Inactivation of group 2 σ factors upregulates production of transcription and translation machineries in the cyanobacterium *Synechocystis* sp. PCC 6803

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We show that the formation of the RNAP holoenzyme with the primary σ factor SigA increases in the Δ*sigBCDE* strain of the cyanobacterium *Synechocystis* sp. PCC 6803 lacking all group 2 σ factors. The high RNAP-SigA holoenzyme content directly induces transcription of a particular set of housekeeping genes, including ones encoding transcription and translation machineries. In accordance with upregulated transcripts, Δ*sigBCDE* contain more RNAPs and ribosomal subunits than the control strain. Extra RNAPs are fully active, and the RNA content of Δ*sigBCDE* cells is almost tripled compared to that in the control strain. Although Δ*sigBCDE* cells produce extra rRNAs and ribosomal proteins, functional extra ribosomes are not formed, and translation activity and protein content remained similar in Δ*sigBCDE* as in the control strain. The arrangement of the RNA polymerase core genes together with the ribosomal protein genes might play a role in the co-regulation of transcription and translation machineries. Sequence logos were constructed to compare promoters of those housekeeping genes that directly react to the RNAP-SigA holoenzyme content and those ones that do not. Cyanobacterial strains with engineered transcription and translation machineries might provide solutions for construction of highly efficient production platforms for biotechnical applications in the future.

Cyanobacteria are eubacteria characterized by oxygenic photosynthesis. They branched early from other eubacteria and are currently found where ever light is available, from oceans to hot springs and fresh water environments to desert crust. These important primary producers are estimated to be responsible for one-third of carbon fixation on Earth. Recently cyanobacterial research has focused on possibilities to use cyanobacteria as bio-factories to produce valuable compounds. Lack of efficient, easily-controllable promoters has been a bottleneck in the application of cyanobacteria in biotechnology. Although many *E. coli* promoters function in cyanobacteria and cyanobacterial promoters in *E. coli*, none of the commonly used *E. coli* production systems functions well in cyanobacteria. These experiences suggest that transcriptional regulation differs notably between cyanobacteria and *E. coli*.

The RNA polymerase (RNAP) has gained specific features in the cyanobacterial lineage. In the vast majority of eubacteria, the RNAP core is composed of two α subunits and a single β, β′ and ω subunit. In the cyanobacterial lineage, the RNAP core has a unique composition with six subunits. The β′ subunit has been split in cyanobacteria, and the N-terminal part is called γ and the C-terminal part retains the name β′. Only the plastid encoded RNAPs of plants and algae share this arrangement. As plastids are descendants of cyanobacteria, the splitting of β′ has obviously occurred only once in evolution. The biological reasons and consequences of β′ splitting remain to be solved. The β′ subunit bears a 600 amino acids long cyanobacteria lineage specific insertion whose physiological role remains unclear as well. The small ω subunit of RNAP is non-essential in cyanobacteria just like in many other eubacteria, and it might play a regulatory role in cyanobacteria.

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For transcription initiation, the RNAP core recruits one of the several $\sigma$ factors to form a transcription initiation-competent RNAP holoenzyme. All $\sigma$ factors compete for the same RNAP core. This competition is affected by numerous factors including the amount of each $\sigma$ factor, affinity of different $\sigma$ factors to the RNAP core, amounts and activities of anti-$\sigma$ factors that prevent recruitment of a particular $\sigma$ factor, and RNAP modifying factors like the small signaling molecule ppGpp that directly binds to the RNAP core and changes the recruitment efficiency of different $\sigma$ factors and affect the promoter selectivity of the RNAP holoenzyme. These processes are not yet well understood in cyanobacteria.

Cyanobacteria typically encode multiple group 2 $\sigma$ factors that closely resemble the essential primary $\sigma$ factor but are non-essential in standard growth conditions. The primary $\sigma$ factor is assumed to be mainly responsible for transcription of housekeeping genes during growth. In a particular stress condition, the recruitment of one or more of the group 2 $\sigma$ factors increases. Accordingly, cells lacking group 2 $\sigma$ factors show acclimation defects in high salt, bright light or heat stresses. In addition to stress responses, the group 2 $\sigma$ factors control metabolic changes during dark-light transitions.

We have constructed a Synechocystis sp. PCC 6803 strain without any functional group 2 $\sigma$ factors. The $\Delta$sigBCDE strain is vulnerable to all tested stress conditions but grows well in standard conditions. Even in the standard conditions almost 20% of genes are at least two-fold up or down regulated in $\Delta$sigBCDE compared to the control strain. Interestingly, many genes encoding subunits of the transcriptional or translational machineries are upregulated in the $\Delta$sigBCDE mutant. In the present paper, we further investigated the regulation of transcriptional and translational machineries in cyanobacteria. The results indicate that in the absence of competition between SigA and group 2 $\sigma$ factors, more RNAP-SigA holoenzyme is formed than in the control strain. Numerous RNAP-SigA holoenzymes enhance the transcription of a particular set of housekeeping genes including those encoding transcription and translation machineries, and $\Delta$sigBCDE produces more RNAPs and ribosomal subunits than the control strain. The extra RNAPs are fully functional, and enhanced transcription leads to a high RNA content per cell whereas extra ribosomal subunits do not form translationally active ribosomes, and $\Delta$sigBCDE cells show similar translation activity and protein content as the control strain. Co-regulation of transcription and translation machineries as well as promoter differences between subgroups of housekeeping genes will be discussed.

Results

Transcripts for transcription and translation machineries are abundant in the $\Delta$sigBCDE strain. We have previously shown that the $\Delta$sigBCDE strain that lacks all functional group 2 $\sigma$ factors grows well in standard growth conditions. DNA microarray analysis of the transcriptome of $\Delta$sigBCDE in the standard growth conditions revealed that many genes encoding proteins for transcription and translation machineries were up-regulated in the $\Delta$sigBCDE strain (Supplementary Table S1). These comprise genes for RNAP core subunits, tRNA synthases, ribosomal protein subunits and translation initiation, elongation and termination factors. Unlike the RNAP core genes, the sigA gene, encoding the primary $\sigma$ factor in Synechocystis, was down-regulated in the stand-control strain13 (Supplementary Table S1). The opposite behavior of genes encoding RNAP core and sigA promoted us to study the amount and activity of RNAP complexes in the mutant strain.

RNAP content and transcription activity are upregulated in $\Delta$sigBCDE. To measure the amount of the RNAP in $\Delta$sigBCDE, total proteins were isolated from cells grown in standard conditions and the amounts of RNAP subunits were detected by western blotting. The RNAP core proteins $\alpha$, $\beta$, $\beta'$, $\gamma$, and $\delta$ were approximately 2.5-fold more abundant in $\Delta$sigBCDE than in the control strain (CS) (Fig. 1A; original Western blots are shown in Supplementary Fig. S1). Similar amount of the ATP synthase $\beta$ subunit in both strains confirmed equal loading of the samples in western blots. Thus increased transcripts of RNAP core genes were indeed used to produce more RNAPs. In addition, the SigA protein was 1.4-fold more abundant in $\Delta$sigBCDE than in CS (Fig. 1A) although sigA transcripts were 6.1-fold down-regulated in $\Delta$sigBCDE (Supplementary Table S1) indicating either post-transcriptional regulation of the sigA gene or higher stability the SigA protein in the mutant than in CS.

To analyze RNAP complexes, isolated protein samples were treated with ServaG blue and separated by blue native gel electrophoresis. ServaG blue binds to protein complexes and proteins, giving a negative charge without disturbing the subunit composition of the complex. After electrophoresis, proteins were transferred to a membrane and then the SigA factor and the $\sigma$ subunit were detected by western blotting. The antibody against the $\alpha$ subunit detected a wide area and a faint band (Fig. 1B). The upper part of the wide area contains the RNAP holoenzyme complex with the $\sigma$ factor, as this part is also recognized by the SigA antibody (Fig. 1C). The lower part of the wide area contains the RNAP core complex without the $\sigma$ factor (Fig. 1D). The faint band contains unassembled $\alpha$ subunits (Fig. 1C). The RNAP-SigA holoenzyme was more abundant in $\Delta$sigBCDE than in the control strain while the amount of free SigA protein did not differ between the strains (Fig. 1C). Also the RNAP core complexes were more abundant in $\Delta$sigBCDE than in CS (Fig. 1B).

The RNAP holoenzyme with the primary $\sigma$ factor is assumed to transcribe housekeeping genes during active growth. Upregulation of RNAP-SigA holoenzyme in $\Delta$sigBCDE might thus enhance transcription. To figure out if this is the case, the RNA content of cells of $\Delta$sigBCDE and CS was analyzed. Cell counting with a flow cytometer confirmed that the cell number per OD730 was similar in both strains (Fig. 1D). The total amount of RNA isolated from 1 ml of cell culture with optical density at 730 nm of 1.0 was approximately three-fold as high in $\Delta$sigBCDE as in CS (Fig. 1E) showing that the extra RNAPs of $\Delta$sigBCDE are active. The vast majority of all cellular RNAs are ribosomal RNAs, and analysis of RNA by agarose gel electrophoresis revealed a high RNA content in $\Delta$sigBCDE. Ribosomal RNA genes form two identical operons in Synechocystis and the final RNAs are matured in a complex process including cleavages of the sugar-phosphate backbone and modification of the bases. Only mature RNAs were detected showing that not only transcription of rRNA operons was upregulated in the mutant strains but these extra RNAs were efficiently processed to mature RNAs.
Upregulation of ribosomal subunits does not enhance translation in ΔsigBCDE.

The synthesis rate of ribosomal proteins in *E. coli* depends on the rRNA content of the cells. We found that also in ΔsigBCDE the high rRNA content was accompanied with a high amount of the Rps1 (subunit of 30S ribosomal particle) and the Rpl1 (subunit of 50S ribosomal particle) proteins (Fig. 2A: original Western blots are shown in Supplementary Fig. S1). However, labelling newly synthesized protein with radioactive 35S-methionine revealed that translation activity was similar in both strains (Fig. 2B) as was also the protein content of cells (Fig. 2C). Thus, an increased amount of ribosomal subunits did not accelerate translation in ΔsigBCDE.

To find reason(s) for the discrepancy between the ribosomal subunit content and translation activity we further analyzed the ribosomes of ΔsigBCDE. To that end, a soluble protein fraction containing ribosomes was isolated and separated by sucrose density gradient centrifugation. To keep 70S ribosomes intact, buffers were supplemented with 10 mM Mg2+. After the centrifugation, nineteen fractions were collected, and RNAs and the Rps1 and Rpl1 proteins were analyzed from each fraction. In CS, all ribosomal subunits (16S and 23S rRNAs and the Rps1 and Rpl1 proteins) were only detected in Fraction 19 (Fig. 3A,B) whereas in ΔsigBCDE all these ribosomal subunits were detected in Fractions 18 and 19 (Fig. 3C,D). Although the overall number of translating ribosomes (Fractions 18–19) was similar in both strains, the results indicate that polysomes (Fraction 19) are more common in CS than in ΔsigBCDE while in ΔsigBCDE monosomes (Fraction 18) are also typical. As the amount of translating ribosomes was similar in both strains but many housekeeping genes produce more copies of mRNA in ΔsigBCDE than in CS, the number of ribosomes reading each mRNA molecule must be lower in ΔsigBCDE than in CS.
region was divided into four transcription units; rpl3 showed lower expression than the other genes (Supplementary Table S2). In an earlier transcriptome analysis, this region comprises 8 genes for 30S ribosomal proteins, 17 genes for 50S ribosomal proteins, the infA gene encoding the translation initiation factor IF-1, the secY gene encoding a subunit of the protein translocase, the adk gene encoding adenylate kinase and the rpl15 gene encoding the β subunit of ATPase was detected as well. Original Western blots are shown in Supplementary Fig. S1. (B) Cells were labelled with radioactive 35S-methionine for 10 or 30 min, as indicated, in standard growth conditions, and total proteins were isolated by autoradiography. (C) Total proteins were isolated from the same amount of cells and the protein content was estimated using the BioRad DC protein assay kit. Three independent biological replicates were measured, Student’s t-test \( P = 0.905 \).

Arrangement of RNAP core genes in Synechocystis sp. PCC 6803 genome. Our results indicate that the production of the main components of transcriptional and translational machineries was upregulated in \( \Delta \text{sigBCDE} \). Interestingly, the gene encoding the α subunit of the RNAP core is surrounded by numerous genes encoding ribosomal proteins and thus arrangement of these genes into an operon could provide a basis for co-regulation of transcriptional and translational machineries. To detect if \( rpoA \) forms an operon with surrounding genes, DNA-free RNA was isolated and cDNA synthesis was performed using random hexanucleotide primers. Specific primer pairs recognizing two adjacent genes were then used in subsequent PCR reactions. Successful amplification of the DNA fragment in a PCR reaction indicates that the adjacent genes are in the same cDNA molecule thus belonging to the same operon.

The \( rpoA \) gene was found to belong to a large gene complex where 30 genes showed positive signals for an operon test (Fig. 4A; original agarose gels are shown in Supplementary Fig. S2). In addition to the \( rpoA \) gene, this region comprises 8 genes for 30S ribosomal proteins, 17 genes for 50S ribosomal proteins, the infA gene encoding the translation initiation factor IF-1, the secY gene encoding a subunit of the protein translocase, the adk gene encoding adenylate kinase and the truA gene encoding the tRNA pseudouridine synthase (Fig. 4A). However, when expression levels of these 30 genes were compared, two genes, \( adk \) and \( \text{infA} \), in the middle of the region, showed lower expression than the other genes (Supplementary Table S2). In an earlier transcriptome analysis, this region was divided into four transcription units, \( \text{rpl3-rpl15} \) (TU837), \( \text{secY-adk} \) (TU836), \( \text{infA} \) (Tu835), \( \text{rpl36-rpl31} \) (TU833). Our RNA secondary structure analysis revealed possible transcription termination loops downstream of the \( \text{rpl15} \) and \( \text{secY} \) genes. Because combining all these results turns out to be complicated, we next performed a Northern blot analysis.
transcript (this size would fit to the predicted TU833 from \textit{rpl36} to \textit{rpl31}) were abundant together with 2700 nt, 2200 nt and 1500 nt long transcripts (Fig. 4B). The longest transcripts became more abundant when transcription initiation was prevented with rifampicin. Thus, actively transcribing cells might mainly produce the numerous short transcripts from \textit{rpl3}-\textit{rpl31} while the full length \textit{rpl3}-\textit{rpl31} transcripts are mainly produced only if transcription initiation inside the operon is not active. The longest transcript was more stable than the shorter ones (Fig. 4B). Our results indicate a complicated transcription pattern for the \textit{rpoA} region including many internal transcription initiation and termination sites, possible regulation via antitermination process resulting in partially overlapping transcripts covering the whole region from \textit{rpl3} to \textit{rpl31}.

The \textit{rpoC2} (the $\beta'$ subunit) and \textit{rpoB} (the $\beta$ subunit) genes form an operon together with the \textit{tatD} gene (subunit of the twin-arginine protein export system) and the \textit{rps20} gene (a subunit of 30S ribosome), while the \textit{rpoC1} gene (the $\gamma$ subunit) produces a monocistronic transcript (Fig. 4C, D). The \textit{rpoZ} gene forms an operon with the \textit{sll1532} gene with an unknown function (Fig. 4E). Transcription of both the \textit{slr1634} gene and the \textit{rpoZ}-\textit{sll1532} operon continues long downstream the actual coding regions so that a great part of this area is transcribed in both directions. The originally sequenced glucose tolerant Kazusa strain of \textit{Synechocystis} sp. PCC 6803 contains a probable transposase gene \textit{slr1635} between the \textit{rpoZ} and \textit{slr1636} genes, but \textit{slr1635} is missing from our CS and from many other sequenced \textit{Synechocystis} strains.

\textbf{Promoter regions of differently regulated housekeeping genes.} Our results revealed that housekeeping genes in \textit{Synechocystis} can be divided to two categories, to those that are upregulated when RNAP-SigA content increases and to those that do not directly respond to RNAP-SigA content. We analyzed \textit{in silico} the promoter regions of those operons/genes that were upregulated in $\Delta$\textit{sigBCDE} and those that were not affected or
were even down-regulated in ΔsigBCDE. Only genes/operons with experimentally verified transcription initiation sites were chosen\(^26,27\). The expression levels of the selected genes in ΔsigBCDE, in comparison to CS, are indicated in Supplementary Table S3. An extended −10 region characterized by TGN just upstream of the −10 region was detected in many genes upregulated in ΔsigBCDE but not in those ones showing similar or lower expression in ΔsigBCDE than in CS (Fig. 5) suggesting that an extended −10 region type promoter can be considered as a consensus promoter for genes that respond directly to an increased amount of the RNAP-SigA holoenzyme. Genes that were not up-regulated in ΔsigBCDE showed high variability in the −35 region and shared only the conserved −10 region with classical *E. coli* promoters (Fig. 5).
RNAP-SigA holoenzyme induces expression of particular housekeeping genes. We selected a collection of genes overproduction of RNAP core and the primary strain.

Synechocystis cells drops drastically in the stationary phase in nitrogen depletion conditions, the growth rate and formation of RNAP-SigA holoenzymes often correlate. For example, in high CO2 the formation of RNAP-SigA holoenzymes is abundant and cells grow fast whereas nitrogen depletion causes cessation of growth and minimal formation of RNAP-SigA holoenzymes. Furthermore, the RNAP-SigB holoenzyme, in addition to the RNAP-SigA holoenzyme, can recognize sigA promoter(s). In standard growth conditions the RNAP-SigB holoenzyme content is low and thus the regulation of the sigA gene by the SigB factor is important only in special conditions like dark-light transitions. The SigA protein indicates regulation at post-transcriptional level. The mechanism of this regulation remains to be solved, but one possibility is that extra SigA protein triggers down-regulation of sigA transcripts.

In ΔsigBCDE, a high amount of SigA leads to extra efficient formation of the RNAP-SigA holoenzyme. However, the recruitment of SigA by the RNAP core does not depend only on the abundance of SigA. The Synechocystis strain missing the non-essential ω subunit of the RNAP core recruits less SigA than CS although the SigA protein is available. During nitrogen starvation, the RNAP-SigA holoenzyme content decreases more prominently than the SigA protein content and simultaneously the recruitment of group 2 σ factors increases. In E. coli, unfavorable conditions induce production of the small signaling molecule ppGpp that binds to the RNAP core and renders the core less favorable to recruitment of σ70 and more favorable to the group 2 σ factor σ54. In addition, stationary phase induces production of the Rsd protein that functions as an anti-σ70 factor in E. coli, thereby decreasing the formation of the RNAP-σ70 holoenzyme. Furthermore, binding of the RNAP-σ70 holoenzyme to the small 6S RNA molecule prevents active transcription in E. coli until favorable conditions are resumed (for 6S RNA regulation, see review). All together, these regulatory mechanisms lower the transcription activity of housekeeping genes in E. coli a lot without large changes in the σ70 content. Unlike in E. coli, the SigA content of cells drops drastically in the stationary phase in Synechocystis, whereas 6S RNA content increases only slightly, suggesting that the dose of the primary σ factor might play a more direct role in controlling the formation of the RNAP holoenzyme in Synechocystis than in E. coli.

RNAP-SigA holoenzyme and growth rate. In eubacteria, all σ factors compete for the same RNAP core to form a transcription initiation competent RNAP-holoenzyme, different σ factors having different affinity to the core. In ΔsigBCDE, an elevated dose of SigA factor and a low amount of competitors result in formation of a high number of RNAP-SigA holoenzymes, over expression of many housekeeping genes and accumulation of extra RNAs. However, the growth rate of the ΔsigBCDE is not elevated, indicating that overall transcriptional enhancement does not induce faster growth and on the other hand that accumulation of extra RNA is not harmful for Synechocystis cells. However, when the growth rate of Synechocystis changes due to changed environmental conditions, the growth rate and formation of RNAP-SigA holoenzymes often correlate. For example, in high CO2 the formation of RNAP-SigA holoenzymes is abundant and cells grow fast whereas nitrogen depletion causes cessation of growth and minimal formation of RNAP-SigA holoenzymes. E. coli cells produce different amounts of RNAP depending on growth medium, and the RNAP level has been shown to be just above the level needed for maximal growth in the particular growth medium. In E. coli, similarly as in Synechocystis, simultaneous overproduction of RNAP core and the primary σ70 factor enhances transcription of many housekeeping genes but does not enhance growth.

Selection of promoters by RNAP-SigA holoenzyme. In the ΔsigBCDE strain, a high content of RNAP-SigA holoenzyme induces expression of particular housekeeping genes. We selected a collection of
upregulated and non-upregulated housekeeping genes in ΔsigBCDE and compared their promoters. Upregulated *Synechocystis* promoters often resembled *E. coli* promoters with an extended −10 region. The promoters of non-upregulated genes only contain a typical −10 region while the rest of the promoter region varies from gene to gene. Thus promoters recognized by RNAP-SigA holoenzyme in *Synechocystis* contain a clear −10 region but otherwise those promoters show high variability, suggesting that housekeeping genes are regulated in a complicated manner that requires other factors in addition to the RNAP holoenzyme. Increased availability of RNAP-SigA holoenzymes directly enhanced the transcription efficiency of genes with extended −10 region promoters in *Synechocystis*, while cyanobacterial-specific promoters of the photosynthetic machinery did not respond to the overdose of RNAP-SigA holoenzyme. Thus, our results suggest that activation of photosynthesis related genes requires more specific regulators than just a high content of RNAP-SigA holoenzyme. Demand for mechanisms strictly regulating expression of photosynthetic genes is obvious, as imbalances in the function of photosynthetic reactions potentially induces production of harmful reactive oxygen species in cyanobacteria. We have previously shown that in the ΔrhoZ strain, a low RNAP-SigA holoenzyme content lowers especially the transcription of genes encoding the carbon concentrating and carbon fixating machineries while expression of many other housekeeping genes remain at the normal level. Patterns of gene expression in the ΔsigBCDE and ΔrhoZ strains show that different functional groups of housekeeping genes respond differently to the dose of the RNAP-SigA holoenzyme. This might be a common phenomenon in bacteria, as a recent study showed that the dose sensitivity of the RNAP-σ70 holoenzyme correlates with the functional category of the σ70 regulated genes in *E. coli*.

**Regulation of translation machinery.** The ΔsigBCDE strain produces more rRNAs than CS. In *E. coli* activity of rRNA operons correlates with growth rate and the presence of thirteen highly active rRNA operons in *Vibrio natriegens* has been used to explain the rapid growth of this bacterium (doubling time of only 10 min). High rRNA content but normal growth of ΔsigBCDE shows that growth rate and RNA content do not directly correlate in *Synechocystis*.

Two major regulatory signals, the concentration of initiator trinucleotides (ATP for six operons and GTP for one) and the amount of the starvation induced ppGpp molecule, set the expression level of the rRNA operons in *E. coli*. The rrr operons of *E. coli* contain two promoters P1 and P2; P2 provides rather constant expression while P1 is highly induced or repressed depending on conditions. The high transcription efficiency from the P1 promoters has been explained by their unique features that lead to formation of a scrutinized open complex, thereby reducing abortive initiation. The two identical rrr operons of *Synechocystis* have GTP as an initia-tor nucleotide and they contain only a single promoter resembling the σ70 consensus promoter with neither an extended −10 region nor a discriminator sequence typical for the P1 promoter (Supplementary Fig. S4), suggesting that cyanobacterial and *E. coli* rrr operons are regulated differently. Furthermore, a Blast search revealed that *Synechocystis* lacks close homologs of the HN-S, DksA or Fis proteins regulating rRNA operons in *E. coli*. In *Mycobacterium tuberculosis* the CardP protein replaces the function of the DksA protein but close homologs of this protein are missing in *Synechocystis* as well.

*Synechocystis* does contain Nus proteins that regulate Rho-dependent attenuation of the rrr operon in *E. coli*, but the Rho factor is missing and thus an *E. coli* type attenuation mechanism cannot function in cyanobacteria. Nus proteins in *Synechocystis* might be involved in rRNA processing in the same way as was recently reported in *E. coli*. At least rRNAs are efficiently processed in ΔsigBCDE, as only 16S, 23S and 5S rRNAs were detected and according to microarray data transcripts of many Nus proteins and rRNA processing enzymes were up-regulated in ΔsigBCDE.

Ribosomal RNA content regulates the production of ribosomal proteins with a feedback mechanism in which ribosomal proteins bind strongly to rRNA and weakly to their own mRNA. In accordance with that, the high rRNA content of ΔsigBCDE is accompanied with high ribosomal protein content in the mutant strain. Further studies are required to reveal the actual mechanism regulating ribosome production in *Synechocystis*.

**Co-regulation of transcription and translation machineries.** Our results show that the production of both transcription and translation machineries are similarly up-regulated in the ΔsigBCDE strain. Interestingly, both the rpoA operon and the rpoB-rpoC2 operon contain ribosomal protein genes in addition to RNAP genes. Actually the long rpoA cluster in *Synechocystis* comprises three ribosomal protein operons of *E. coli* (Supplementary Fig. S5). Gene order near the rpoA gene seems to be conserved. Analysis of circa 5000 bacterial genomes reveals that in eubacteria, the arrangement of rps13, rps11, and rpl17 genes next to the rpoA gene is highly conserved, and in many cases also rps4 is part of the gene cluster (Supplemental Table S5). In *Synechocystis*, the rps4 gene forms a monocistronic transcript in a separate location, and the rpoA operon encodes numerous other ribosomal genes in addition to the rps13, rps11, and rpl17 genes. High conservation of rps13-rps11-(rps4)-rpoA-rpl17 gene cluster suggests that the rpoA operon might play a central role for simultaneous production of transcriptional and translational machineries. The biogenesis of the RNAP core starts with the formation of the σ70 dimer but the actual roles of Rps13, Rps11 and Rpl17 proteins in the formation of functional ribosomes during the complicated assembly of ribosomes (for a review see) remains to be elucidated.

**Conclusions**

Our findings about cyanobacterial regulation of transcriptional and translational machineries are summarized in Fig. 6. Inactivation of group 2 σ factors increases the content of the principal σ factor SigA and the formation of the RNAP-SigA holoenzyme, which leads to an increase in the transcription of many housekeeping genes including genes for RNAP itself and the translational machinery. Extra transcripts of the RNAP subunits are used to produce high amounts of transcriptionally active RNAP, which enhances transcription and triples the RNA content of the cell. Ribosomal subunits are also produced in excess in ΔsigBCDE, but translation activity and the total protein content of the control and ΔsigBCDE strains are similar. The amount of functional ribosomes
ΔsigBCDE remains similar as in the control strain, pointing to post-translational regulation of the number of active ribosomes. A possibility to engineer a cyanobacterial strain with high transcription and translation capacity without a high growth rate might offer new solutions for the use of cyanobacteria for efficient production of valuable compounds in future.

Materials and Methods

Strains and growth conditions. The glucose-tolerant strain of Synechocystis sp. PCC 6803 (CS) and ΔsigBCDE were grown in BG-11 medium buffered with 20 mM Hepes, pH 7.5, in normal air at 32 °C under constant illumination at the photosynthetic photon flux density of 40 µmol m⁻² s⁻¹ as described earlier. The BG-11 agar plates of ΔsigBCDE were supplemented with appropriate antibiotics but for the experiments, liquid cultures were grown without antibiotics.

Cell content of the culture. The cell content was detected by measuring OD_{730}, dense cultures were diluted to OD_{730} = 0.4 before measurements. To determine the cell number of the CS and ΔsigBCDE cultures, the OD_{730} was set to 0.6 and cells were calculated with a flow cytometer BD LSRRortessa (BD Biosciences).

Analyses of RNA polymerase complexes. To analyze the amounts of the RNAP subunits, 30 mL of culture (OD_{730} was 1.0) grown in standard conditions was harvested at 4 °C by centrifugation at 4500 g for 6 min. Then total proteins were isolated as described earlier and protein concentration was measured with Bio-Rad DC protein assay kit. Then 15 µg of proteins were solubilized and separated with 10% NEXT GEL® SDS-PAGE (Amresco), transferred to Immobilon-P membrane (Millipore) with Trans-Blot® (BioRad) according to the manufacturer's instructions. The RNAP core proteins and the primary σ factor SigA were detected by Western blotting as described. Immuno-bLOTS were quantified using a FluorChem image analyzer (Alpha Innotech Corp). As a
loading control, ATPaseβ subunit was detected with a specific antibody (Agrisera). Three independent biological replicates were analyzed.

The RNAP complexes were analyzed by blue native gel electrophoresis. Cells were grown and harvested as described above. The cell pellet was washed with BN isolation buffer (50 mM TrisHCl pH 7.5, 150 mM NaCl, 10 mM NaEDTA pH 8.0, supplemented with Protease Inhibitor Cocktail (Roche)). Then 180 μL of BN isolation buffer and the pellet volume of acid-washed 150–212 μm glass beads (Sigma) were added, and cells were broken by vortexing 8 × 1 min at 4°C. Cell debris and membranes were removed by two centrifugation steps, first at 10 000 g for 5 min and then 18 000 g for 15 min.

To separate protein complexes, 60 μg of soluble proteins were solubilized with BN solubilization buffer (Serva G Blue 5 mg mL⁻¹, 2% glycerol, 90 mM sucrose, 2.5 mM BisTris) for 5 min on ice, and separated using 5–12% gradient gels (acrylamide:bis-acrylamide 32:1) with 4% of stacking gel using Mini protein gel system (BioRad) at 4°C. The anode buffer was 50 mM BisTris pH 7.0 and the cathode buffer 50 mM Tricine, 15 mM BisTris pH 7.0 supplemented with 0.01% Serva G; the voltage was increased after 30 min of run from 75 V to 100 V and again after 30 min to 150 V. Then after 30 min, the cathode buffer was changed to a similar buffer without Serva G and the run was continued at 200 V until the blue color ran out of the gel. The gel was soaked in a transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% methanol) for 30 min and thereafter protein transfer and immunodetection was performed as described above. Two independent biological replicates were analyzed.

**RNA content of cells.** Total RNAs was isolated from 1-mL cell culture (OD₇₃₀ was 1) with the hot phenol method as described earlier⁴⁸, and any DNA contamination was removed with TURBO DNA-free™kit (Ambion). The RNA content was measured with a spectrophotometer at 260 nm (Biodrop). To visualize RNA, a sample of isolated RNA from the same amount of the cells was separated on 1.2% agarose gel and stained with ethidium bromide. The DNA microarray data⁶ is available in GEO accession GSE69981.

**Operon analysis.** DNA-free RNA was extracted from CS as described earlier⁴. Reverse transcription (RT) was performed for 900 ng RNA using SuperScript III kit (Invitrogen) according to the manufacturer’s instructions. Then 2 μL of RT reaction was used in sequent PCR amplification with Phusion HeatShock II (Thermo Scientific). The PCR primers are listed in Supplemental Table S5. To detect PCR products, 5 μL of the PCR products were separated on 1% agarose gel and stained with ethidium bromide.

**Northern blot analysis.** The cell culture (30 mL; OD₇₃₀ was 1) was supplemented with rifampicin (500 μg mL⁻¹) and cells were collected by centrifugation after 0, 5 or 15 min of incubation in the standard growth conditions. RNA was isolated with the hot-phenol method⁴⁸. Samples containing 8 μg of total RNA were denatured with the glyoxal system, separated on 1.2% agarose gel in phosphate buffer and transferred to a Hybond-N membrane⁴⁸. The rpoA gene was amplified from genomic DNA using specific primers (Supplementary Table S5) and labelled with α-³²P-dCTP 10 mCi mL⁻¹ (Perkin Elmer) with the Prime-a-gen labelling system (Promega) according to the manufacturer’s instructions. After prehybridization in 6xSSC, 1xDenhardt’s, 0.1% SDS, 100 μg mL⁻¹ Herring sperm DNA for 1 h at 67°C, denatured probe was added and hybridized overnight at 67°C. Then membrane was washed once with 2xSCC, 0.1% SDS at 60°C for 15 min and once with 1xSCC, 0.1% SDS at 60°C for 15 min and autoradiographed.

**Promoter analysis.** Two groups of promoters of highly expressed genes were selected for sequence analysis. The first group comprises genes up-regulated in ΔsigBCDE and the second group consists of genes that either show similar expression in ΔsigBCDE and CS or are down-regulated in ΔsigBCDE (Supplementary Table S3). Each of the selected genes contained a well-defined transcriptional start site, obtained from previous studies⁶⁷,⁶⁸, and the sequence 1–60 nt upstream of each start site was retrieved. For modeling of the –10 region, the sequences were aligned, maximizing the information content in a 6-nt window ending 8 nt upstream of the transcription start site. In the optimization of the alignment, each sequence was allowed to move by 1 nt to the left and to the right. The –35 motif was modeled by aligning the sequences initially according to the –10 region, and then maximizing the information content of a 6-nt window located 15–17 nt upstream of the –10 region. The alignments were optimized with custom software used earlier in⁶.

**Ribosome analysis.** Cells (100 mL; OD₇₃₀ was 1) were collected from standard conditions by centrifugation and ribosomes were isolated at 4°C. The cell pellet was rapidly washed with 20 mM Na-EDTA, 20 mM Tris-HCl pH 8 and then with isolation buffer (20 mM NH₄Cl, 10 mM MgCl₂, 5 mM dithiothreitol, 20 mM Tris-HCl pH 8.0, and protease inhibitor cocktail (Roche)). The pellet was resuspended in 2 mL of isolation buffer and the cells were broken using a French press (Constant Systems Ltd) treating the samples twice at 2.7 kPa. Cell debris was removed by centrifugation at 10 000 g for 10 min, and the supernatant was centrifuged again at 30 000 g for 30 min. The supernatant was collected and protein concentration was determined. Two mg of proteins were loaded onto the top of 5% to 45% linear sucrose density gradient in isolation buffer and the samples were centrifuged in TH-641 rotor (Sorval) at 107 000 g for 18 h at 4°C. After centrifugation, 19 fractions, 620 μL of each, were collected. Three independent biological replicates were analyzed.

Ribosomal proteins were analyzed by western blotting. A 24-μL sample of each fraction was solubilized and proteins were separated on 12% SDS-PAGE gels with 4% stacking gel (acrylamide:bis-acrylamide 37:5:1) using Protean® II (BioRad). Proteins were transfer to the membrane and immunodetected as described above. The Rps1 promoter analysis was optimized with custom software used earlier in⁶.
volumes of ethanol. RNAs were collected by centrifugation at 18 000 g for 15 min and the RNA pellet was washed once with 70% ethanol and dissolved into 20 μL of water. Fifteen μL samples were separated on 1.2% agarose gels in TAE buffer and stained with ethidium bromide.

**Detection of newly synthesized proteins.** Cells were grown in standard growth conditions. Five μL of radioactive 35S-methionine (35S L-Met 185 MBq, Perkin Elmer) was added to 20 mL of cell culture (OD730 was 1) and cells were labelled for 10 or 30 min under the standard growth conditions. Cold L-Met was added to a final concentration 0.4 mg mL⁻¹, samples were rapidly cooled and cells were harvested by centrifugation at 4500 g for 6 min at 4 °C and total proteins were isolated as described. Proteins (60 μg) were separated by 10% NEXT GEL SDS-PAGE, transferred onto membrane and autoradiographed. Two independent biological replicates were performed.

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**Author Contributions**

S.K. and T.T. designed research, S.K. performed operon and ribosome analysis, K.H. measured translation activity and cell, RNA and protein contents, J.K. analyzed RNA polymerase and performed Northern blots, E.T. constructed sequence logos and T.T. wrote the article with contributions of all authors.

**Additional Information**

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