminal end of the γ subunit of the RNAP, the pMA-T-His-tag-Cm plasmid (Koskinen et al. 2016) was used to transform the GT-T to produce RNAP-His strain (Koskinen et al. 2016). To construct the SigB-oe + RNAP-His strain, the RNAP-His strain was transformed with the pUC57-sigB plasmid, as described above; for selection, BG-11 plates were supplemented with 10 mg/mL chloramphenicol. Segregation of the SigB-oe + RNAP-His strain was verified with PCR (Figure S2) using the *psbA1_ forward* and *psbA1_ reverse* primers (Table S1), and the replacement of *psbA1* gene was finally confirmed by sequencing.

Cultures were grown in our standard growth conditions in BG-11 medium buffered with 20 mM Hepes-NaOH pH 7.5 at 32°C under continuous light, with a photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}, in ambient air. The light source was a mixture of fluorescent tubes, light colors 865 and 840 (Osram/Philips), the spectrum is shown in Figure S3A. Mutant plates were always supplemented with appropriate antibiotics, but liquid cultures (30-ml culture in 100-ml Erlenmeyer flask, shaken at 90 rpm) were grown without antibiotics. Storage cells were prepared by collecting cells from 1 mL of culture grown to $OD_{730} = 0.6$, resuspending them to BG-11 medium supplemented with 20% glycerol and stored at −80°C.

2.2 | Growth measurements

To measure growth, OD_{730} of the cell culture was set to 0.060, and 30-ml cell cultures were grown in 100-ml Erlenmeyer flasks, shaken at 90 rpm, in our standard conditions. Growth was monitored by measuring OD_{730} daily with ThermoScientific Genesys 10S spectrophotometer. Dense cultures were diluted so that the measured OD_{730} did not exceed 0.4, and the dilution was taken into account when the results were calculated.

High light stress was induced by treating cells at PPFD 500 μmol m^{-2} s^{-1} (Heliospectra L4A illuminator); the spectrum of high light is shown in Figure S3B. For high-temperature treatments, cultures were grown at 40°C. Oxidative stress was induced chemically as described earlier (Hakkila et al. 2019): superoxide production was induced with 4 μM methyl viologen and singlet oxygen production with 5 μM methylene blue. Addition of 0.25% butanol (v/v) was shown to decrease growth of *Synechocystis* cells (Kämäräinen et al. 2012), and we tested tolerance of *Synechocystis* strains against 1-butanol by adding 0.5% 1-butanol (v/v) to cell cultures. All growth experiments were performed using at least three independent biological replicates.

2.3 | Co-cultivation experiments

Cultures were prepared by mixing 15 mL of GT-T ($OD_{730} = 0.060$) and SigB-oe ($OD_{730} = 0.060$) cell suspensions. Co-cultures were grown in standard conditions, at 40°C or in standard conditions after addition of 4 μM methyl viologen. Samples were taken after 0, 24, 48 and 96 h of incubation, OD_{730} was set to 0.060, and then samples were diluted 1:1000 and 1:5000, and ten 10 μL droplets of each dilution were pipetted onto two BG-11 plates, one of which was supplemented with 10 μg/mL nourseothricin and one without antibiotics. After one week, the colonies were counted, and colonies on plates with and without nourseothricin were used to estimate the percentage of the SigB-oe cells in the co-culture. Three independent experiments were done.

2.4 | Survival rate

GT-T and SigB-oe cultures were grown for one day in standard conditions. OD_{730} of the culture was set to 0.50 ± 0.01, and 1 mL samples were incubated in 48°C water bath for 15 or 20 min under PPFD 20 μmol m^{-2} s^{-1}. From heat-treated and untreated cultures, dilutions of 1:1000 to 1:50000 were prepared, and eight 10 μL droplets from each dilution were pipetted on BG-11 plates. The number of colonies was counted and used to determine the percentage of viable cells after the 48°C treatments.

2.5 | Measurement of total RNA content

15 mL of cell culture ($OD_{730} \approx 0.6$) was collected from standard conditions, and total RNA was extracted with NucleoZOL reagent (Macherey-Nagel™) according to manufacturer's instructions. RNA concentration was measured with BioDrop μLITE spectrophotometer (Montreal Biotech Inc.). The total amount of RNA in the sample was divided with OD_{730} of the cell culture to determine the RNA content per OD unit of the original cell culture.

2.6 | Real-time reverse transcriptase PCR

RNA was extracted with the hot-phenol method (Pinto et al. 2009) from GT-T, SigA-oe and SigB-oe cultures (30 mL, $OD_{730} \approx 0.6$) grown in standard conditions. After DNase treatment (TURBO DNA-free™, Ambion), 1 μg of RNA was used for cDNA synthesis using Superscript III Reverse transcriptase kit (Invitrogen) according to manufacturer's instructions. The RT-qPCR reactions were performed with Sensifast™ SYBR & Fluorescein Kit (Bioline) using specific primers for *sigA*, *sigB* genes and the *rnpB* reference gene, as listed in Table S1. Amplification reactions were run with IQ5 machine (BioRad). Three biological replicates with three technical replicates per strain were measured. The possibility of DNA contamination was excluded by running controls lacking reverse transcriptase in cDNA synthesis.

2.7 | Western blotting

Cells were collected by centrifugation at 11000 *g*, 5 min, at 4°C from 30-ml cultures grown for 3 days in standard conditions, high light or after cells were treated for 24 h with 4 μM methyl viologen. The cell pellet was washed with STNE buffer (10 mM Tris–HCl pH 8.0, 0.4 M sucrose, 10 mM NaCl, 20 mM EDTA) and then resuspended into 250 μL of STNE buffer. An equal volume of 150–210 μm glass beads (Sigma Aldrich) to the cell pellet was added, and cells were lysed by vortexing for 6 min at 4°C. Glass beads were removed by two centrifugations at 3000 *g* for 4 min at 4°C. The total cell lysate was centrifuged at 18 000 *g* for 15 min at 4°C to separate membrane pellet and soluble proteins. Protein samples were solubilized in Laemmli buffer (Laemmli 1970) and run on Mini-Protean TGX 4–15%

acrylamide gels (BioRad). Proteins were transferred to Immobilon-P PVDF membrane (Millipore) by semi-dry transfer (BioRad Trans-blot), and each protein was detected by specific primary antibodies (Table S2). Primary antibodies were detected by goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Zymed) and CDP-star Chemiluminescence reagent (Perkin-Elmer) and visualized on Super RX-N x-ray film (Fujifilm). The bands were quantified with FluorChem8000 software.

2.8 | Isolation of RNAP complexes and detection of the RNAP-SigB holoenzyme

RNAP-His and SigB-oe + RNAP-His strains (30 mL cultures in 100 mL Erlenmeyer flasks) were grown in the standard growth conditions to $OD_{730} \sim 0.6$. Cells were collected from 60 mL of culture by centrifugation at 4500 g for 6 min at 4°C; samples were cooled using precooled centrifuge tubes on ice. Some samples were treated at 42°C for 15 min or 1 h before the collection of the cells, as indicated. Isolation of soluble proteins and the His-tag pulldown of RNAP complexes were performed as described in Koskinen et al. (2016). Samples containing 15 μL of RNAP pulldown complexes (isolated from 0.44 mg of soluble proteins) were solubilized with Laemmli's solubilisation buffer for 10 min at 75°C, then proteins were separated with 12% SDS-PAGE gels and transferred to Immobilon-P-PVDF-membrane (Millipore) with Trans-blot® (Bio-Rad) device. SigB, SigC and SigD proteins were detected using a group 2 σ factor-specific primary antibody (Koskinen et al. 2016), which was detected as described above. Thereafter, the membrane was re-probed with the antibody against the α subunit of RNAP core.

2.9 | Measurements of *in vivo* absorption spectra

Cell cultures were grown for three days, and samples normalized to OD_{730} of 0.200. Three independent replicates were used to measure the absorption spectra at the 370–750 nm range with Olis Clarity Beam 14 spectrophotometer (OLIS, Inc). In some experiments, as indicated, superoxide stress was induced by adding 4 μM methyl viologen to samples 6 h prior to absorption spectra measurements; samples were kept in standard growth conditions during the methyl viologen incubation. The ratio of carotenoids to chlorophyll *a* was determined by dividing carotenoid absorption peak at 485 nm by the chlorophyll absorption peak at 680 nm.

2.10 | Photosynthesis measurements

77 K fluorescence was measured in liquid nitrogen with QEPro spectrometer (Ocean Insight) from 50 μL whole cell samples set to $OD_{730} = 0.25$. Phycobilisome excitation at 580 nm (10 nm band-pass filter, Newport) was used for illumination. Liquid culture samples were collected directly from culture conditions (3-day-old cultures from standard conditions or from high light, PPFD 500 μmol photons $m^{-2}\ s^{-1}$) and immediately frozen with liquid nitrogen. Three biological replicates were measured from each condition with three technical replicates each. The spectra were normalized to the PSI peak at 725 nm.

NPQ was measured using Multi Color PAM fluorescence analyzer (Walz). OD_{730} of cultures was set to 0.50 and 1 mL samples were incubated in darkness for 5 min prior to NPQ induction. NPQ was induced with 480 nm light of PPFD 300 μmol $m^{-2}\ s^{-1}$ for 7 min. Samples for NPQ measurements were grown in standard growth conditions, or in high light conditions (PPFD 500 μmol photons $m^{-2}\ s^{-1}$) or under superoxide stress, which was induced by 24 h incubation with 4 μM methyl viologen. The wavelength of the measuring beam was 590 nm, and 10 measurements were taken during the induction and one in darkness. The sample cuvette was kept at 32°C and the sample was continuously mixed with a magnetic stirrer. NPQ was calculated as $NPQ = (Fm\text{-}Fm')/Fm'$, where Fm is the maximum fluorescence measured after dark incubation and Fm′ is the maximum fluorescence measured in the light.

2.11 | Production of a heterologous protein

GT-T and SigB-oe were transformed with the pDF-lac2(S3)-sYFP2-CmR (Thiel et al. 2018) production plasmid, as described earlier. Liquid cultures were grown in standard conditions in the presence of 10 μg/mL chloramphenicol and 25 μg/mL spectinomycin. 30 mL cultures were started from $OD_{730} = 0.060$ and cells were grown for 3 days in standard conditions or in high light (PPFD 500 μmol $m^{-2}\ s^{-1}$) or 5 days after induction of superoxide stress with 4 μM methyl viologen or singlet oxygen stress with 5 μM methylene blue. Cultures were diluted to $OD_{730} = 0.060$ and divided into 15 mL batches: one with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and one without.

Fluorescence was measured using a Tecan infinite 200 PRO microplate reader with 495 nm emission and 535 nm excitation wavelengths in 24 h intervals. Three technical replicates of 150 μL intact cells from each culture were measured on PerkinElmer Isoplate-96 F black frame, clear well polystyrene plates. Fluorescence count was measured with 4 reads per well with 25 flashes each. For each treatment, at least three independent biological replicates were analyzed.

3 | RESULTS

3.1 | A 3.3-fold increase in the *sigA* transcripts enhanced SigA protein only 1.6-fold

The enhanced growth rate of cyanobacteria could be beneficial for many biotechnological applications; for example, when cyanobacteria are used for collecting nutrients from wastewaters and then utilized as bio-fertilizers. In *E. coli* cells, growth rate positively correlates with the formation of RNAP-σ^{70} holoenzyme (Gummesson et al. 2009) and active ribosomes (Dai et al. 2016). We have earlier shown that simultaneous inactivation of all four group 2 σ factors increases the formation of RNAP-SigA holoenzyme and enhances the production of ribosomal subunits, including 3-fold up-regulation of rRNA content (Koskinen et al. 2018). However, neither the content of active ribosomes nor the growth rate of the ΔsigBCDE strain increased (Koskinen et al. 2018). Furthermore, the ΔsigBCDE strain lacking all group 2 σ factors is extremely vulnerable to any stress conditions (Koskinen et al. 2018).

In the present paper, we tested if growth enhancement can be achieved by overexpressing the primary σ factor, SigA. To that end, we inserted an additional copy of the *sigA* gene into the genome of our glucose-tolerant GT-T substrain of *Synechocystis* sp. PCC 6803 (Koskinen et al. 2023) using homologous recombination. The extra copy of the *sigA* gene was expressed under the strong native *psbA2* promoter. Complete segregation of the mutant strain was confirmed by colony PCR (Figure S2A). The amount of *sigA* transcript was measured with RT-qPCR and found to have increased 3.3-fold in the SigA-oe strain compared to the GT-T strain (Figure 1A). However, the amount of SigA protein in the SigA-oe strain was only 1.6-fold higher than in the GT-T control strain (Figure 1B), suggesting that post-transcriptional mechanism(s) are involved in the regulation of the SigA protein content in *Synechocystis*. The enhancement of the SigA content in the SigA-oe strain did not affect the growth rate in standard conditions (Figure 1C). In the ΔsigBCDE strain, high RNAP-SigA content is accompanied with high RNA content (Koskinen et al. 2018), but, in the SigA-oe strain, the RNA content was similar as in the GT-T strain (Figure 1D). This might suggest that the formation of the RNAP-SigA holoenzyme is not highly up-regulated in the SigA-oe strain. Taken together, our results indicate that the formation of RNAP-SigA holoenzyme is a strictly regulated process and does not directly reflect changes in the amount of *sigA* transcripts.

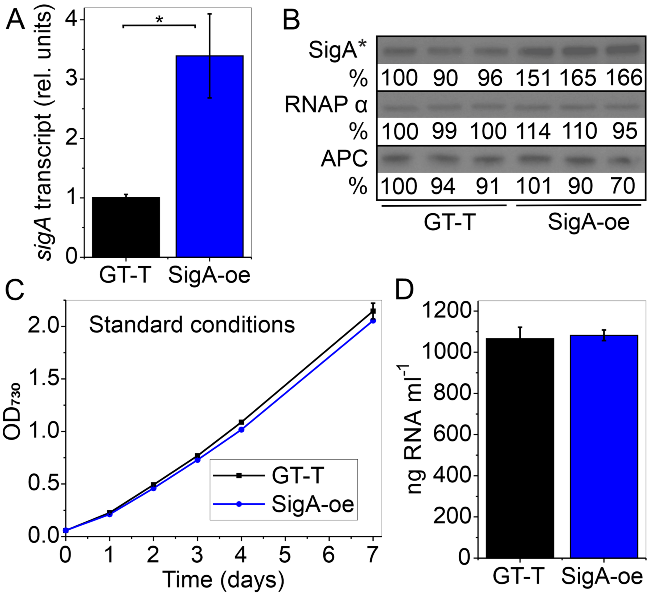

FIGURE 1 Properties of SigA-oe and GT-T strain in standard conditions (BG-11 medium with 20 mM Hepes-NaOH pH 7.5; 32°C; continuous light, 40 µmol photons m^{-2} s^{-1}; ambient air). (A) The amount of the *sigA* mRNA in SigA-oe and GT-T strains. (B) The amount of the SigA, RNAP α subunit and allophycocyanin (APC) proteins in the SigA-oe and GT-T strains. Soluble proteins (30 µg for SigA and RNAP α, 2 µg for APC) were isolated from cultures in standard conditions, separated by SDS-PAGE and detected by specific antibodies. Results for three independent biological replicates for both strains are shown and the numbers indicate quantified amounts relative to the first replicate of GT-T. (C) Growth of SigA-oe and GT-T in standard conditions. (D) The amount of total RNA in 1 mL of cell culture with $OD_{730} = 1.00$. All measurements were performed with three biological replicates (A-D), and the error bars denote SE; statistically significant differences ($p < 0.05$ according to Student's t-test) are marked with *.

3.2 | Overexpression of the *sigC* gene is lethal for *Synechocystis* cells

In some biotechnological applications, host cells with negligible growth but strong production capacity would be beneficial. Formation of high amounts of RNAP-SigC holoenzyme has been connected to no growth/slow growth phenotypes (Asayama et al. 2004; Antal et al. 2016; Heilmann et al. 2017). Despite multiple trials, we could not generate a strain overproducing *sigC* under the strong *psbA2* promoter. After transformation, only a few colonies appeared on the first selection plate, and all colonies died rapidly during selection. Obviously, too strong expression of the *sigC* gene is lethal for *Synechocystis*.

3.3 | The SigB-oe strain grows normally in the standard conditions

The SigB factor has been reported to provide protection against multiple stresses, including high temperature, high salt and oxidative stress (Tuominen et al. 2006; Nikkinen et al. 2012; Hakkila et al. 2019). To study if overproduction of the SigB protein would be beneficial for biotechnological applications by providing extra robustness to the host cells, we constructed a SigB-overproduction strain by expressing an extra copy of the *sigB* gene under the strong *psbA2* promoter (Figure S1C). Complete segregation of the mutant strain was verified with PCR analysis (Figure S2B). In standard growth conditions, the *sigB* transcripts were 4-fold more abundant in the SigB-oe strain than in the GT-T strain (Figure 2A). The amount of SigB protein in the standard growth conditions is too low to be reliably measured with Western blotting. To overcome that problem, we added a His-tag to the γ subunit of RNAP polymerase in the GT-T control strain [the RNAP-His strain (Koskinen et al. 2016)] and in the SigB-oe strain (the SigB-oe + RNAP-His strain, Figure S2C) and purified RNAP complexes to compare the amounts of the RNAP-SigB holoenzymes. The amount of RNAP-SigB was only slightly higher in the SigB-oe + RNAP-His strain than in the RNAP-His strain in the standard growth conditions (Figure 2B). The group 2 σ factor-specific antibody (see Koskinen et al. 2016) recognized also SigC and SigD factors, whose amounts were similar in both strains; the amount of SigE was below detection limit (Figure 2B). The original Western blot with three biological replicates is shown in Figure S4A.

The SigB-oe and GT-T strains grew similarly as separate cultures in standard growth conditions (Figure 2C), as well as in co-cultivation experiments (Figure 2D). Comparison of the *in vivo* absorption spectra showed that the carotenoid-to-chlorophyll *a* ratio in SigB-oe (0.56 ± 0.02) was 7.7% higher ($p = 0.03$; Student's t-test) than in the GT-T (0.52 ± 0.005) strain (Figure 2E).

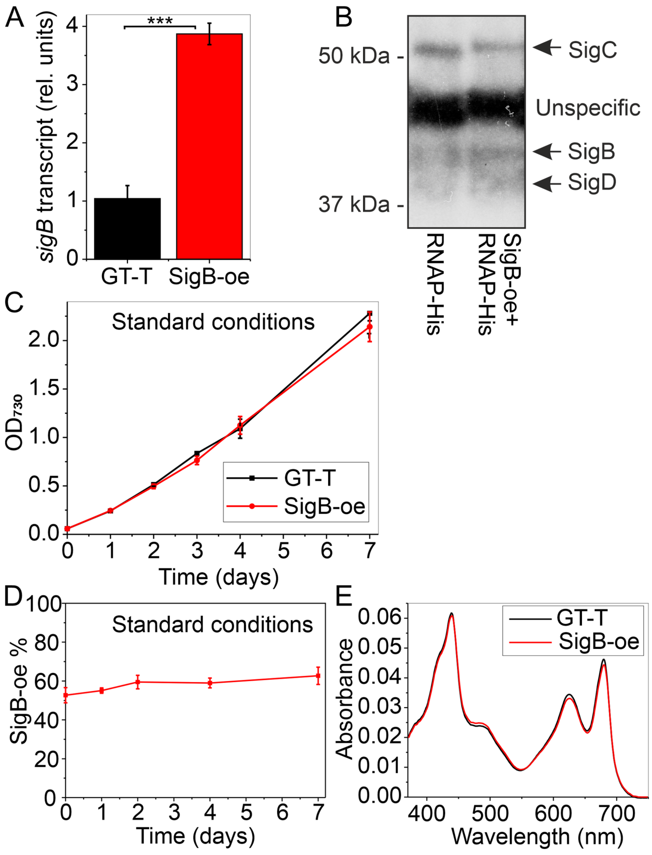

FIGURE 2 Comparison of SigB-oe and GT-T strains in standard conditions (BG-11 medium with 20 mM Hepes-NaOH pH 7.5; 32°C; continuous light, 40 μmol photons m^{-2} s^{-1}; ambient air). (A) The amount of the *sigB* mRNA in SigB-oe and GT-T strains. (B) Amounts of RNA polymerase bound SigB, SigD and SigC factors in GT-T and SigB-oe strains in standard conditions. Soluble proteins were isolated from RNAP-His and SigB-oe + RNAP-His cultures (a His-tag in the γ subunit of RNAP), and RNAPs were collected from soluble proteins with cobalt-coated magnetic beads. Proteins pulled down from 0.44 mg of soluble proteins were separated with SDS-PAGE, and RNAP-bound SigB, SigC and SigD were detected with the group 2 σ factor-specific primary antibody. (C) Growth of SigB-oe and GT-T strains in standard conditions. (D) Percentage of SigB-oe cells in a co-cultivation experiment in standard conditions. An equal amount of GT-T and SigB-oe cells were mixed to start a liquid culture, and the percentages of SigB-oe cells were monitored for a week. (E) *In vivo* absorption spectra measured from cultures grown in standard conditions. The results are means of three biological replicates (A, C, D) and the error bars denote SE; statistically significant differences (p < 0.001 according to Student's t-test) are marked with ***.

3.4 | SigB-oe strain tolerates elevated temperatures better than the control strain

We continued by testing the acclimation of the SigB-oe strain to mild and severe heat stresses. After a long exposure to mild heat stress at 40°C, SigB-oe cells grew faster than the GT-T cells as high-temperature-induced growth deceleration after four days was more prominent in the GT-T strain than in the SigB-oe strain (Figure 3A). To evaluate *sigB* expression during the 40°C growth experiment, the amount of *sigB* mRNA was measured with quantitative real-time PCR. A 3-fold upregulation of *sigB* transcripts was detected in the GT-T strain during the first day of high-temperature treatment, but afterwards the amount of *sigB* transcripts dropped

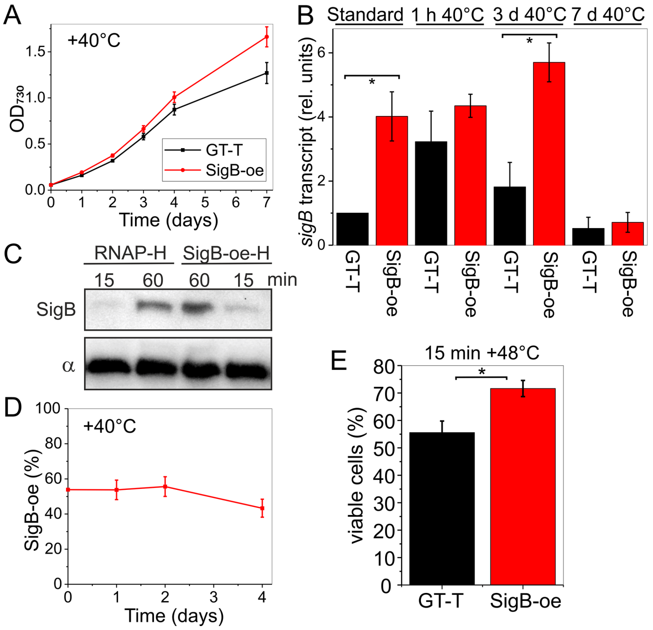

FIGURE 3 Properties of SigB-oe and GT-T strains at high temperatures. (A) Growth of SigB-oe and GT-T at 40°C (BG-11 medium with 20 mM Hepes-NaOH pH 7.5, continuous light of 40 μmol photons m^{-2} s^{-1}, ambient air). (B) The amount of *sigB* mRNA in standard growth conditions and after 1 h, 3 days and 7 days of high-temperature treatment at 40°C. The amount of *sigB* mRNA in GT-T strain at standard conditions was set to 1, and all other samples were compared to that. (C) The amount of the RNAP-SigB holoenzyme in the RNAP-His (RNAP-H) and SigB-oe + RNAP-His (SigB-oe-H) cultures after 15 min and 60 min of high-temperature treatments. RNAPs collected from 0.44 mg of soluble proteins were separated with SDS-PAGE, and the amount of SigB was detected with the group 2 σ factor specific primary antibody. After the SigB detection, the same membrane was re-probed with the antibody against the α subunit of the RNAP core. (D) Percentage of SigB-oe cells in a co-cultivation experiment at 40°C. Equal amounts of GT-T and SigB-oe cells were mixed to start a liquid culture, and the percentages of SigB-oe cells were determined over 4 days. (E) Cell viability after 15 min heat treatment at 48°C, determined by counting colony forming units before and after treatment. Results are shown as means of three biological replicates and the error bars denote SE; statistically significant differences (p < 0.05 according to Student's t-test) are marked with *.

back to the level of standard growth conditions (Figure 3B). Unlike in the GT-T strain, the amount of *sigB* transcripts remained high in the SigB-oe strain for at least 3 days at high-temperature (Figure 3B). We also measured 1.3 times higher content of the RNAP-SigB holoenzyme in the SigB-oe + RNAP-His strain than in the RNAP-His strain after 1 h of high-temperature treatment (Figures 3C and S4B). Co-cultivation experiments at 40°C confirmed that the growth difference between the SigB-oe and GT-T strains was not seen during the first 4 days of mild high-temperature treatment (Figure 3D).

In addition to long-term mild heat stress, we tested the response to a short extreme heat shock at 48°C. The survival rate of SigB-oe cells after 15 min heat shock at 48°C was 72%, whereas it was only 56% in the GT-T strain (Figure 3E). The treatment at 48°C is lethal for *Synechocystis*, and hardly 1% of cells survived after 20 min treatment. According to these results, overexpression of the SigB factor could especially benefit cells exposed to severe heat stress for short periods.

3.5 | Acclimation of SigB-oe to high light

In bright light (PPFD 500 µmol m^{-2} s^{-1}), SigB-oe and GT-T strains grew similarly (Figure 4A). To probe the function of the phycobilisome antenna and photosystems, we measured 77 K emission spectra using phycobilisome excitation at 580 nm. In the standard growth conditions (40 µmol photons m^{-2} s^{-1}), SigB-oe cells showed higher PSII and phycobilisome peaks than GT-T cells (Figure 4B). To investigate if this was due to differences in the abundance of the photosynthetic complexes, the amounts of the PSI reaction center protein PsaB, PSII core proteins D1 and CP43, and allophycocyanin and phycocyanin of the phycobilisome antenna were measured by Western blotting (Figures 4C and S5). In standard conditions, the amounts of PsaB, D1, CP43 and phycocyanin were similar in both strains, but the amount of allophycocyanin was 25% higher in SigB-oe than in GT-T. The increased amount of allophycocyanin in SigB-oe might partly explain the high phycobilisome fluorescence of SigB-oe in standard conditions, but as the amounts of other proteins did not differ, 77 K fluorescence results rather suggest functional differences in the energy transfer reactions between the strains.

Upon high-light treatment, the amounts of light-harvesting allophycocyanin and phycocyanin decreased in the GT-T strain, as did also PsaB,

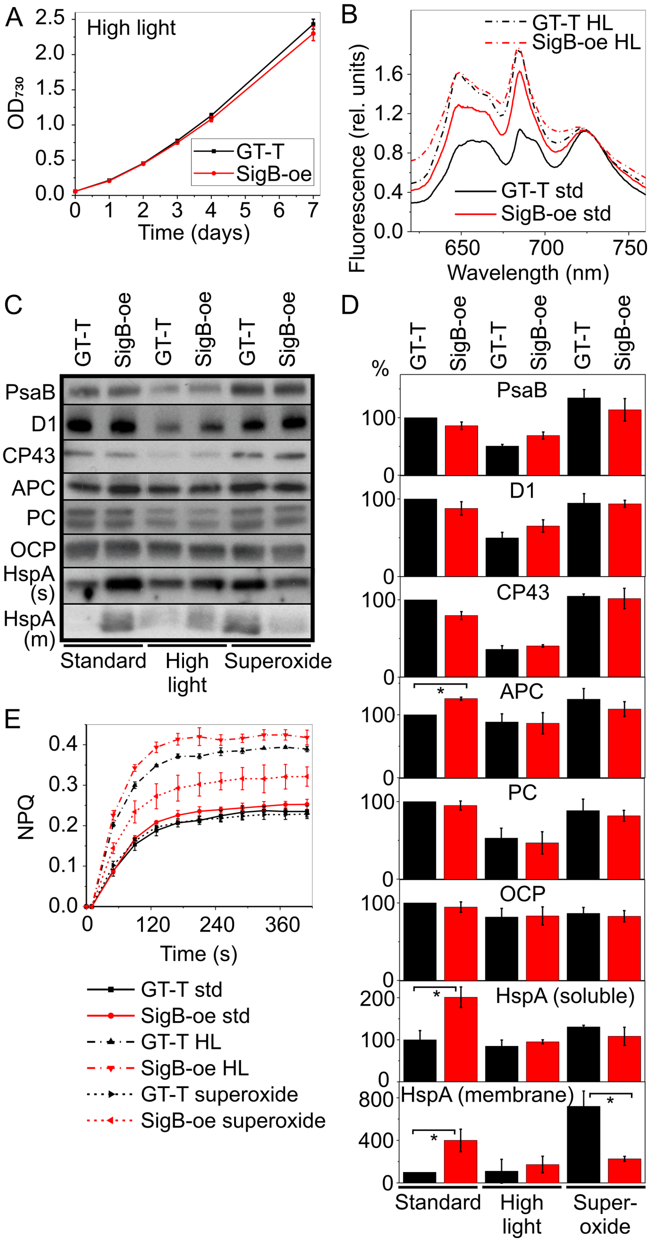

FIGURE 4 Photosynthetic properties of the SigB-oe and GT-T strains. (A) Growth of SigB-oe and GT-T in high light, PPFD 500 µmol photons m^{-2} s^{-1}. (B) 77 K fluorescence spectra excited with phycobilisome specific light of 580 nm, measured from cultures grown in standard conditions or in high light. Graphs were normalized to Photosystem I fluorescence peak at 725 nm. (C) Amounts of photosynthetic and protective proteins in SigB-oe. Amounts of PsaB, D1, CP43, phycocyanin (PC), allophycocyanin (APC), orange carotenoid protein (OCP) and HspA heat shock protein were detected with Western blotting. Soluble (PC, APC, OCP, HspA) and membrane (PsaB, D1, PsaB, HspA) protein samples were extracted from cells grown in the standard conditions, in high light or after one day treatment with 4 µM methyl viologen (superoxide stress), as indicated. Protein samples containing 1 µg (PC and APC), 2.5 µg (D1, CP43), 5 µg (PsaB), 10 µg (OCP and soluble HspA) or 50 µg (membrane-associated HspA) of proteins were separated on SDS-PAGE gels and specific antibodies were used for detection. One representative blot is shown, all three replicates are shown in Supplemental Figure S5. (D) Quantification of Western blots. The protein content in each sample was compared to the protein content of the GT-T sample in the standard conditions. Samples showing a significant difference between the SigB-oe and GT-T strains are marked with * ($p < 0.05$; Student's t-test). (E) Non-photochemical quenching induced with blue light at 480 nm (PPFD 300 µmol $m^{-2s}s^{-1}$) for 7 min after a 5 min dark adaptation. Samples were grown in standard conditions or in high light or superoxide stress was induced with 4 µM methyl viologen for one day. The results are means of three independent biological replicates (A, B, D, E), and error bars denote SE.

D1 and CP43 proteins (Figure 4C,D). Photosynthetic complexes were reduced in SigB-oe strain similarly as in the control strain in high light (Figure 4C,D), and 77 K fluorescence measurements did not reveal differences between the strains (Figure 4B). Interestingly, the 77 K fluorescence of SigB-oe in standard conditions actually resembled an intermediate state between the low-growth light and the high light-acclimated states of GT-T (Figure 4B). These data can be interpreted to indicate that the energy transfer from the phycobilisome to the reaction center in the SigB-oe strains is pre-acclimated to the high-light state already in low-light. The HspA protein has been suggested to have a dual role; it functions as a chaperone helping in the re-folding of proteins together with the other chaperones in the cytosol, whereas membrane-associated HspA protects membrane integrity (Török et al. 2001). In standard conditions, only traces of HspA were membrane-associated in the GT-T strain (Figure 4C,D). The accumulation of the HspA protein, both soluble and membrane-associated forms, was elevated in the SigB-oe strain in standard conditions (Figure 4C,D), further confirming a stress pre-acclimated state of SigB-oe cells in standard conditions.

Non-photochemical quenching (NPQ) is an important photoprotective mechanism in cyanobacteria. NPQ thermally dissipates light energy absorbed by the phycobilisome antenna via the orange carotenoid protein (OCP) in high-light (Wilson et al. 2008). The amount of OCP was similar in SigB-oe and GT-T cells both in standard and high-light conditions (Figure 4C,D), but more active NPQ was observed in SigB-oe than in GT-T cells (Figure 4E). NPQ is regulated by the fluorescence recovery protein (Boulay et al. 2010), and it is tempting to speculate that regulatory differences cause higher NPQ in SigB-oe than in GT-T strain. Similar low amounts of the soluble and membrane-associated HspA protein were measured in both strains in high-light conditions. Although SigB has been shown to play a role in high light acclimation (Pollari et al. 2009), the SigD factor is the most important σ factor for high light acclimation (Pollari et al. 2008, Koskinen et al. 2016, Hakkila et al. 2019). The *hspA* gene is not included in the SigD regulon (Turunen et al. 2022), and the HspA protein might thus play only a minor role in high light acclimation.

3.6 | SigB-oe cells are resistant to oxidative stress

Subsequently, we decided to ascertain if increased heat tolerance and active photoprotection also improve the tolerance of SigB-oe cells to oxidative stress. Superoxide stress was induced by addition of methyl viologen. The addition of methyl viologen resulted in growth arrest of the GT-T strain after one day of cultivation, whereas the SigB-oe strain was less affected and reached higher optical density during the experiment (Figure 5A). In agreement, co-cultivation experiments also demonstrated that SigB-oe cells tolerated superoxide stress better than GT-T cells, as SigB-oe cells comprised 75% of all cells after one day and 85% of all cells after two days of culture (Figure 5B). In superoxide stress, the amounts of chlorophyll *a* and phycocyanobilin were lower in SigB-oe cells than in GT-T cells (Figure 5C), and the carotenoid to chlorophyll *a* ratio was 10.5% greater in SigB-oe than in GT-T (0.60 ± 0.02 and 0.54 ± 0.004, respectively). Furthermore, the SigB-oe strain was able to activate NPQ more efficiently than GT-T in superoxide stress (Figure 4E). According to Western blot measurements, superoxide stress increased the amount of membrane-associated HspA two-fold in the SigB-oe strain and eight-fold in the GT-T strain (Figures 4C,D and S5). Complete growth arrest indicated severe stress symptoms of the GT-T strain in oxidative stress, and we suggest that membranes of GT-T become so seriously damaged that even the high accumulation of the protective HspA protein in the membrane fraction can't rescue membrane integrity. The SigB-oe strain can acclimate better to oxidative stress than the GT-T strain, which indicates that a moderate increase of the membrane-associated HspA protein in the SigB-oe strain is sufficient to keep membranes functional in oxidative stress.

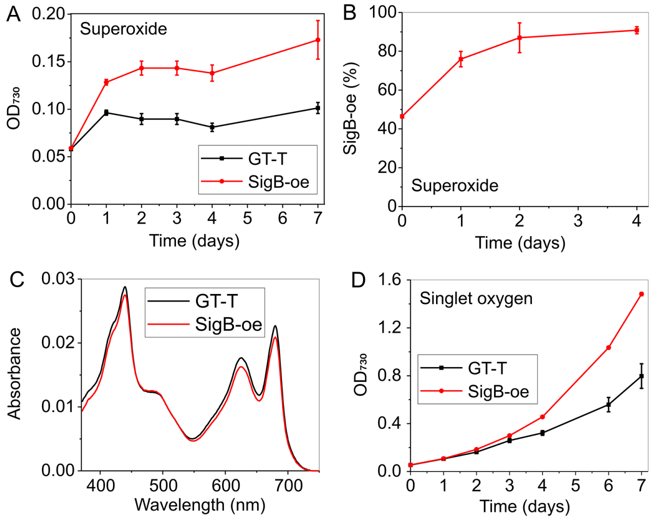

FIGURE 5 Oxidative stress tolerance of the SigB-oe and GT-T strains. (A) Growth of SigB-oe and GT-T strains under superoxide stress induced by adding 4 μM methyl viologen to the cultures in the standard conditions. (B) Percentage of SigB-oe in a co-cultivation experiment. Equal amounts of GT-T and SigB-oe cells were mixed to start a liquid culture, and the percentage of SigB-oe cells was determined once a day. (C) *In vivo* absorption spectra of SigB-oe and GT-T strains measured 6 h after 4 μM methyl viologen was added to induce superoxide stress. (D) Growth of SigB-oe and GT-T strains under singlet oxygen stress, induced by adding 5 μM methylene blue in the standard growth conditions. Each result in (A-D) represents the mean of three biological replicates and the error bars denote SE.

We also tested the performance of the SigB-oe strain in chemically induced (5 mM methylene blue) singlet oxygen stress. Singlet oxygen reduced growth in both strains, but the reduction was more prominent in the GT-T strain than in the SigB-oe strain (Figure 5D; growth in standard conditions Figure 2B).

3.7 | Potential of SigB-oe as a production host strain

To test the tolerance of the SigB-oe strain towards a model biotechnological product of industrial interest, we monitored the growth of the SigB-oe and GT-T strains in the presence of 0.5% 1-butanol for one week (Figure 6A). The growth of GT-T strain was completely

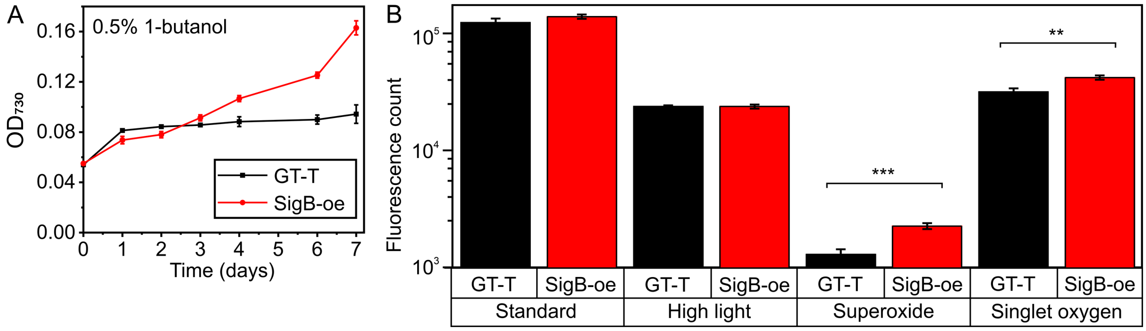

FIGURE 6 Bioproduction using GT-T or SigB-oe as a host strain. (A) Growth of SigB-oe and GT-T strains under butanol stress induced by supplementing the growth medium with 0.5% of 1-butanol in the standard growth conditions. (B) Production of yellow fluorescence protein by the GT-T and SigB-oe strains transformed with pDF-lac2(S3)-sYFP2-CmR plasmid. Cells were pre-cultured either in standard conditions for 3 days, or in the stress conditions for 5 days, thereafter OD_{730} set to 0.060 and production of YFP was induced by adding 1 mM IPTG. PPFD of high light was 500 μmol m^{-2} s^{-1}, superoxide stress was induced with 4 μM methyl viologen and singlet oxygen stress with 5 μM methylene blue. Total fluorescence count is shown per 150 μL of culture 24 h after IPTG induction, except for singlet oxygen stress, where fluorescence was measured 48 h after IPTG addition. Each result represents the mean of at least three biological replicates, and the error bars denote SE. Statistically significant differences are marked with ** ($p < 0.01$) or *** ($p < 0.001$), both according to Student's t-test.

arrested one day after the 1-butanol supplementation, indicating severe toxicity. However, although butanol addition reduced the growth of the SigB-oe strain, cells resumed growth after three days and continued to grow until the end of the experiment, unlike the GT-T strain. These results suggest that SigB-oe strain as a host strain might provide resistance against toxic end-products.

The ability of the SigB-oe strain to express heterologous protein under various conditions was investigated using yellow fluorescent protein (YFP) as a quantitative reporter. A modified broad-host-range plasmid for expressing YFP under lactose-inducible promoter (Thiel et al. 2018) was transformed into SigB-oe and GT-T strains. After antibiotic selection of the plasmid-containing lines SigB-oe + pDF-lac2-(S3) sYFP2-CmR and GT-T + pDF-lac2-(S3)sYFP2-CmR, the expression of YFP was induced with IPTG, and quantitated based on fluorescence. Similar YFP fluorescence levels were measured in SigB-oe and GT-T host strains in standard growth conditions (Figure 6B). An increase in light intensity to PPFD 500 μmol m^{-2} s^{-1} did not cause any differences between SigB-oe and GT-T strains, but decreased the total amount of YFP produced during induction (Figure 6B). The induction of oxidative stress also reduced the YFP fluorescence levels but revealed differences between the two strains. The expression of YFP was higher in SigB-oe background than in the GT-T background when cells were exposed to superoxide or singlet oxygen stresses. This result is in accordance with increased tolerance of the SigB-oe strain towards oxidative stress.

4 | DISCUSSION

4.1 | Selection of *psbA2* promoter as the overexpression promoter and the *psbA1* and *psbA2* genes as insertion target sites in the genome

The *psbA2* promoter has been widely used as a strong promoter to produce various proteins in *Synechocystis* without any reported side effects on the photosynthetic performance of the cells (Osanai et al. 2011; Gunnelius et al. 2014; Hasunuma et al. 2014; Englund et al. 2015). In accordance, amounts of the D1 protein were similar in the SigB-oe strain as in the GT-T control strain both in standard growth conditions and in different stress conditions (Figure 4C,D), indicating that the extra copy of the *psbA2* promoter does not disturb the normal regulation of the D1 protein.

The *psbA1* gene was selected as insertion target because the *psbA1* gene is known to be practically silent, and the ΔpsbA1 strain does not show any detectable phenotype, except under anaerobic conditions where the *psbA1* gene is expressed and the D1* protein is produced (Mohamed and Jansson 1989; Summerfield et al. 2008). As the *psbA1* gene is only expressed in anaerobic conditions, we consider it as a neutral insertion target for this study. The *psbA2* and *psbA3* genes produce identical D1 proteins, and previous studies have shown that either *psbA2* or *psbA3* gene alone is sufficient for normal, light-responsive, production of the D1 protein in *Synechocystis* cells (Nixon and Diner 1992; Mohamed et al. 1993; Tyystjärvi et al. 1994). Furthermore, we have replaced the coding region of the *psbA2* gene with the coding region of the *rpoZ* gene in the ΔrpoZ strain to produce a complementation strain; the photosynthetic properties of that complementation strain are similar to those of the non-mutated control strain (Gunnelius et al. 2014, Kurkela et al. 2017), indicating that the *psbA2* gene is a good insertion target.

4.2 | Growth rate control by RNAP manipulation is not yet possible

Demand for biotechnological host strains with either increased or reduced growth rates is obvious. However, we do not yet understand the regulation of cyanobacterial growth so well that we could easily pick the correct targets for bioengineering to construct optimal host strains. We tested if manipulation of σ factor content could be used to adjust the growth rate of *Synechocystis* cells. Our aim was to enhance growth by overexpressing the primary SigA factor. RNAP-SigA holoenzyme transcribes household genes (Tuominen et al. 2003; Srivastava et al. 2020) and formation of extra RNAP-SigA holoenzyme could, in theory, lead to an enhanced growth rate. Furthermore, we have previously shown that, in the ΔsigBCDE strain, the lack of group 2 σ factors leads to extra efficient formation of RNAP-SigA holoenzyme and 3-fold upregulation of rRNA and also enhanced amounts of ribosomal proteins (Koskinen et al. 2018). However, in the SigA-oe strain, RNA content was normal and growth was not up-regulated. In SigA-oe strain, the enhancement of *sigA* transcripts was stronger than that of the SigA protein, indicating that the amount of SigA protein must be under post-transcriptional regulation. One possible regulator could be a *sigA*-antisense RNA (Kopf et al. 2014) that might repress the translation of *sigA* transcripts, but further experiments are needed to identify the mechanism(s) actually controlling the abundance of the SigA protein. In comparison to ΔsigBCDE strain, SigA-oe still has all group 2 σ factors that can, by competition, lower the formation of RNAP-SigA holoenzyme. When post-transcriptional regulatory mechanisms of the *sigA* gene have been identified, manipulation of regulatory mechanisms might offer solutions to construct faster-growing *Synechocystis* cells.

Overproduction of the growth-restricting SigC factor under the strong *psbA2* promoter appeared to be detrimental to the cells as overexpression lines could not be isolated. This could possibly be circumvented by lowering the expression level with a tuneable promoter that allows the expression to be adjusted to levels that enable slow growth without significant harm to the cell.We have recently shown that the formation of RNAP-SigC holoenzyme is controlled by an anti-σ factor/anti-σ factor antagonist pair (Kurkela 2020), and manipulation of this regulation might also offer a solution to construct a slowly growing production host strain.

4.3 | SigB overexpression improves multiple stress responses

By overexpressing the group 2 σ factor SigB, we managed to construct a strain with a broad range of resistances, including tolerance to increased temperatures, high light intensities, oxidative stress and end-product toxicity. In the control strain, high *sigB* expression is only transiently induced by different stress treatments (Tuominen et al. 2003). Formation of RNAP-SigB holoenzyme in standard conditions might prepare cells for stress conditions prior to stress by enhancing carotenoids, heat shock proteins and NPQ already in standard conditions. On the other hand, constitutive maintenance of high *sigB* expression may be a key to competitive advantages. When GT-T cells encounter heat stress, the *sigB* gene is transiently up-regulated and strongly forms RNAP-SigB holoenzyme, but only after a few hours the expression returns to the normal low background level (Tuominen et al. 2006; Koskinen et al. 2016), whereas the *sigB* gene expression stays at a high level for days in the SigB-oe strain.

The regulatory targets of SigB in heat stress include the chaperones HspA and ClpB1-2 (Singh et al. 2006; Tuominen et al. 2006), which are important for acclimation to heat. In the standard conditions, the SigB-oe strain indeed had increased levels of HspA. In agreement with a previous study showing that the survival rate of the ΔsigB strain was 90% lower than that of the GT-T strain after short exposure to 48°C (Tuominen et al., 2006), the SigB-oe strain survived at 48°C better than the GT-T strain. Thus, our current work underlines the importance of SigB in both an extended exposure to moderately increased temperatures and in short-term exposure to an extreme temperature.

SigB also regulates high light acclimation including the function of the PSII repair cycle, where the D1 protein is replaced with a newly synthetized copy after photodamage (Mulo et al. 2012). Either SigB or SigD is needed for the normal high-light induction of *psbA2* and *psbA3* genes; upregulation of *psbA* genes is essential for the function of the D1 repair cycle in high light (Pollari et al. 2009). In addition, the normal expression of proteases CtpA and FtsH degrading the damaged D1 protein is dependent on RNAP-SigB (Singh et al. 2006; Turunen et al. 2022).

Besides the repair of the damaged PSII centers, SigB is also involved in the regulation of photoprotective mechanisms. The ΔsigCDE strain, containing SigB as the only functional group 2 σ factor, overproduces photoprotective carotenoids (Nikkinen et al. 2012; Hakkila et al. 2013), just like the SigB-oe strain. In addition, both SigB-oe and ΔsigCDE strains show increased NPQ (Hakkila et al. 2013) that dissipates excess light energy collected by phycobilisomes. Even though the absolute amount of OCP is not increased in SigB-oe, NPQ is activated in SigB-oe faster and more effectively across all studied conditions, which suggests regulatory differences between the strains. NPQ is induced when light-activated OCP binds to the allophycocyanin core to dissipate excitation energy as heat, and relaxes when OCP is detached by the fluorescence recovery protein (Muzzopappa and Kirilovsky 2020). A favorable conformation of the phycobilisome rods may also be required for OCP binding and action (Domínguez-Martín et al. 2022). This suggests that either decreased fluorescence recovery protein activity or another mode of regulation, like conformational differences, is taking place in SigB-oe to increase NPQ.

Both excessive high light and heat induce oxidative stress in cells. Natively, the SigB regulon varies greatly in high light and heat stress, and many of the target genes have yet unknown functions (Turunen et al. 2022). Altered expression patterns show that the SigB factor also functions as a part of regulatory networks with other regulators. Some of them are known, such as the CIRCE/HrcA system along with SigB in heat stress response (Singh et al. 2006), whereas some are likely yet to be discovered.

The most relevant reactive oxygen species (ROS) in *Synechocystis* are superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH·). *Synechocystis* can scavenge ROS enzymatically by superoxide dismutase, catalase and peroxiredoxin, or by reactions with carotenoids and glutathione as antioxidants (Latifi et al. 2009; Cameron and Pakrasi 2010; Hakkila et al. 2014). In natural conditions, especially under high light, PSII can produce singlet oxygen by transferring excitation energy to oxygen (Latifi et al. 2009). In this study, we chemically induced singlet oxygen stress, and found that SigB-oe grows faster in those conditions than the GT-T strain.

This might be explained, at least in part, by the elevated carotenoid levels of the SigB-oe strain, as carotenoids are known as efficient protectants against singlet oxygen stress (Schäfer et al. 2005). The other ROS species, superoxide, is mainly formed in cyanobacteria cells when PSI donates one electron to oxygen (Latifi et al. 2009; Kozuleva et al. 2021). We induced superoxide stress with methyl viologen and found that SigB-oe cells tolerate superoxide stress better than GT-T cells. The formation of the RNAP-SigB holoenzyme is enhanced by superoxide stress and SigB has been suggested to be the most important σ factor for acclimation to superoxide stress (Hakkila et al. 2019). The *sigB* gene is also induced in H_2O_2 stress, but the regulatory mechanism remains unknown, as the *sigB* gene does not belong to the signaling cascades involving the histidine kinases Hik33, Hik34, Hik16 or Hik41, or to the signaling cascade involving the transcription factor PerR (Kanesaki et al. 2007), which have been connected to H_2O_2 stress earlier. In context with our current findings, the observation that ΔsigCDE strain with SigB as the only remaining group 2 σ factor tolerates H_2O_2 better than GT-T (Hakkila et al. 2019), supports the role of SigB as an important regulator in different types of oxidative stress responses in *Synechocystis*.

4.4 | SigB-oe as a production host

The multiple stress adaptations linked to SigB overexpression could be beneficial for biotechnological applications, as improved host strain performance under suboptimal conditions increases system robustness and thereby the overall bioprocess efficiency. *Synechocystis* has already been engineered for the production of different chemicals, such as organic solvents and biofuels as renewable replacements for petroleum-derived chemicals now in use (Agarwal et al. 2022). Industrially relevant hydrocarbons or alcohols such as 1-butanol (Liu et al. 2019, 2022), however, are often toxic (Kämäräinen et al. 2012). 1-butanol, for example, may decrease the integrity of the lipid membranes and interfere with photosynthesis by producing reactive oxygen species (Anfelt et al. 2013) already at relatively low concentrations. The observed increase in the overall stress tolerance of the SigB-oe strain prompted us to investigate the effects in response to supplemented 1-butanol, and revealed that increased levels of SigB protected the strain and enabled growth in the presence of 0.5% 1-butanol. Although the current study calls for a systematic investigation of SigB-oe strain tolerance towards biotechnologically interesting chemicals, it emphasizes the versatile effects of SigB in protecting the cell from different types of environmental stress factors, as well as its potential applications.

To test the SigB-oe strain as a host for producing recombinant proteins, we employed YFP as a quantitative reporter, as it is nontoxic and easy to monitor. Our results demonstrated similar YFP production in SigB-oe and GT-T in the standard growth conditions, which indicates that the SigB-oe strain could be used as a host strain in favorable conditions without loss of productivity. However, in long-term oxidative stress, the end titers of YFP are greater in the SigB-oe strain than in the GT-T strain, indicating that overproduction of the stress-responsive SigB factor might be used to increase the robustness of biotechnological host strains.

5 | CONCLUSIONS

We have tested if engineering sigma factors, regulatory units of the bacterial RNA polymerase, could be used for generating better host strains of the model cyanobacterium *Synechocystis* sp. PCC 6803. Depending on practical applications, strains with a modified growth rate or stress tolerance would be highly beneficial for industrial-scale bio-production. In theory, overexpression of the primary σ factor SigA could lead to a fast-growing host strain. However, post-transcriptional regulation of the *sigA* gene prevents the construction of a fast-growing strain via simple overexpression of *sigA* transcripts. To overexpress the SigA protein, post-transcriptional regulation mechanisms should first be understood and then manipulated. To produce a slowly growing strain, the growth-restricting SigC factor might be the right target, but production of SigC should be under tunable control, as overproduction of the *sigC* gene under a strong promoter is lethal. SigB-oe cells tolerate heat, oxidative and end-product stresses better than the control strain. Bio-production of the heterologous YFP protein in the SigB-oe strain was similar in standard conditions and better during chemically induced oxidative stress. Taken together, SigB-oe might offer a more robust production host strain than the GT-T control strain.

AUTHOR CONTRIBUTIONS

T.T. and J.K. designed and supervised this work; P.K. provided expression plasmid and supervised yellow fluorescent protein measurements. O.T. performed the majority of the experiments and analyzed the results, T.S. performed His-tag experiments; T.T. and O.T. wrote the manuscript with the help from T.S., J.K. and P.K. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data can be found in the manuscript or in the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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