6S RNA – an old issue became blue-green

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INTRODUCTION

6S RNA was initially discovered in Escherichia coli in 1967 (Hindley, 1967), and some years later was shown to exist as a ribonucleoprotein complex in the cell (Lee et al., 1978). No function could be assigned until the year 2000, when a major breakthrough was made by the discovery that the protein associated with 6S RNA is RNA polymerase and that this small non-coding RNA apparently regulates transcription (Wassarman & Storz, 2000). This finding initiated broad interest, and since then a plethora of studies were confined to the E. coli system, and we know that 6S RNA in that organism accumulates in stationary phase, forming stable complexes with RNA polymerase holoenzyme containing the sigma factor 70, responsible for exponential growth. This leads to the inhibition of transcription for many but not all 70-dependent promoters, and facilitates the adaptation to stationary phase and environmental stress (Cavanagh et al., 2008; Gildehaus et al., 2007; Trotochaud & Wassarman, 2004, 2006). Additional functions for 6S RNA have been discovered by genome-wide transcriptome studies, indicating that the molecule is important for central steps in the metabolism of the cell, such as growth phase adaptation or carbon and purine metabolism (Geißen et al., 2010; Neußer et al., 2007; Trotochaud & Wassarman, 2004, 2006).

6S RNA from Escherichia coli acts as a versatile transcriptional regulator by binding to the RNA polymerase and changing promoter selectivity. Although homologous 6S RNA structures exist in a wide range of bacteria, including cyanobacteria, our knowledge of 6S RNA function results almost exclusively from studies with E. coli. To test for potential structural and functional conservation, we selected four predicted cyanobacterial 6S RNAs (Synechocystis, Synechococcus, Prochlorococcus and Nostoc), which we compared with their E. coli counterpart. Temperature-gradient gel electrophoresis revealed similar thermodynamic transition profiles for all 6S RNAs, indicating basically similar secondary structures. Subtle differences in melting behaviour of the different RNAs point to minor structural variations possibly linked to differences in optimal growth temperature. Secondary structural analysis of three cyanobacterial 6S RNAs employing limited enzymic hydrolysis and in-line probing supported the predicted high degree of secondary structure conservation. Testing for functional homology we found that all cyanobacterial 6S RNAs were active in binding E. coli RNA polymerase and transcriptional inhibition, and had the ability to act as template for transcription of product RNAs (pRNAs). Deletion of the 6S RNA gene in Synechocystis did not significantly affect cell growth in liquid media but reduced fitness during growth on solid agar. While our study shows that basic 6S RNA functions are conserved in species as distantly related as E. coli and cyanobacteria, we also noted a subtle degree of divergence, which might reflect fundamental differences in transcriptional regulation and lifestyle, thus providing the first evidence for a possible physiological role in cyanobacteria.
2010). Our previous studies also revealed that there is a link between 6S RNA and the global regulator ppGpp, which affects growth rate, ribosome synthesis and the translational capacity of the cell (Cavanagh et al., 2010; Neußer et al., 2010). A particularly striking discovery was the observation that 6S RNA can serve as a template for the de novo transcription of small RNAs [de novo RNAs (dnRNAs) or product RNAs (pRNAs)] when stationary cells encounter better nutritional conditions (Gildehaus et al., 2007; Wassarman & Saecker, 2006; Wurm et al., 2010). This finding implies that the small RNA acting as transcriptional regulator templates its own regulatory RNA (Kugel & Goodrich, 2007).

The widespread nature of 6S RNA among eubacteria has raised the question whether 6S RNA is a universal regulator and if there is functional conservation of 6S RNAs from different organisms. With the exception of a partial characterization of 6S RNA from Legionella pneumophila, indicating its involvement in virulence (Faucher et al., 2010), and 6S-1 RNA from Bacillus subtilis implicated in transcriptional fine-tuning (Beckmann et al., 2011), almost nothing is known about the function of 6S RNAs in other organisms. Here we were especially interested in the molecular functions of 6S RNA within cyanobacteria because they are different in many features from other bacteria.

The phylum cyanobacteria is highly diverse, with at least 10,000 species known to date populating all regions on Earth such as freshwater, oceans and hot springs, or living in symbioses with plants or marine sponges. Fossil and molecular studies suggest that the archetype of cyanobacteria thrived successfully for billions of years (Schopf, 1993). Photosynthesis evolved early in ancient cyanobacteria, enriching our atmosphere in oxygen.

For two freshwater species, Synechococcus PCC 6301 and Synechocystis PCC 6803, a 6Sa RNA (encoded by the ssaA gene) was described for the first time (Watanabe et al., 1997) without recognizing that the cyanobacterial 6Sa RNA is the orthologue of the γ-proteobacterial 6S RNA. Later on, extensive sequence comparisons revealed that the highly stable secondary structure of 6S RNA in cyanobacteria is different from other bacteria.

The expression of 6S RNA of cyanobacteria have revealed different results. In contrast to E. coli, which 6S RNA accumulation can be observed during entry into stationary phase, in Synechococcus PCC 6301 the opposite has been found (Watanabe et al., 1997). In Prochlorococcus MED4, two 6S RNA transcripts exist with different lengths. Maximal accumulation is observed either at high cell densities (220 nt transcript) or earlier (332 nt transcript), probably during entry into stationary phase (Axmann et al., 2007). The expression of 6S RNA of Prochlorococcus MED4 is not affected by other conditions employed, and the RNA has been shown to be very stable (half-life >1 h) (Axmann et al., 2005). The existence of two differentially expressed 6S RNAs, 6Sa (6S-2 RNA) and 6Sb (6S-1RNA), has already been shown for B. subtilis, in which 6Sb is the orthologue of E. coli 6S RNA, and 6Sa has diverged functionally (Barrick et al., 2005).

This study was conducted to test the structural and functional conservation of 6S RNA in cyanobacteria and to compare their properties with the characteristic functions of E. coli 6S RNA, which had already been analysed in more detail. The study should contribute to understanding cyanobacterial regulation by small RNAs and enable more specific investigations in future.

**METHODS**

**Bacterial strains and growth conditions.** The Δssp E. coli strain KS-1, an MG1655 derivative, constructed by a one-step homologous recombination (Datsenko & Wanner, 2000), was a friendly gift of K. Shanmugarajah. Liquid cultures of Synechocystis PCC 6803 wild-type and the ΔssaA mutant strains were grown at 30 °C in BG11 medium (Rippka et al., 1979) under continuous illumination with white light of 80 μmol photons·m⁻²·s⁻¹ and a continuous stream of air. The medium for the mutant strains was supplemented with 25 μg kanamycin ml⁻¹.

**Mutagenesis.** The complete Synechocystis sequence region ssaA encoding the 6S RNA was deleted and replaced by a resistance marker gene, which enables the isolation of generated mutants. For homologous recombination a construct was generated which included a kanamycin-resistance cassette flanked by 700 bp regions upstream and downstream of the ssaA gene (Fig. S7). Primers for the two-step overlap extension PCR are listed in Table S1, available with the online version of this paper. This construct was used to transform Synechocystis as described elsewhere (Ernokova et al., 1993).

**Complementation of the E. coli 6S RNA deletion strain with cyanobacterial homologues.** For the complementation of the E. coli Δssp strain KS-1 we inserted the PCR-generated 6S RNA genes of Synechocystis, or Synechococcus flanked by EcoRI/Msal restriction sites, into the vector pKK223-3 under the control of the tac promoter. PCR primers are listed in Table S1. Cloning resulted in the vectors pKK-6803-6S and pKK-7942-6S. The vector pKK-6S containing the E. coli 6S RNA gene was used as a control. Competent E. coli KS-1 cells were transformed and positive transformants selected by their resistance to ampicillin.

**Preparation of 6S RNAs.** The different 6S RNA sequences were isolated as PCR fragments from genomic DNA of the respective cyanobacteria and transcribed by T7 RNA polymerase (AmpliScribe T7-Flash Transcription kit; Epicentre). Primers for the amplification of the cyanobacterial 6S RNA genes are presented in Table S1. E. coli 6S RNA was prepared by in vitro transcription with purified T7 RNA polymerase and linearized pUC18-T7 derivatives, which contained the respective 6S RNA gene sequence behind the T7 Φ10 promoter (Gildehaus et al., 2007).

**Preparation of E. coli RNA polymerase.** E. coli RNA polymerase holoenzyme was purified according to published procedures (Burgess & Jendrisak, 1975; Gonzalez et al., 1977).

**Temperature-gradient gel electrophoresis (TGGE).** Temperature-dependent structural transitions of in vitro-transcribed 6S RNAs were analysed by TGGE, as described by Rosenbaum & Riesner (1987). Prior to electrophoresis RNAs were heated in 50 mM sodium cacodylate, pH 7.2, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT for 10 min at 70 °C and slowly refolded (1 °C min⁻¹). Each RNA (1–1.5 μg) was
RESULTS

Given the bioinformatics prediction of 6S RNA genes in almost all sequenced cyanobacteria and the experimental evidence for several different strains that these genes are expressed (Axmann et al., 2005, 2007; Watanabe et al., 1997) (see also Fig. S1) we wished to know whether predicted cyanobacterial 6S RNA molecules exhibit properties and/or functions similar to those demonstrated for the well-characterized E. coli 6S RNA (Gildehaus et al., 2007; Wassarman, 2007; Wassarman & Storz, 2000; Wassarman & Saecher, 2006; Wurm et al., 2010). For the analysis we selected four examples [Synechocystis sp. PCC 6803, Synechococcus elongatus PCC 7942, Prochlorococcus MED4 and Nostoc sp. PCC 7120 (named below Synecho-
cystis, Synechococcus, Prochlorococcus and Nostoc, respectively)], which were distantly related on the phylogenetic map (Fig. S1). Interestingly, the 6S RNA genes are often located downstream of purK (encoding phosphoribosyl-
minoimidazole carboxylase), which is reminiscent of the situation in many entero- and γ-proteobacteria, where the 6S RNA gene is co-transcribed with the ygA gene, also involved in purine metabolism (Barrick et al., 2005; Jeanguenin et al., 2010).

Comparison of the secondary structures and thermodynamic stabilities of 6S RNAs from cyanobacteria

The secondary structure predictions for the selected cyanobacterial 6S RNAs are generally in agreement with the respective E. coli 6S RNA secondary structure, which consists of a largely single-stranded central bulge flanked by two non-contiguous helical stem regions (Figs 1b and S3). This structure has been experimentally verified (Barrick et al., 2005; Gildehaus et al., 2007; Trotchaud & Wassarman, 2005). One exception results from a 5′ sequence extension of the Prochlorococcus 6S RNA, which can be transcribed from two different promoters, giving rise to a long (332 nt) and a short form (220 nt) in the cell (Axmann et al., 2007). Both RNAs are significantly longer than E. coli 6S RNA or the other cyanobacterial 6S RNAs (generally below 200 nt), slightly obscuring structure comparison.

The secondary structure of E. coli 6S RNA gives rise to a very characteristic melting behaviour, which can readily be observed by altered mobilities during TGGE (Wagner, 2006). To test the reliability of the predicted cyanobacterial 6S RNA structures and to analyse their thermodynamic stabilities we performed TGGE experiments (Fig. 1). A characteristic, partly irreversible transition to slower gel mobility was visible at 46 °C for the E. coli 6S RNA (Fig. 1a), very likely reflecting the cooperative melting of the structure, which initiates at the two helical arms flanking the central bulge and proceeds into the neighbouring stem regions (see scheme in Fig. 1b). The resulting change in mobility can be taken as a specific signature for typical 6S RNA secondary structures. In fact, all four cyanobacterial 6S RNAs analysed exhibited similar electrophoresis.
patterns on the TGGE gels (Fig. 1c–f). However, compared with the *E. coli* 6S RNA, the transition temperature representing cooperative melting of the secondary structure was lower and differed slightly for the individual cyanobacterial 6S RNAs. The characteristic melting temperatures derived from the TGGE gels (indicated by arrows in Fig. 1) varied between 28 °C (*Prochlorococcus*) and 36 °C (*Nostoc*) and, interestingly, the order of melting temperatures correlated with the optimal growth temperature of the different bacteria tested. In the case of *Synechococcus* (Fig. 1e) and *Synechocystis* (Fig. 1d), additional bands are visible on the high-temperature side of the gel, which merge with the main band. These bands likely indicate coexisting structures of the 6S RNA molecules, which disappear at higher temperature. As opposed to the rather discontinuous mobility transitions of 6S RNAs from *E. coli*, *Synechocystis*, *Synechococcus* and *Nostoc*, the transition for *Prochlorococcus* was continuous, indicating an ordered, more reversible melting process. This is consistent with the prediction of a different closing stem structure for *Prochlorococcus* 6S RNA that prevents the complete disruption of the 5′ and 3′ ends in one step, and rather causes a consecutive melting of the stem structures involved. While a general common secondary structure appears to be conserved for 6S RNAs, the results also indicate subtle structural differences, possibly reflecting specific adaptation to the individual lifestyles of different organisms.

**Structural probing analysis of 6S RNAs from *Synechocystis*, *Synechococcus* and *Nostoc***

Limited enzymic hydrolysis and spontaneous cleavage (inline probing) of 6S RNAs labelled with [32P]ppCp at the 3′ ends was employed to verify and compare the secondary structures of the different cyanobacterial 6S RNAs (Soukup & Breaker, 1999; Wagner, 2006). Enzymic reactions were performed with guanosine-specific RNase T1, which preferentially cleaves non-base-paired structures, and the double strand-specific RNase V1 from cobra venom was used to map helical and base-paired regions. Single-stranded structures were additionally identified by the inline probing method based on their reduced chemical stability (Soukup & Breaker, 1999). Results are shown in Fig. 2, where the probing patterns of 6S RNAs from *Synechocystis*, *Synechococcus* and *Nostoc* after separation on
Fig. 2. Structural probing of 6S RNAs from Synechocystis, Synechococcus and Nostoc. Results from enzymic secondary structure probing are shown. (a) Short gel electrophoretic separation of 6S RNA samples from Synechocystis (PCC 7803), Synechococcus (PCC 7942) and Nostoc (PCC 7120) (from left to right) after limited enzymic hydrolysis. T1, RNA after hydrolysis with guanosine-specific RNase T1 (left, 20 mU; right, 10 mU); V1, cobra venom RNase (double strand-specific); OH−, alkaline ladder; C, unreacted control RNA. Characteristic sequence positions are depicted at the margin of each panel (left, RNase T1; right, V1). (b) For a better resolution of the 5′ sequence a longer gel electrophoretic separation of the same analysis as in (a) is shown. (c) Results from in-line probing (ILP) are presented. In C and OH−, unreacted control RNAs and samples after alkaline hydrolysis are separated, respectively. The left panel Synechocystis (PCC 6803) contains a T1 lane to indicate relevant guanosine positions, and characteristic positions prone to in-line attack are marked in the right margin. In the mid and right panels, sequence length positions for the different Synechococcus (PCC 7942) and Nostoc (PCC 7120) 6S RNA hydrolysis products are indicated.

Cyanobacterial 6S RNAs bind specifically to RNA polymerase from E. coli

It is known that 6S RNA from E. coli specifically interacts with the σ70 holoenzyme of RNA polymerase, which is responsible for transcription of the housekeeping genes during exponential growth. Contact sites on the RNA have been identified for E. coli (Gildehaus et al., 2007) and the RNA polymerase β, β′ and σ70 subunits have been shown to be in contact with the 6S RNA from E. coli and Haemophilus influenzae (Gildehaus et al., 2007; Wassarman & Storz, 2000). Moreover, a detailed analysis has identified sites within the σ70 subunit which likely interact with E. coli 6S RNA (Klocko & Wassarman, 2009). No direct interaction could be shown with the isolated sigma factors and only weak, possibly non-specific, binding was observed to the RNA polymerase core enzyme. Generally, bacterial RNA polymerases are highly conserved with respect to structures and function. RNA polymerases from cyanobacteria, however, are known to contain an additional γ subunit, which represents a split β′ gene (Schneider et al., 1987; Xie et al., 1989). Based on in vitro transcription studies it has also been reported that the two enzymes from Calothrix PCC 7601 and E. coli are not completely interchangeable (Schyns et al., 1998). However, despite subtle differences in the RNA polymerase architecture, a general recognition of heterologous E. coli or Nostoc promoters and transcription initiation could be demonstrated for the different enzymes (Schneider et al., 1987). Encouraged by the latter observation and considering the general functional conservation of RNA polymerases among bacteria we performed heterologous binding experiments with the different cyanobacterial 6S RNAs and the purified RNA polymerase holoenzyme from E. coli. Binding assays were performed with 32P end-labelled 6S RNA and increasing concentrations of RNA polymerase holoenzyme Eσ70 under standard conditions (see Methods). Complex formation was analysed by gel retardation, as described previously (Wurm et al., 2010), and the results are presented in Fig. 3. Since direct 3′ end-labelling of the Prochlorococcus 6S RNA turned out to be inefficient we analysed binding of this RNA species by a competition assay (Fig. 3b). 6S RNAs from Synechocystis, Synechococcus and Nostoc were able to form specific complexes with E. coli RNA polymerase with affinities close to that of the homologous E. coli 6S RNA (Fig. 3a). Binding of 6S RNA from Prochlorococcus to RNA polymerase could also be demonstrated by efficient competition for pre-formed RNA polymerase complexes with 6S RNA from Synechocystis (Fig. 3b). The presence of heparin in all binding experiments assured the specificity of the complexes formed. It is also evident from Fig. 3 that more than one complex is formed, particularly for 6S RNAs from Synechocystis and Synechococcus. Although we cannot exclude the putative existence of multiple conformations of RNA–holoenzyme complexes, formation of more than one complex on native polyacrylamide gels has also been
reported for RNA polymerase and 6S RNA from *E. coli*, and in that case it was shown that the band with the higher mobility represented core RNA polymerase–6S RNA complexes (Gildehaus et al., 2007). Since the RNA polymerase preparation contained some free core enzyme it is likely that the two complex bands observed also represent core and holoenzyme complexes. In summary, the experiment demonstrates that all four investigated cyanobacterial 6S RNAs are capable of forming specific complexes with *E. coli* RNA polymerase in much the same way as the homologous RNA, underlining their potential involvement in transcription regulation.

**Cyanobacterial 6S RNAs inhibit transcription in vitro**

It has been shown that 6S RNA from *E. coli* is able to inhibit *in vitro* transcription from a variety of promoters (Gildehaus et al., 2007). Therefore we compared the ability of the different cyanobacterial 6S RNAs to interfere specifically with transcription under the same conditions. Transcription reactions were performed with a multipromoter template harbouring a set of different *E. coli* promoters, which differ in regulatory properties and promoter strength (*rrnB P1, tac, bolA, RNA 1 and hisL*). Each promoter gives rise to a transcript of defined length due to the *rrnB* tandem terminators, which are positioned at a defined downstream site. The results from multiple-round *in vitro* transcription reactions performed in the presence of increasing concentrations of the respective 6S RNAs are shown in Fig. 4. The specificity of 6S RNAs regulating transcription was tested in a control reaction, where instead of 6S RNA, increasing amounts of tRNA were added. The analyses revealed similar inhibition patterns for all four cyanobacterial 6S RNAs in comparison with the *E. coli* 6S RNA, with slightly weaker inhibition for 6S RNA from *Prochlorococcus* and *Synechococcus*. The amount of 6S RNA to reach half-maximal inhibition varied slightly for the different 6S RNAs and was also not identical for the individual promoters tested. The observed differences in the degree of inhibition for the different cyanobacterial 6S RNAs can be explained by subtle differences in the affinities of the RNAs for *E. coli* RNA polymerase, but may also reflect an unidentified promoter-specific component, a property which is also known for *E. coli* 6S RNA (Cavanagh et al., 2008; Gildehaus et al., 2007; Neußer et al., 2010). The addition of increasing concentrations of tRNA did not affect the transcription of any promoters notably, underlining the specificity of the 6S RNAs to act as transcriptional repressors. In summary, we conclude that cyanobacterial 6S RNAs, like their *E. coli* counterpart, are able to act as specific transcriptional inhibitors. Moreover, as demonstrated for *E. coli*, inhibition seems to depend on specific promoter properties.

**Cyanobacterial 6S RNAs serve as templates for the de novo synthesis of small pRNAs**

The most striking property of 6S RNA is its function as an RNA polymerase template, directing the synthesis of small RNAs, which themselves serve as regulators of 6S RNA activity (Gildehaus et al., 2007; Wassarman & Saecker, 2006; Wurm et al., 2010). 6S RNA-directed *de novo* transcription of small RNAs (pRNAs) occurs in the cell at high concentrations of substrate NTPs, for instance, when cells recover from stationary phase after a nutritional
upshift. The reaction has been analysed in detail in *E. coli*, but 6S RNA-directed pRNA transcripts have also been reported in other bacteria, such as *B. subtilis* and *Helicobacter pylori*, mainly based on deep sequencing approaches (Beckmann et al., 2011; Irnov et al., 2010; Sharma et al., 2010). The existence of 6S RNA-templated small RNAs has also been observed from whole-transcriptome sequencing analysis of several different cyanobacteria. For instance, a potential pRNA transcript, slightly larger (~30 nt) than the ones observed in *E. coli*, was detected by genome-wide mapping of transcription start sites in *Synechocystis* (Mitschke et al., 2011). In principle, the small RNAs may also arise as antisense transcription products directed from a possible convergent promoter. In fact, a sequence with reasonable similarity to cyanobacterial promoters can be found on the *ssaA* antisense strand in *Synechocystis*. To verify that the observed small RNA derives from a 6S RNA-templated transcription we performed *in vitro* reactions with purified RNA polymerase, NTP substrates and cyanobacterial 6S RNAs in the absence of any DNA under conditions that lead to the well-characterized synthesis of pRNAs in *E. coli*. In Fig. 5, the generation of small 6S RNA-derived transcripts at increasing RNA polymerase concentrations is exemplified for 6S RNAs from *E. coli* and *Synechocystis*. In the case of *E. coli* 6S RNA the characteristic products of 15 to 20 nt starting from sequence position U44 can be seen (Wurm et al., 2010). A similar reaction pattern is apparent for *Synechocystis* 6S RNA, although a series of longer transcripts, up to 30 nt, consistent with the results from genome-wide sequencing, is formed (Fig. 5a). We take this as evidence that the *E. coli* RNA polymerase can substitute for the homologous enzyme in this reaction. Considering the length of the pRNA transcripts and the deep sequencing data (Mitschke et al., 2011), the deduced start site of the transcript matches with the single-stranded U47 and reads into the irregular helix of the closing stem (Fig. 5b).

To confirm that the *in vitro*-generated products are indeed identical to the genuine pRNA sequence we tested the identity of the sequences by hybridization to specific DNA

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*Fig. 4.* Inhibition of transcription by cyanobacterial 6S RNAs. (a) Gel electrophoretic separation of multiple-round *in vitro* transcription products obtained in the presence of increasing amounts of 6S RNAs (0, 10, 50, 100, 250 and 500 nM) from different species. Transcription reactions were performed with *E. coli* RNA polymerase and a multi-promoter vector (Methods). Transcription products originating from the different promoters are depicted in the left margin.

*Fig. 5.* Cyanobacterial 6S RNAs serve as templates for the synthesis of small pRNAs. (a) 6S RNA-templated synthesis of pRNAs in the absence of DNA with increasing concentrations of *E. coli* RNA polymerase (0, 50, 100, 200 and 500 nM). *E. coli* and *Synechocystis* 6S RNA at 300 nM each was employed as indicated. An NTP mix (300 μM each of ATP, GTP, UTP and 5 μM CTP) containing 133 nM [α-32P]CTP was used for product labelling. Bands representing *de novo* transcription products are indicated on the right. Size markers are shown next to the left panel. (b) Secondary structure arrangement of *Synechocystis* 6S RNA, with the start point and direction of pRNA transcription indicated by an arrow.
oligonucleotides. Results for the products obtained from *Synechocystis* and *Synechococcus* clearly confirmed the correctness of the *in vitro*-generated pRNAs (Fig. S5). Experiments performed with the other cyanobacterial 6S RNAs revealed that in all cases small RNAs were formed, although they differed in yield and length distribution (Fig. S6). The results also demonstrate that the small RNAs identified by deep sequencing are very likely products derived from RNA-templated transcription and not from any antisense promoter. Moreover, we conclude that formation of 6S RNA-templated pRNAs seems to be a common mechanism not only in *γ*-proteobacteria and firmicutes but also in the phylogenetically distant cyanobacteria. The length difference noted and a possible specific influence of the pRNA sequences are matters for more detailed future studies.

**6S RNA-directed de novo synthesis of small pRNAs causes disintegration of RNA polymerase–cyanobacterial 6S RNA complexes**

It has been demonstrated that synthesis of pRNAs in *E. coli* is a key reaction to disintegrate the stable RNA polymerase–6S RNA complexes, which sequester almost all RNA polymerase molecules in the cell during stationary phase. Rapid recovery from this inhibition is necessary when the nutritional conditions improve and active transcription of housekeeping genes is required. The synthesis of pRNAs is triggered in the cell by an increase in substrate NTPs as a consequence of nutritional upshift (Wassarman & Saecher, 2006; Wurm et al., 2010). The situation can be mimicked *in vitro*, and RNA polymerase–6S RNA complexes start to transcribe small RNAs when the NTP concentration is raised above 10 μM (Wurm et al., 2010). Hence, we analysed the stability of pre-formed inhibitory complexes between RNA polymerase and cyanobacterial 6S RNAs under conditions of increasing concentrations of substrate NTPs. As has been demonstrated before for *E. coli*, not only do increasing NTP concentrations give rise to small RNA synthesis but also a concomitant decay of the RNA polymerase 6S RNA complexes can be observed for cyanobacterial 6S RNAs from *Synechocystis, Synechococcus* and *Nostoc* (Fig. 6). It is also apparent that the small RNAs formed during the 6S RNA-directed transcription remain stably associated with the template 6S RNA and migrate as an RNA–RNA complex during gel electrophoresis, in the same way as that shown for *E. coli* (Wurm et al., 2010). The results show that synthesis of pRNAs in cyanobacteria reverses the inhibitory effect of the 6S RNA template. Furthermore, the data indicate a common mechanism for all 6S RNAs, possibly involving similar structural changes that trigger the disintegration of the inhibitory complex.

**Construction and phenotypic characterization of a Synechocystis 6S RNA deletion mutant**

Although 6S RNA affects transcription of many genes *in vivo* (Neußer et al., 2010), deletion of the 6S RNA gene (ssrS) in *E. coli* does not reveal a notable phenotype when analysed under rich growth conditions, and only subtle differences could be detected when mutants were observed under long-term stress or starvation (Trotchraud & Wassarman, 2004, 2006), indicating that 6S RNA improves the fitness of cells under stress conditions. To test whether similar observations can be made for 6S RNA deletions in cyanobacteria, we constructed a *Synechocystis* 6S RNA deletion strain. Deletion of the *Synechocystis* ssaA gene was achieved by homologous recombination with a kanamycin-resistance cassette flanked by upstream and downstream sequences of the ssaA gene (Fig. S7). Two positive clones (del6S-K5 and del6S-K7) were verified by PCR and Northern blot analysis and further used for growth characterization. No significant change in growth behaviour was detected due to the ssaA deletion, and both mutants exhibited similar growth curves in liquid cultures, with marginally faster growth compared with the wild-type (Fig. S8a). A difference in fitness was apparent, however, when the two ssaA mutants were grown on agar plates after serial dilutions (Fig. S8b). The number of living cells taken from an early exponential culture (OD750 0.2) appeared to be drastically reduced for the two deletion mutants, and this was visible after transfer to an agar plate (Fig. S8b, left panel). Likewise, during later growth (OD750 0.6), serial dilution revealed that both deletion mutants were still significantly reduced in the number of living cells (Fig. S8b, right panel). Possibly, cells which are growing as a layer on an agar surface are directly exposed to the light, such that the standard light conditions (80 μmol photons m⁻² s⁻¹) can become light-stress. In contrast, cells in a liquid culture may shadow each other. We conclude from this preliminary

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**Fig. 6.** 6S RNA–RNA polymerase complex decay at high NTP concentrations. Complexes between RNA polymerase (50 nM) and 15 mM of each radiolabelled 6S RNA from *E. coli*, *Synechocystis, Nostoc* and *Synechococcus* (indicated below the gel) were incubated with increasing concentrations of all four NTPs (0, 5, 50, 100 and 500 nM). Reaction mixtures were separated on 5% native polyacrylamide gels. Bands representing the free 6S RNAs, the hybrid of the complementary pRNAs and the 6S RNA, as well as the 6S RNA–RNA polymerase complexes are indicated in the left margin.
observation that, as observed for *E. coli*, cyanobacterial 6S RNAs may function to provide better fitness under certain growth conditions, in this case light.

**DISCUSSION**

In this study we have analysed the functions of a selection of cyanobacterial 6S RNAs and compared the results with the known properties of 6S RNA from *E. coli*, for which detailed functional information has already been collected. The secondary structures among different 6S RNA molecules, which are predicted under conditions that allow suboptimal structures according to Pánek et al. (2011), already suggested a certain functional conservation for these RNA regulators. TGGE analyses demonstrated that the cyanobacterial 6S RNAs share the characteristic thermodynamic melting transition with the *E. coli* homologue, consistent with a common overall secondary structure. The results from structural probing analyses of three cyanobacterial 6S RNAs largely supported the predicted structures (Fig. S3). Distinct sequence variations between the individual RNAs result in slightly different melting temperatures of the major transition, and may indicate a specialized adaptation to the optimal growth temperature and a different lifestyle. It is tempting to speculate that in addition to differences in thermal stability small structural variations may also contribute to specific stability within the cell. This could be an explanation for the differences in steady-state concentrations of 6S RNAs from *Synechocystis* and *Synechococcus* during heterologous expression in *E. coli* (Fig. S9). Since both RNAs are expressed from identical vectors with the same copy number per cell, we infer that the difference in concentration is very likely the result of a structure-dependent difference in turnover at the growth temperature for *E. coli* (37 °C).

All tested 6S RNAs were able to bind specifically to the *E. coli* RNA polymerase holoenzyme, and to inhibit in vitro transcription from a set of *E. coli* promoters. It is interesting to note in this respect that the first systematic studies of transcription initiation sites (TISs) for cyanobacteria revealed general features of the cyanobacterial promoter architecture not too divergent from *E. coli*: a conserved −10 region (TAnntT) about 6 bp upstream of the TIS, which is a purine in most cases. Also, elements with similarity to the enterobacterial −35 box (5′-TTGnnn-3′) have been detected (Mitschke et al., 2011; Vogel et al., 2003).

Most notably, we demonstrated that all tested cyanobacterial 6S RNAs were able to serve as templates for the synthesis of small pRNAs. We could show, furthermore, that the latter reaction induced the decay of the inhibitory complex between 6S RNAs and RNA polymerase. This reaction has been shown to be of physiological importance for *E. coli* in re-establishing a functional population of RNA polymerase during outgrowth from stationary phase.

Even so, the 6S RNA interaction with cyanobacterial RNA polymerase should be tested in the future because of the presence of a split subunit β′ encoded by two separate genes, *rpoC1* and *rpoC2* (Xie et al., 1989). Thus, future experiments using RNA polymerase from cyanobacteria might uncover further details of gene transcription in these organisms.

The subtle structural differences among the diverse cyanobacterial 6S RNAs are reflected in a corresponding modulation of the activity for the characteristic functions analysed in this study. In several of our assays, the 6S RNA from Nostoc exhibited a higher degree of functional homology with *E. coli*. This is noticeable for the similarity of the TGGE pattern, the efficiency in inhibiting transcription from DNA promoters, and the specificity of the pRNA products. 6S RNA from *Prochlorococcus*, however, which deviates considerably in length, gives rise to a noticeably different secondary structure prediction and only displays similar folding with the other 6S RNAs when suboptimal structures are considered. It is not surprising that 6S RNA from *Prochlorococcus* shows the largest functional deviation with respect to the other cyanobacterial 6S RNAs. This was observed, for instance, in the melting transition and the reduced capacity to inhibit in vitro transcription from the strong *Ptac* or *rrnB P1* promoters. The somewhat aberrant structure may also explain the failure of 3′-end-labelling *Prochlorococcus* 6S RNA by a ligation reaction. One might speculate that the specific structure and length of *Prochlorococcus* 6S RNA could be an adaptation to its particular niche, the upper layer of the ocean. Moreover, the 6S RNA expression pattern is very distinct for marine *Prochlorococcus* and *Synechococcus* strains, and thus might correlate with their habitat. In a previous study, all *Prochlorococcus* strains investigated, including MED4, that are adapted to high-light conditions exhibited two signals for 6S RNA, at approximately 200 and 300 nt, whereas RNA from the low-light-adapted *Prochlorococcus* and *Synechococcus* strains analysed gave only a single signal at approximately 180 nt (Axmann et al., 2007).

A particularly interesting observation is the striking genetic organization of many cyanobacterial 6S RNA genes directly adjacent to the *purK* gene, which encodes an enzyme involved in purine metabolism (Fig. S1). A similar genetic link is found in many enterobacteria and *γ- e*-proteobacteria, where 6S RNA genes are often co-transcribed with genes involved in purine metabolism (Sharma et al., 2010). This genetic co-localization obviously represents a functional link, at least in *E. coli*, where a differential expression for several enzymes involved in the biosynthesis and salvage of purines has been reported in a 6S RNA-deficient mutant (Neußer et al., 2010). It has been shown that *E. coli* 6S RNA is functionally connected not only to purine metabolism but also to the basal concentration of the global regulator ppGpp (Cavanagh et al., 2010; Neußer et al., 2010), which reflects a stress signal and mirrors changes in the nutritional status of the cell. In cyanobacteria, ppGpp is known to
accumulate in response to a sudden reduction in the incident light intensity (Akinyanju & Smith, 1982), which might thus suggest a coupling between regulation by light and/or the circadian clock and ppGpp accumulation. It is tempting to speculate, therefore, that 6S RNA in cyanobacteria might also be linked to the light–dark cycle of these organisms (Axmann et al., 2007), controlling metabolic and genetic functions. This would add another facet to the spectrum of 6S RNA regulation. Further studies in this direction are required to answer this interesting question.

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