Crosstalk regulation among group 2- Sigma factors in Synechocystis PCC6803

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Abstract

Background: The cyanobacterium Synechocystis PCC6803 contains one group 1 (sigA) and four group 2 (sigB, sigC, sigD and sigE) sigma factors. The activity of these multiple sigma factors determines the transcriptional program of this bacterium. We wanted to study the role of the group 2 sigma factors in Synechocystis. We have therefore constructed mutants of each of the group 2 sigma factors and investigated their crosstalk.

Results: We used quantitative RT-PCR analysis to measure the relative abundance of the sig mRNAs in the four sigma mutants. Our data indicate that a network of mutual transcriptional regulation links the expression of the sigma genes. Accordingly, an environmental stress acting on only one of the sigma factors will indirectly modify the expression of most of the other sigma factors. This was confirmed by the transcriptional analysis of the sig mRNAs as a function of nitrogen starvation.

Conclusion: Taken together, our observations suggest that the crosstalk regulation between all group 1 and group 2 genes could be important for the adaptation of the bacterium to different environmental and physiological conditions.

Background

Bacterial sigma subunits of RNA polymerase are global regulators of gene expression, conferring specificity to the recognition of promoters by the core enzyme. Two broad families of sigma factors have been identified: the σ70 type, and the σ54 type factors [1]. The σ54 family regulates a variety of genes such as those involved in chemotaxis, synthesis of structural components of flagella and enzymes involved in the response to nitrogen starvation [2]. The σ70 family is subdivided into three groups [1]. Group 1 comprises the primary sigma factors that control the transcription of housekeeping genes, and these sigma factors are therefore essential for cell viability. Groups 2 and 3 include the so-called alternative sigma factors that coordinate the regulation of gene expression in bacteria on a global level. They direct the transcription of a specific genetic program that allows bacteria to cope with particular environmental changes and stress conditions. Group 2 sigma factors are similar in sequence to primary sigma factors and include proteins such as the stationary-phase-specific sigma factor, RpoS [3]. Group 3 sigma factors show less sequence similarity with those of group 1 and
include proteins required for the heat shock response [4] and motility [5]. The inactivation of a gene encoding a particular groups 2 or group 3 sigma factor usually produces growth defects or other phenotypes under specific physiological or environmental conditions. An E. coli rpoS mutant, e.g., has a pleitropic phenotype: it shows a loss of viability in stationary phase and a decreased resistance to some stresses such as the osmotic stress [6]. In Synechocystis PCC6803, inactivation of the sigF gene, encoding a group 3 sigma factor, leads to the loss of motility and pilus formation [7]. In Synechococcus four mutants of rpoD genes show defects in the circadian expression of the psbA1 gene, encoding the protein D1 of the photosystem II reaction center [8].

The unicellular cyanobacterium Synechocystis sp. strain PCC6803 possesses one group 1 sigma factor, sigA (slr0653), four group 3 sigma factors (sll0687, sll0856, slr1545, slr1564) and four group 2 sigma factors, sigB to sigE (sll0306, sll0184, slr2012, sll1689) [9]. SigE is involved in the response to nitrogen stress [10] and a contribution of the SigB/SigD factors to the dark/light adaptation has been reported recently [11]. The synthesis of the other alternative sigma factors is also modulated in response to particular stresses [12-14].

In order to assess the role of alternative sigma factors in Synechocystis, we have chosen to study the group 2 sigma factors. We have analyzed the transcription of all members of this family of sig genes as well as the transcription of the group 1 sigma factor in Synechocystis PCC6803 wild type strain and mutants lacking the group 2 sigma genes. Based on our results we suggest that these sigma factors are linked by a network of mutual regulation that could allow them to act in concert in the global transcriptional control of this bacterium.

Results and discussion

Construction and growth of sigma mutants

We have constructed null mutant strains lacking either sigB, sigC, sigD, or sigE gene as described in Materials and Methods. These four mutants segregated completely (data not shown). We were not able to inactivate the sigA gene, suggesting that it is necessary for the viability of the organism. The growth of the sigB, sigC, sigD, or sigE mutants compared to the wild type strain was examined under normal growth conditions. The results of Figure 1 indicate that all four mutants have a similar growth rate as the wild type strain during the exponential phase, showing that these sigma factors are dispensable for growth under our culture conditions and confirming their classification as group 2 sigma factors.

Expression of the group 2 sigma genes during normal growth

Recent studies have shown that all four group 2 sigma genes are expressed in normally growing cells [12]. We wanted to assess if their transcription changes in response to a gradual physiological modification of the internal and external environment of the bacterium. Our analysis is based on quantitative RT-PCR. We quantified the amount of the sigB, sigC, sigD and sigE transcripts during three stages of growth: mid log, early stationary phase and late stationary phase. The level of the transcripts was normalized to the level of rpoA transcription as described in Material and Methods. The results presented in Figure 2 show that all four group 2 sigma factors are transcribed in all growth phases. However, the relative abundance of the different sigma factors changes all along the growth of the culture. The sigB transcript was maximum during exponential phase, and decreased as cells grew into stationary phase. sigC and sigD transcription decreased upon entry into stationary phase and increased as cells were adapted to this stage of growth, sigE had the weakest variation. Imamura et al. [12] have shown that the protein concentrations of three (SigC, SigD, SigE) sigma factors vary in a similar manner as in our transcriptional analysis, suggesting that, at least for these factors, RNA levels correlate well with protein amounts.

These expression profiles show that the transcript levels of at least three of these sigma factors depend on the cell density (A750 value) of the culture and suggest that they could be important for the global physiology of this bacterium at all stages of growth.

All sigma factors are expressed in many environmental conditions tested, such as iron and sulfur starvations, and heat and osmotic shocks (data not shown). Similar results were previously obtained in Synechococcus elongatus PCC7942 [15] where all sigma genes were found to be active under many growth conditions. Recently Imamura et al. [12] measured the concentration of all five sigma factors during normal growth of Synechocystis PCC6803. They found the amounts of these proteins to vary between 1 and 10 fmoles/µg of total protein. These data suggest that all five factors are important for the cellular physiology of Synechocystis PCC6803 under standard conditions. This conclusion is somewhat surprising since four of the five sig genes can be mutated, indicating that neither of them is essential for viability under these conditions. One possible way to reconcile these two divergent observations would be to suppose that the function of the different sigma factors is redundant. According to this model, most genes would be transcribed by more than one sigma factor. Indeed, the sigma genes themselves are transcribed from multiple promoters [12]. Furthermore, it is even possible that the different sigma factors recognize the
same promoter. This hypothesis is supported by data obtained from in vitro transcription experiments in which all three different sigma factors (RpoD1, RpoD3, RpoD4) could initiate the transcription of the rRNA, cpcB1A1 and P1a promoters of Synechococcus sp. strain PCC7942 [15]. This specificity crosstalk among sigma factors is also revealed by an in vivo analysis of psbA1 promoter activities in *S. elongatus* where the principal sigma factor, as well as each group 2 sigma factor, all recognize the psbA1 promoter of this bacterium [16].

**Transcription of sig genes in σ mutants**

Since the sig genes are transcribed by RNA polymerase holoenzyme, they necessarily regulate each other's transcription. In other systems it is well documented that the transcription of alternative sigma factor genes is controlled by other σ factors [17-19]. In order to investigate this regulatory network, we measured the transcription of each of the five sig genes in all four σ mutants during exponential growth. Our method can detect transcripts of all sig genes in all of the mutants because the cDNA synthetized during the RT-PCR used primers that anneal upstream of the inactivating chloramphenicol cassette. The results shown in Figure 3 confirm the existence of complex regulatory connections between the different sigma factors and suggest a highly interconnected network: (i) mutation in the sigB gene leads to a 6-fold decrease of sigA, sigC and sigE genes (Figure 3), (ii) in the sigD mutant, transcription of the sigA, sigB, sigC and sigE genes decreased about 3 to 4-fold (Figure 3), (iii) sigE mutation leads to a strong decrease (about 20-fold) of the transcription of the sigA and sigB genes and to a 5-to 3-fold decrease of the

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**Figure 1**

Growth of the sig mutants (solid circle) compared to the wild type strain (open circle) under normal conditions. a: sigB mutant, b: sigC mutant, c: sigD mutant, d: sigE mutant. Wild type and mutant strains were grown in BgllI medium. Growth was monitored by measuring the optical density at 750 nm (A750).
expression of the sigC and sigD genes (Figure 3). (iii) mutation in the sigC gene does not negatively affect the transcription of any of the 4 sigma genes tested (Figure 3).

SigE seems to be a particularly important sigma factor because it controls the expression of three other sig genes. Mutation of the sigE gene had the strongest effects among all mutants inactivating sigma genes, affecting particularly the housekeeping gene sigA and sigB. The role of the housekeeping sigma factor, SigA, remains less well defined. Since inactivation of this gene is lethal, we will have to investigate its role using conditional mutants or biochemical methods.

By quantifying the sigma transcripts in different sigma mutants we have shown that the transcription of the sig genes is controlled by a network of mutual connections between the sigs. Previous studies in related organisms had also shown a mutual transcriptional regulation of sigma factors. In *Synechococcus* PCC7942, the rpoD1 gene is transcribed by RpoD3 and RpoD4 factors [15] and SigC factor has a negative effect on SigB expression [11]. In *Borrelia burgdorferi*, for example, RpoN regulates the expression of rpoS [18].

Sigma factors transcription under nitrogen starvation
According to the network of mutual transcriptional regulation of the sigma factors, we speculated that an environmental stress acting on only one of the sigma factors will indirectly modify the expression of most of the other sigma factors. We tested this hypothesis by analyzing the transcription profiles of the group 1 sigma gene and the four group 2 sigma factors under nitrogen starvation. We measured the mRNA concentration of the group 1 sigma gene and the four group 2 genes by quantitative RT-PCR under nitrogen starvation. As shown in Figure 4a, sigE expression was induced, as expected, about 5-fold with respect to the reference gene rpoA. The transcription of the other four sigma genes was also induced (about 2- to 6-fold). Nitrogen starvation does not only lead to the over-expression of the sigE gene but rather provoked a readjustment of the relative abundance of the sigma factors. This global change of the expression of all sigma genes in response to this particular environmental condition agrees with our hypothesis that the cross-talk regulation among the sigmas could lead to the transmission of one particular signal to all of them. The transcription of a structural gene involved in this stress should be affected by more than one sigma factor. *glnN*, encoding the GSIII glutamine synthase, known to be highly expressed under conditions of nitrogen deficiency [10,17] was chosen as a target gene for the nitrogen starvation. We have analyzed its transcription in the wild-type and mutant strains after one week of nitrogen starvation. Induction of *glnN* transcription was observed in the wild type strain. This induction was abolished when SigB, SigC or SigE factors were inactivated (Figure 4b). These results clearly demonstrated that more than one sigma factor affects the transcription of the *glnN* gene.

Our analysis is based on measuring the first level of control of sig genes expression. In other systems, post-transcriptional modifications of sigma factors can occur and may not correlate with transcriptional profiles. The recent data of Imamura et al [12] suggest however that the intracellular concentration of the sigma factors correlates well with the transcriptional control of these sigma factors (with the possible exception of SigB). The same authors demonstrated that a negative effect of SigC on sigB transcription correlates with the reduction of SigB protein levels in this mutant [12]. These data suggest that transcriptional regulation of sig genes is not drastically modified by post-transcriptional control. This hypothesis is actually under investigation in our laboratory.

Conclusion
*Synechocystis* PCC6803 possesses multiple sigma factors and its transcriptional program is largely determined by the activity of these multiple sigma factors. Our study has explored the relationship among all members of the group 2 sigma factors and their connection with the housekeeping sigma factor. We have shown that the transcription of the sig genes is controlled by a network of mutual connections. The strongest effects are compiled in

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**Figure 2**
Trascription of sig genes during normal growth. Measurement of relative concentrations of sigB, sigC, sigD and sigE mRNAs in *Synechocystis* by quantitative RT-PCR during three stages of normal growth: mid log phase in black (A750 = 1); early stationary phase in white (A750 = 8); and late stationary phase in grey (A750 = 12.6). Each sample was measured in triplicate and the standard deviation is indicated by error bars. Values were normalized to the rpoA transcript.
Figure 5, where the thickness of the arrows is proportional to the effect of a given mutation on the expression of the sigma genes. For example, mutating the \( \text{sigE} \) gene results in the reduced expression of four sigma genes, with a more pronounced effect on the \( \text{sigA} \) and \( \text{sigB} \) genes. A mutation in the \( \text{sigB} \) gene results in a small reduction of the transcription of the \( \text{sigA} \), \( \text{sigE} \) and \( \text{sigC} \) genes. Expression of the group 1 sigma gene (\( \text{sigA} \)) is affected by mutations of three group 2 sigma genes. Our study has explored the relationship among all members of one family of sigma factors in eubacteria. We assume that their mutual connections are part of a more extended regulation network. In fact, it is tempting to speculate that all sigma factors in a cell control each other. A possible connection to group 3 sigma factors, as well as the relationships among sigma factors and sensor and regulatory proteins in the cell remain to be elucidated.

**Methods**

**Culture and growth conditions**

*Synechocystis* sp. strain PCC6803 was obtained from the Pasteur culture collection. Wild-type and mutant strains were grown at 30°C with continuous illumination at approximately 20 \( \mu \text{E} \text{ m}^{-2} \text{ s}^{-1} \), with 3% \( \text{CO}_2 \) in air, in BG11 medium [20], buffered with 5 mM Hepes-KOH, pH8. Growth was monitored by measuring the optical density at 750 nm (\( A_{750} \)).

For nitrogen starvation, BG11 medium lacking the nitrogen source (\( \text{NaNO}_3 \)) was buffered with 20 mM N-
tris(hydroxymethyl)methyl-2-aminoethanosulfonic acid (TES) buffer, pH 7.5. Strains used in this condition were grown to an A750 = 1, transferred to the nitrogen-depleted medium and incubated for one week. All cyanobacterial strains were grown on BG11 plates containing 1.5% Difco Bacto Agar. When needed, chloramphenicol was added to a concentration of 10 μg/ml. Growth rates of mutants were compared to a *Synechocystis* strain carrying the same antibiotic resistance cassette inserted into an inessential gene, *ureA*. We call this strain the wild-type for our experiments.

**DNA manipulation and RNA isolation**

Molecular techniques were performed according to standard procedures [21]. *Synechocystis* genomic DNA was prepared according to the method of Tandeau de Marsac et al. [22]. RNA was extracted from pelleted cells, broken by freezing in liquid nitrogen, and using the RNeasy kit (Qiagen) according to the manufacturer’s specifications. Chromosomal DNA was removed by treating RNA preparations with 1 μl of DNAse (at 2U/μl) (Ambion) for 1 hour at 37°C. The concentration of RNA was determined spectrophotometrically.

**Gene inactivation**

The *sigB* (*sll0306*), *sigC* (*sll0184*), *sigD* (*sll2012*) and *sigE* (*sll1689*) genes [9] were cloned using the TOPO-TA cloning kit (Invitrogen) and the following primers: *sigB*-1 and *sigB*-2, *sigC*-1 and *sigC*-2, *sigD*-1 and *sigD*-2, *sigE*-1 and *sigE*-2.

These genes were then subcloned into pBluescript SK-plasmid (Stratagene) between the *ApaI* and *SpeI* sites. A
chloramphenicol cassette was inserted at the unique site BglII in sigC, SmaI in sigB, and BamH1 both in sigE and sigD. The cassette was obtained from the pACYC184 plasmid [23] by PCR amplification. The primers used were cat-1 and cat-2; they add BamH1 and SmaI restriction sites at each end of the amplified sequence.

The SK- vector derivatives containing each interrupted gene were used to transform *Synechocystis*. Chloramphenicol-resistant transformants that were also ampicillin sensitive were selected and subsequently screened for replacement of the wild-type gene allele with the corresponding mutant. Genomic DNA isolated from individual CmR transformants was verified by PCR.

**Reverse transcription**

For each reaction, 1 µl of antisens primer mix at 2.5 µM of each of the primers and 200 ng of total RNA were denatured at 95°C and chilled quickly on ice. A mix consisting of 4 µl of 5x buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2, 50 mM DTT), 0.5 µl of RNase Inhibitor (40U/µl), 1 µl of 5 mM dNTP and 1 µl of MMLV reverse transcriptase enzyme (200U/µl) was added in a total volume of 20 µl, followed by one hour of incubation at 37°C. The composition of the primer mix used in each reverse transcription reaction was dependent on the genes analyzed in the quantitative PCR experiments (for example when *rpoA* and *glnN* genes were quantified, the reverse transcription reaction used *rpoA*-RT and *glnN*-RT primers). The primers used were: *rpoA*-RT; *sigB*, *C*-RT; *sigA*, *D*, *E*-RT; *glnN*-RT.

The sequences of all the primers used in this study are listed in table 1.

**Real-time quantitative PCR**

PCR conditions were identical for all reactions. The 25 µl reaction mixture consisted of 1x master mix buffer (Eurogentec), 0.75 µl of SYBR Green I Dye (Eurogentec), and 1 µl of each primer (2 µM). 5 µl aliquots of the diluted reverse transcription reaction were used as template. PCR amplifications (2' at 50°- 10' at 95° – 40 X [15 '' at 95° – 1' at 60°]) were carried out in a Gen Amp 5700 sequence detection system (Applied Biosystems). The forward (F) and reverse (R) primers used in these PCR reactions were designed using the Primer Express software (Applied Bio-

| Primer | Sequence |
|--------|----------|
| *sigB*-1 | CGGAATTCTTGGGTATCTTTTACG |
| *sigB*-2 | CCGAAGCTTGGGCAACTACCTGG |
| *sigC*-1 | CCGAATTCAAGCTGGCATGGG |
| *sigC*-2 | CCGAAGCTTGGCTGCTAACCAGATAG |
| *sigD*-1 | CCGAATTCTTGGATCTTTTACG |
| *sigD*-2 | CCGAAGCTTGGCAGCTTAAAGGC |
| *sigE*-1 | CCGAATTCTTGGGAAATCTAAATAG |
| *sigE*-2 | CCGAAGCTTGGCTGCTAACCAGATAG |
| *cat*-1 | CCGGATTCGGCAGGAAATTACCCG |
| *cat*-2 | CCGGATTCGCGCCCGAGGAGCTAAGGAAGCTA |
| *rpoA*-RT | TAACCTA |
| *sigB*, *C*-RT | TAACCTT |
| *sigA*, *D*, *E*-RT | TAACCTT |
| *glnN*-RT | CCATCGTC |
| *sigA* forward | TGGAGTTGAAACCG |
| *sigB* reverse | GACTGACACATTGTCTTGG |
| *sigB* forward | AGAAATGGCCCAGCTATCC |
| *sigB* reverse | GCCGCCAGTGTAAAAAGG |
| *sigC* forward | TGGAGTGGAAACCG |
| *sigC* reverse | GACTGACACATTGTCTTGG |
| *sigD* forward | GATGGCCCTGCGAGG |
| *sigD* reverse | TTGCCTTCTGATATTCTGG |
| *sigE* forward | CGGGCCGAGAATCC |
| *sigE* reverse | CCAACTCCTGGCAGATC |
| *rpoA* forward | GAGTTCCGCGACTTTCTAGGC |
| *rpoA* reverse | TTGGATCAATAACCTCCACCTC |
| *glnN* forward | GATTTACCAGGAGCCG |
| *glnN* reverse | CAAAACCGTTCAGTGAAGGC |
Table 2: rpoA Cₜ values for the different growth conditions tested in this study. The reported values represent the average of 6 measurements obtained from two separate RNA preparations.

| Culture condition or genetic background | rpoA Cₜ value per 20 ng total RNA |
|-----------------------------------------|-----------------------------------|
| Normal                                  | 28.5 ± 1.03                       |
| Nitrogen starvation                     | 29 ± 0.27                         |
| Exponential phase                       | 29.44 ± 0.03                      |
| Early stationary phase                  | 28.60 ± 0.52                      |
| Late stationary phase                   | 28.15 ± 0.04                      |
| Wild type strain                        | 30 ± 0.91                         |
| sigB mutant                             | 30.29 ± 0.25                      |
| sigC mutant                             | 29.66 ± 1.06                      |
| sigD mutant                             | 29.76 ± 0.8                       |
| sigE mutant                             | 29.85 ± 0.69                      |

A L conceived the study, constructed the mutants and helped in writing the manuscript.

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