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6S RNA, A Global Regulator of Transcription

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Abstract

6S RNA is a small RNA regulator of RNA polymerase that is present broadly throughout the bacterial kingdom. Initial functional studies in *Escherichia coli* revealed that 6S RNA forms a complex with RNA polymerase resulting in regulation of transcription, and cells lacking 6S RNA have altered survival phenotypes. The last decade has focused on deepening the understanding of several aspects of 6S RNA activity including: 1. Addressing questions of how broadly conserved 6S RNAs are in diverse organisms through continued identification and initial characterization of divergent 6S RNAs; 2. The nature of the 6S RNA-RNA polymerase interaction through examination of variant proteins and mutant RNAs, crosslinking approaches, and ultimately a cryo-EM structure; 3. The physiological consequences of 6S RNA function through identification of the 6S RNA-regulon and promoter features that determine 6S RNA sensitivity; 4. The mechanism and cellular impact of 6S RNA-directed synthesis of pRNAs (i.e. pRNA synthesis). Much has been learned about this unusual RNA, its mechanism of action and how it is regulated; yet, much still remains to be investigated, especially regarding potential differences in behavior of 6S RNAs in diverse bacteria.

INTRODUCTION

It is now well established that small RNAs (sRNAs) have diverse and widespread roles in regulating gene expression in all organisms (1–4). Mechanisms of action are varied, but can be broadly classified into three categories: 1. sRNAs that act by basepairing to target RNAs; 2. sRNAs that act to modulate protein activity through direct RNA-protein interaction; and 3. sRNAs that have intrinsic function (e.g. catalytic). Two well studied sRNA families that modulate protein activity include sRNAs that regulate CsrA protein (see Chapter X) and 6S RNA, which regulates RNA polymerase and is the focus of this chapter as well as several reviews (5–8). 6S RNA was first identified in *Escherichia coli* (9, 10), which remains the best understood model of 6S RNA function, although identification of 6S RNAs and their roles in diverse bacterial species has been an active area of research in the past decade. Here, information for *E. coli* will be presented first, followed by discussion of similarities and differences known or postulated for 6S RNAs in diverse species.

E. coli 6S RNA (*Ec*6S RNA) was first discovered as a highly abundant, stable RNA more than 50 years ago (9), but it was not until the discovery that *Ec*6S RNA formed a complex with RNA polymerase more than 30 years later that its biological function and mechanism of action began to be revealed (11). Bacterial RNA polymerase is a multisubunit enzyme, consisting of a core (E: $\alpha\alpha\beta\beta$') that is transcriptionally competent but requires the addition of a specificity factor (sigma: σ) to form the holoenzyme (Eσ) needed to recognize

promoters and initiate transcription (12, 13). All bacteria contain a housekeeping σ, usually referred to as σ^{70} or σ^{A}, which is highly abundant and required at all stages of growth. Different bacterial species have varied numbers of alternative sigma factors, ranging from zero to several dozen. *Ec*6S RNA interacts specifically and very tightly with the housekeeping holoenzyme form of RNA polymerase ($E\sigma^{70}$ in *E. coli*) (11, 14). Early studies demonstrated that *Ec*6S RNA binding to $E\sigma^{70}$ resulted in downregulation of σ^{70} -dependent transcription at a tested promoter (*rsd*P2), and that cells lacking *Ec*6S RNA have altered survival phenotypes (11, 15, 16). These findings led to the suggestion that the physiological role of 6S RNA, at least in *E. coli*, is to contribute to regulation of gene expression in response to poor nutrient environments. Research over the past decade has brought considerable insight into 6S RNA function, but also raised many questions for future work. This chapter focuses on several active areas of inquiry including the identification of 6S RNAs in diverse bacterial species, the details of 6S RNA-RNA polymerase interactions, the identification of promoters regulated by 6S RNA, the physiological consequences of 6S RNA-dependent regulation, and finally the discovery and impact of pRNA synthesis, a process in which 6S RNA serves as a template for synthesis of a product RNA (pRNA). The biogenesis of *Ec*6S RNA has also been an area of interest and active research, but will not be discussed in this chapter. For information about the biogenesis of 6S RNA see (5, 17–24)

6S RNAS ARE WIDESPREAD THROUGHOUT THE BACTERIAL KINGDOM

Identification Of 6S RNA And Candidate 6S RNAs

During the past 15 years, much work on 6S RNA has focused on its interaction with $E\sigma^{70}$ and its presence and potential impact in diverse bacteria. Biochemical and phylogenetic approaches demonstrated that overall secondary structure was critical for 6S RNA-$E\sigma^{70}$ interactions (14, 25). Specifically, 6S RNA is primarily double-stranded with minor disruptions of bulged nucleotides in addition to a large, central, single-stranded region [Figure 1]. It is this secondary structure, rather than sequence identity, that is required for *Ec*6S RNA to bind $E\sigma^{70}$ and has been the basis for identification of most 6S RNAs and 6S RNA-encoding genes in diverse genomic sequences. Alternative approaches to identify 6S RNAs have included identification of sRNAs that co-immunoprecipitate with RNA polymerase or through identification of 6S RNA-associated pRNA in RNA-sequencing data. Altogether, 6S RNAs have been identified or predicted in a wide range of bacteria (26), making it one of the very highly conserved sRNAs along with tmRNA, SRP RNA and the Csr/Rsm family of RNAs (27).

Identification of additional 6S RNAs is an ongoing process as more genome sequences become available and as RNAseq approaches to examine RNAs globally are applied to more organisms. However, identification of 6S RNAs globally remains non-trivial as the parameters defining the structural requirements for interaction with RNA polymerase are not as fixed as structural requirements of some other sRNAs (e.g. tmRNA interaction with ribosomes and alanyl-tRNA synthetases (28)), and recent work suggests there may be more variation in specific contacts in divergent bacteria than previously predicted. Nevertheless, identification and characterization of diverse 6S RNAs continues to provide important information about 6S RNA and its cellular role generally and in specific species.

Some species have multiple 6S RNAs—Some genomes encode more than one 6S RNA, most notably *Bacillus subtilis* where the two 6S RNAs (*Bs*6S-1 and *Bs*6S-2 RNAs) (14, 25) have been studied in some detail. *Legionella pneumophila* also has been reported to express two 6S RNAs, *Lp*6S RNA and *Lp*6S-2 RNA, and two candidate 6S RNA encoding genes were identified in *Hydrogenivirgia* (29–32). The presence of two 6S RNAs in divergent bacteria raises interesting questions regarding how common it is to have multiple 6S RNAs, how they are independently regulated, if their activities are redundant or overlapping, and so forth. In *B. subtilis* and *L. pneumophila*, the two 6S RNAs act independently of each other, have different accumulation profiles, and at least for *B. subtilis*, are known to regulate different genes (14, 25, 33–36).

6S RNA accumulation profiles - insight into function?—One of the first steps in characterization of many of the newly identified 6S RNAs has been to look at their expression profiles, especially during growth phase or under specialized growth conditions. *Ec*6S RNA is present at all times of growth in *E. coli*, but is at lower levels in early exponential phase (<1,000 copies per cell) and gradually accumulates during growth until it reaches maximal levels (~10,000 copies per cell) several hours after transition into stationary phase (11). Thus, much work on *Ec*6S RNA has focused on its role in stationary phase, where it contributes to cell survival, although it should be noted that *Ec*6S RNA is present in exponential phase, is bound to $E\sigma^{70}$ and can regulate transcription at this time (14, 37, 38). In contrast, many 6S RNAs in divergent species have different expression profiles, which may provide insight into predictions of different physiological roles for these RNAs.

*Bs*6S-1 RNA is an example of a 6S RNA that accumulates in stationary phase with expression profiles similar to *Ec*6S RNA, and *Lp*6S RNA also accumulates post-exponentially (29, 39). In contrast, Bs6S-2 RNA levels change only modestly throughout growth (< 2–3 fold) with maximal levels observed in late exponential phase, although the precise expression profile reported for Bs6S-2 RNA has varied between different studies, likely due to differences in strains and growth media and timing of “stationary” phase examined (14, 25, 40). The *Lp*6S2 RNA expression profile is more complex but is not similar to *Ec*6S RNA (31). The differences in accumulation of *Bs*6S-1 and *Bs*6S-2 RNAs or *Lp*6S and *Lp*6S2 RNAs suggest that they likely have different physiological roles, in agreement with observations that gene expression and proteomic profiles for cells lacking *Bs*6S-1 and *Bs*6S-2 RNAs are different (33, 36), and observed mutant phenotypes (i.e. altered timing of sporulation and cell density changes in stationary phase) are associated specifically with the loss of *Bs*6S-1 RNA (34, 36). *Streptomyces coelicolor* 6S RNA also accumulates with a profile similar to *Ec*6S RNA and influences growth rate (41).

Other 6S RNAs accumulate with different profiles that hint at interesting cellular roles. For example, within identified Cyanobacteria 6S RNAs there is an array of different accumulation patterns. Levels of *Prochlorococcus* MED4 6S RNA are cell-cycle dependent and change with light, suggesting 6S RNA may contribute to the high light adaptation of this strain (42). 6S RNA in *Synechocystis* PCC6803 has also been suggested to contribute to light stress as well as recovery from nitrogen depletion (6, 43). Alternatively, 6S RNA in *Synechococcus* sp. PCC6301 changes during growth suggesting a response to nutrient

status, although in this case 6S RNA is abundant in exponential phase and reduced in stationary phase, perhaps more similar to *Bs*6S-2 RNA (44).

Examples of several alpha-proteobacteria that associate with host cells exhibit differential expression dependent on host association. For example, the plant symbiont *Bradyrhizobium japonicum* 6S RNA is higher in root nodules compared to free living cells (45, 46). *Wolbachia* 6S RNA levels change with host identity (i.e. 6S RNA levels were higher in germ-line cells compared to somatic cells) (46, 47). It has been suggested the *Wolbachia* 6S RNA accumulation increases during fast replication, in contrast to *Ec*6S and *Bs*6S-1 RNAs that accumulate during slow growth (i.e. stationary phase). *Rhodobacter sphaeroides* and *Caulobacter crescentus* (free living alpha-proteobacteria) 6S RNA accumulation patterns change with cell growth, although the change between exponential and stationary phase levels is rather modest (~3 fold) (33, 48, 49). *R. sphaeroides* 6S RNA has been associated with high salt stress survival (48).

Several 6S RNAs from pathogenic bacteria or close relatives have been shown to increase under stress, suggesting a potential role in pathogenesis or in host survival. Examples include *Burkholderia coenocepacia* 6S RNA that increases during oxidative stress (50), *Yersinia pestis* 6S RNA that has altered levels during lung infection (51), *Rickettsia* 6S RNA that accumulates many hours post-infection and correlates with intracellular growth kinetics (52), *Coxiella burnetii* 6S RNA that accumulates in the stress resistant cellular form (small cell variant; SCV) suggesting a role in stress (53), *Salmonella enterica serovar Typhimurium* 6S RNA that accumulates at low pH resulting in altered invasion and stress survival (54), *Clostridium acetobutylicum* 6S RNA that increases in response to general stress and is reported to promote butanol tolerance (55), and *Borrelia burgdorferi* 6S RNA that accumulates in ticks with timing suggesting a role in persistence (S. Samuels, L. Hall and D. Drecktrah, personal communication).

Additional 6S RNAs have been observed to be expressed under at least one condition (e.g. *Bordetella pertussis* (14), *C. crescentus* (56), *Clostridium difficile* (57), *Helicobacter pylori* (58), *Pseudomonas aeruginosa* (59), *Rhodospeudomonas palustris* (46)), and many others have been predicted from genomic sequences with minimal additional information about expression patterns or function yet available. In fact, 6S RNA candidates have been predicted in the majority of bacterial genomes; see (26) for a recent update on the distribution of 6S RNAs throughout bacteria.

Many questions remain about 6S RNAs from diverse bacteria, and future work is anticipated to focus on providing further understanding of their physiological roles in different biological circumstances. In addition, there is the potential that some of these 6S RNAs and candidate 6S RNAs may expand on known mechanistic activities.

How to define divergent 6S RNA candidates as 6S RNAs?—Of particular importance as these divergent 6S RNAs are further studied and as additional 6S RNAs candidates are identified, is the question of whether these candidates are all true 6S RNAs and what defines a 6S RNA. We have previously suggested 6S RNAs should be defined as RNAs that bind their cognate primary holoenzyme form of RNA polymerase in a manner

resembling promoter DNA binding; a definition likely to capture a class of RNAs that are mechanistically similar and thereby providing a functionally useful definition (5). It has been suggested to include "directing pRNA synthesis" in the definition (36), but it is not included here as pRNA synthesis is not required for 6S RNA to regulate transcription (see (35)) and there may be examples where 6S RNAs rely on other mechanisms to cycle off of RNA polymerase but retain similar mechanisms of regulation of transcription. Nevertheless, either definition requires detailed information about RNA polymerase binding, a characteristic that is harder to test, especially in divergent systems where tools are not readily available. In some tested cases, however, there has been quite a large variation in the fraction of 6S RNA that is bound to RNA polymerase (>75% for *Ec*6S RNA, *Bs*6S-1 RNA and *Bs*6S-2 RNA compared to <10% for *Lp*6S RNA) (11, 14, 29), which may be due to technical reasons, but could represent critical differences in 6S RNA function. As more details about 6S RNA-RNA polymerase interactions in diverse bacteria become available, it will be important to revisit the definition of 6S RNAs, in particular whether there are additional classes of small RNAs that interact with RNA polymerase to function using different mechanisms.

At least one RNA identified based on its structural similarity to 6S RNA (Ms1 RNA in *Mycobacterium smegmatis*) does not bind to the holoenzyme form of RNA polymerase, although it does interact with core RNA polymerase (60, 61). This example serves as a cautionary tale in relying too heavily on secondary structure as the predictor of 6S RNA candidates, but also raises the intriguing possibility that there are additional classes of sRNAs that function through interaction with RNA polymerase using different mechanisms. Future work must focus on characterization of 6S RNA candidates beyond secondary structure predictions for definitive 6S RNA identification.

THE 6S RNA-RNA POLYMERASE COMPLEX

*Ec*6S RNA-Eσ^{70} Interactions

The past decade has revealed much about the nature of the 6S RNA-RNA polymerase interaction, including a cryo-EM structure of the *E. coli* 6S RNA-Eσ^{70} complex in the past year (62). As noted above, it has been appreciated for some time that the secondary structure is the key element required for binding, and that there is minimal if any sequence specificity to the interaction (14, 25, 63). It was demonstrated that the central bubble of *Ec*6S RNA resides near the active site similar to promoter DNA during transcription initiation when the DNA surrounding the start site of transcription is melted to form the "open complex" (64). Consistent with this hypothesis, the *Ec*6S RNA can be used as a template by Eσ^{70} to generate product RNA (pRNA) in a process called "pRNA synthesis" (64). More about pRNA synthesis below, but the ability to use 6S RNA as a template for pRNA synthesis in vitro strongly supported similar binding of 6S RNA and promoter DNA to Eσ^{70} globally. Site-directed crosslink mapping (62) and Fe-BABE cleavage mapping (65) further supported a strong correlation between the path of RNA and DNA when bound to Eσ^{70}. The cryo-EM structure provided direct visualization of the 6S RNA:Eσ^{70} complex and its overall similarity to open complexes (62). However, in spite of the overall similarity of architecture,

closer inspection also revealed regions where *Ec*6S RNA and promoter DNA binding were quite different.

Template strand central region—One area of great interest is the template strand of the central region in 6S RNA and how similarly it is positioned in RNA polymerase to the open complex DNA template bubble (66), as this is the region where RNA synthesis initiates during both pRNA synthesis and transcription. Perhaps unsurprisingly, the path of RNA and DNA are quite similar here, as demonstrated by crosslinking to the same residues and directly visualized in the cryo-EM *Ec*6S RNA:Eσ^{70} structure compared to open complex structures, although the most upstream single-stranded region of 6S RNA was not as well resolved in the *Ec*6S RNA:Eσ^{70} structure as the rest of the RNA (62).

Non-template central region—The cryo-EM structure also provided information about the path of the non-template central region of the 6S RNA. Although most of the non-template single-stranded region was similar between *Ec*6S RNA and open complex DNA, one noted difference is that A131 in *Ec*6S RNA, the position equivalent to −11A in promoter DNA, was not flipped and thus did not make interactions with the σ^{70} pocket (67). 6S RNA is a premelted bubble and thus is not expected to require this interaction to initiate and maintain melting, in contrast to open complex formation where flipping of −11A to interact with σ^{70} is thought to initiate melting and recognition of the −10 element during open complex formation (68). Interestingly, the position equivalent to A131 in *Ec*6S RNA is highly conserved in identified and predicted 6S RNAs raising questions about whether there is another role for this nucleotide or if there are conditions when it does interact with σ^{70} region 2. However, A131 is not required for *Ec*6S RNA activity as mutation of this residue has no detectable effect on the kinetics of binding to Eσ^{70} nor on the efficiency of pRNA synthesis (62).

Other contacts between the non-template bubble region of *Ec*6S RNA (e.g. U135, G136 and G143) and Eσ^{70} were similar to contacts between DNA and Eσ^{70} in the open complex (62). Intriguingly however, these contacts likewise are not required for 6S RNA binding to Eσ^{70} nor for efficient pRNA synthesis initiation (62), raising questions about whether they play a role in other aspects of 6S RNA function/activity. Sequences in this region do contribute to timing of pRNA-synthesis mediated release of *Ec*6S RNA from Eσ^{70} through a releasing structure.

Upstream stem interactions with σ^{70} region 4.2—One area where *Ec*6S RNA interactions are quite different than promoter DNA open complexes is the nucleic acid interaction with region 4.2 of σ^{70} [Figure 2]. The fact that this interaction was distinct between DNA and RNA was first revealed by differential binding of Eσ^{70} variants with alanine substitutions in region 4.2 of σ^{70}, which suggested a larger binding surface for 6S RNA (69). The cryo-EM structure provides a high resolution view of the *Ec*6S RNA interaction with Eσ^{70} in this region and correlates well with the biochemical analysis (62). The cryo-EM structure also demonstrates an unusual structure for the *Ec*6S RNA in this region, an area where secondary structure mapping and mutagenesis had been largely uninformative (33), consistent with the fact that these techniques are best at predicting

canonical structures. The importance of this region of *Ec*6S RNA for binding to Eσ^{70} was also highlighted by random mutagenesis studies (63).

Aspects of 6S RNA structure allow it to adopt an overall architecture that follows the B-form DNA helix of the open complex—Of particular interest from an RNA structure perspective is the region of RNA between the central bubble and the contacts with region 4.2 of σ^{70}, which follows the path and overall architecture of double-stranded, B-form helix DNA rather closely (62). Double-stranded RNA is typically an A-form helix, but the 6S RNA uses a combination of short A-form helices that have some B-form characteristics, in addition to gaps and bulged nucleotides to adopt this unusual structural mimic of double-stranded DNA.

6S RNA-RNA Polymerase Interactions In Diverse Bacteria—Although understanding of the *Ec*6S RNA-Eσ^{70} interaction has increased substantially over the past decade and has provided much insight into how 6S RNAs function mechanistically, detailed biochemical analysis of 6S RNA-RNA polymerase interactions in diverse bacteria remains largely unexplored. In many organisms, 6S RNA-RNA polymerase complexes have been detected by co-immunoprecipitation or in vitro analysis (e.g. *B. subtilis* (14, 35, 70), *L. pneumophila* (29), *C. burnetii* (53), *Aquifex aeolicus* (71), and *Streptomyces coelicolor* (41)) supporting a general similarity to *Ec*6S RNA. However, detailed understanding of interactions in other organisms is likely to require biochemical or structural analysis to uncover any potential differences from *Ec*6S RNA. Directed in vitro studies have been done for *B. subtilis* 6S RNAs, revealing many interesting aspects of these RNAs, much of which is focused on pRNA synthesis initiation and release. Most studies to date have assumed that key features of *Ec*6S RNA-Eσ^{70} interactions are conserved; however, some observations suggest this assumption may be premature. Some 6S RNAs, notably from alpha-proteobacteria and *A. aeolicus*, have a shortened upstream stem and therefore are lacking the region critical for *Ec*6S RNA interactions with σ^{70} region 4.2 (25, 69, 72). However, pRNAs have been detected for *A. aeolicus* and *R. sphaeroides* 6S RNAs strongly supporting an RNA polymerase interaction, and the *Aa*6S RNA has been demonstrated to bind *B. subtilis* Eσ^{A} in vitro (48, 71). The *H. pylori* 6S RNA appears to bind RNA polymerase in either the "forward" or "reverse" orientation based on evidence of pRNAs templated from both strands of the central region (58), in contrast to *Ec*6S RNA that binds quite specifically in one orientation and directs pRNA from one strand of the central region (64). Orientation of *Ec*6S RNA binding to Eσ^{70} has been proposed to be directed through upstream stem contacts with σ^{70} region 4.2; thus lack of orientation also suggests a change in the requirement or nature of interactions with σ^{70} region 4.2. Additionally, work on 6S RNA-dependent changes in transcription appear to be different in several tested organisms compared to *E. coli*, perhaps suggesting key or substantial differences in binding that impact regulation of transcription. Likewise, until the potential differences and similarities between divergent 6S RNAs are understood, caution is advised for studies using noncognate 6S RNA-RNA polymerase matches for in vitro studies, as species-specific behaviors of RNA polymerase in 6S RNA function have been observed (73).

6S RNA REGULATION OF TRANSCRIPTION

Regulation Of Transcription In *E. coli*

The binding of 6S RNA to Eσ^{70}, the major transcriptional machinery of the cell, strongly suggested 6S RNA would regulate σ^{70} -dependent transcription as initially confirmed at one tested promoter (*rsdP2*) in vivo (11). However, somewhat surprisingly at the time, further experiments revealed that 6S RNA dependent regulation of transcription is promoter specific (i.e. some promoters are downregulated in the presence of 6S RNA while others are insensitive to 6S RNA), even during late stationary phase when *Ec*6S RNA levels are maximal and Eσ^{70} is essentially saturated by *Ec*6S RNA (11, 15). Work in the last decade on 6S RNA-dependent regulation of transcription has taken two approaches: 1. Study of reporter genes with minimal core promoters and mutants to reveal promoter features that determine 6S RNA sensitivity; and 2. Identification of the 6S RNA regulon using global approaches.

Identification of promoter features th at determine regulation by *Ec*6S RNA—

The similarity of binding of *Ec*6S RNA and promoter DNA to Eσ^{70} strongly suggested a direct competition model for regulation of transcription, and in vitro binding studies demonstrated that *Ec*6S RNA blocks binding of promoter DNA (64), as also observed in *B. subtilis* for both *Bs*6S-1 and *Bs*6S-2 RNAs (74). However, a set of promoters reported to respond to changes in RNA polymerase concentrations (75) were not uniformly sensitive to *Ec*6S RNA nor did 6S RNA sensitivity correlate with affinity for RNA polymerase binding (38). Furthermore, mutagenesis of several studied promoters revealed that strength of the −35 element or the presence of an extended −10 element specifically determined 6S RNA sensitivity, and that changing these parameters alone could interconvert sensitive and insensitive promoters (38). Strength of the core −10 element did not contribute to 6S RNA sensitivity, in conflict with a model of direct competition of 6S RNA and promoter DNA for free Eσ^{70}. Future work will be required to provide a better understanding of the mechanistic details of 6S RNA regulation of transcription in *E. coli* and other bacteria.

Often, in vitro transcription assays are used to provide mechanistic insight. However, studies examining *Ec*6S RNA regulation of transcription in vitro, with purified components or in cell lysate, demonstrated a lack of correlation between in vitro and in vivo observations, which also was observed for *Bs*6S-1 and *Bs*6S-2 RNAs. Specifically, in vitro, all tested promoters were strongly inhibited in the presence of *Ec*6S RNA, *Bs*6S-1 or *Bs*6S-2 RNAs (14, 74, 76). However, in vivo, some promoters were modestly down-regulated (~2–5 fold) in the presence of *Ec*6S RNA while others were insensitive to *Ec*6S RNA (15, 38, 77); similar results were observed in *B. subtilis* (33, 36). In vitro assays in the *E. coli* system also exhibited a strong order of addition effect, and both *Ec*6S RNA and promoter DNA bind to Eσ^{70} very tightly, which led to the hypothesis that standard in vitro transcription assays do not represent dynamic exchange that must be occurring in vivo. Whether the difference between in vivo and in vitro observations are due solely to differences in dynamics, or whether other factors influence *Ec*6S RNA regulation in vivo that are lacking or inactive in purified and lysate systems, remains to be determined.

Identification of the 6S RNA regulon in *E. coli*—Global expression studies revealed that many hundreds of mRNA levels were altered in cells lacking *Ec*6S RNA compared to wild type cells in exponential phase, early stationary phase and late stationary phase (38, 77). Global studies identify both those genes altered through direct 6S RNA action as well as those altered through secondary effects. Nevertheless, 6S RNA-dependent changes in late stationary phase genes containing mapped promoters correlated fairly well with the promoter features determined through reporter analysis (i.e. weak −35 element or extended −10 element for sensitive promoters) (38). Changes earlier in growth did not correlate well with these identified promoter features (77), which may be due to a decreased response when 6S RNA levels are not maximal, or potentially due to more secondary effects as several targets of *Ec*6S RNA regulation are regulators of transcription (e.g. Crp) or generate molecules that regulate transcription (e.g. RelA, a ppGpp synthetase) at these times (37, 38, 77). Additionally, both global studies identified genes that were increased in the presence of 6S RNA, which are likely to be secondary effects and may include 6S RNA influences on alternative σ factor utilization.

Biological role for 6S RNA function—Of note is that both direct and secondary effects of 6S RNA action are relevant for understanding the physiological consequences of regulation, as opposed to mechanistic understanding of 6S RNA function for which only direct effects are relevant. Phenotypes associated with lack of *Ec*6S RNA are subtle, in part contributing to the long delay between identification of 6S RNA and the first reports of its function in regulating RNA polymerase (9, 11). However, *Ec*6S RNA has been shown to contribute to competitive survival in stationary phase (time scale of days) as well as survival in long term stationary phase when not in competitive growth (time scale of weeks) (15). Additionally, 6S RNA-dependent changes in transcription of one target gene, *pspF*, have been shown to alter survival at high pH in stationary phase (16), suggesting a connection between cell survival and stress response as a primary role for 6S RNA. Results from the large-scale gene expression studies similarly suggest that 6S RNA is integrated into global pathways including regulation of factors that impact transcription (e.g. Crp, FNR, ppGpp via RelA) and general translation machinery (37, 38, 77). Detailed understanding of which gene changes are important for various phenotypes awaits future work.

6S RNA Regulation Of Transcription In Diverse Bacteria—Global gene expression or proteomic studies to address the role of 6S RNA in transcription have been done in other species (e.g. *B. subtilis* (33, 36), *L. pneumophila* (29), *Synechosystis* sp PCC6803 (43)). Intriguingly, there have been some differences in observations from these studies and the *E. coli* studies (38, 77). Of note, there were many fewer mRNAs with altered levels (e.g. 135 for *L. pneumophila*, fewer in *B. subtilis* and *Synechosystis*) than observed in *E. coli* (>800). Whether this large difference represents a true difference in regulatory impact, a difference in timing of analysis, a difference between maximal 6S RNA levels and maximal binding to RNA polymerase or a potential difference in direct versus secondary effects remains to be tested. The other key difference was a preponderance of mRNAs that were higher in wild type cells compared to cells lacking 6S RNA (e.g. 127 out of 135 in *L. pneumophila*), in contrast to *E. coli* where most were decreased in wild type compared to mutant cells consistent with an inhibitory role for 6S RNA. However, in all cases both increased and

decreased mRNAs were observed as expected when examining both direct and secondary effects. It remains an open question whether the mechanism of regulation of transcription is common for all of these reported 6S RNAs, although in all cases to date, the changes in gene expression observed suggest a biological role for 6S RNA in response to environmental conditions.

6S RNA – A TEMPLATE FOR pRNA SYNTHESIS

Perhaps one of the most exciting discoveries about *Ec*6S RNA in the past 15 years was that it not only structurally resembled open complex DNA but could be used as a template by RNA polymerase to generate product RNA (pRNA) (64, 76), a process referred to as "pRNA synthesis". When bound to 6S RNA, the DNA-dependent RNA polymerase is converted to a specialized RNA-dependent RNA polymerase. Detection of pRNA synthesis in vitro provided strong evidence for the similarity of *Ec*6S RNA and open complex DNA interactions with Eσ^{70}. However, it was the determination that pRNA synthesis occurs in vivo, along with subsequent work, that demonstrated that pRNA synthesis is one mechanism to regulate *Ec*6S RNA by contributing to the off-rate of 6S RNA from RNA polymerase (35, 64, 78, 79). For bacteria beyond *E. coli* and *B. subtilis*, the specific role of pRNA synthesis has not been investigated directly, but pRNAs have been detected in vivo for additional bacterial species (e.g. *H. pylori* (58), *R. sphaeroides* (48), *A. aeolicus* (71)) suggesting pRNA synthesis from 6S RNAs is widespread and potentially ubiquitous.

Mechanism Of pRNA Synthesis

Detailed biochemical experiments have examined mechanistic aspects of pRNA synthesis, primarily for *E. coli* and *B. subtilis* 6S RNAs (35, 64, 70, 73, 74, 76, 78, 80–82). Initiation of pRNA synthesis resembles transcription initiation in many ways, including the potential to generate abortive initiation intermediates and release of σ^{70} prior to completion of RNA synthesis. However, unlike transcription, the 6S RNA:RNA polymerase complex is not able to transition into a stable elongation complex, but instead dissociates. In fact, the 6S RNA and pRNA are released as a hybrid (6S RNA:pRNA). This released 6S RNA:pRNA hybrid is unable to rebind RNA polymerase, thereby providing a release mechanism that cannot be reversed without further action. The fact that both regulation of transcription by 6S RNA, and regulation of 6S RNA by pRNA synthesis are mediated by the positioning of 6S RNA within the active site of RNA polymerase provides an elegantly simple mechanism to respond to environmental signals both positively and negatively.

Features of *E. coli* and *B. subtilis* 6S RNAs that contribute to mechanisms of release of 6S RNA:pRNA hybrids from RNA polymerase also have been addressed biochemically (74, 80–82). As pRNA synthesis proceeds, the RNA central bubble is extended, which reveals additional single stranded RNA sequence that participates in an alternative structure. This structure change facilitates the release of 6S RNA from RNA polymerase as a 6S RNA:pRNA duplex. The details of the alternative structures are not the same for *E. coli* and *B. subtilis* 6S RNAs, but the consequences remain the same: destabilization of the interaction with RNA polymerase resulting in release, and contributing to the length of pRNA generated and the timing of release. *A. aeolicus* 6S RNA forms a release structure

similar to *Bs*6S-1 RNA (71), suggesting conservation of this release mechanism, although more information about diverse 6S RNAs is needed before strong conclusions can be made.

Differences in pRNA synthesis have been observed between *E. coli* and *B. subtilis*, most notably in preference for initiating nucleotide identity (73). Although *E. coli* $E\sigma^{70}$ has a preference to initiate pRNA synthesis with a purine, as is also observed generally for transcription, this enzyme will initiate readily with any nucleotide in both pRNA synthesis and transcription. In contrast, *B. subtilis* $E\sigma^{A}$ demonstrates a much stronger preference for initiating pRNA synthesis with GTP over ATP, UTP or CTP, although *B. subtilis* contains promoters that direct initiation with any of the four nucleotides suggesting this trend does not extend to transcription. *Bs*6S-1 RNA directs pRNA synthesis initiation with GTP while *Bs*6S-2 RNA directs initiation with ATP, leading to a large difference in efficiency of pRNA synthesis, at least as measured in vitro (73). A similar preference for initiation with GTP by *B. subtilis* $E\sigma^{A}$ was observed in vitro and in vivo on *E. coli* 6S RNA, which directs initiation with ATP, compared to a mutant *E. coli* 6S RNA, 6S (iGTP) RNA, that directs initiation with GTP (73). Thus, the difference in preference for initiating nucleotide in pRNA synthesis between *E. coli* and *B. subtilis* originates from differences in RNA polymerase, although the mechanistic details await further study. The impact of the observed difference in pRNA efficiency in vitro on in vivo function and regulation of *Bs*6S-1 and *Bs*6S-2 RNAs is likely to continue to be an area of active research (35, 83).

Biological Role For pRNA Synthesis

A burst of pRNA synthesis from *Ec*6S RNA and *Bs*6S-1 RNA occurs in vivo within minutes of diluting stationary phase cells into fresh medium (outgrowth) (35, 64, 70, 78), suggesting a role for this process in the transition out of stationary phase to allow restart of growth. The released 6S RNA remains basepaired to the pRNA, thus preventing rebinding to $E\sigma^{70}$ or $E\sigma^{A}$ due to the altered structure (35, 64, 74, 78, 80). The majority (>90%) of *Ec*6S and *Bs*6S-1 RNAs are degraded during outgrowth, presumably as a consequence of release from RNA polymerase, suggesting pRNA-synthesis may play an important role in determining *Ec*6S RNA and *Bs*6S-1 RNA accumulation profiles. *Ec*6S and *Bs*6S-1 directed pRNA levels were not detected in late stationary phase in one study (35), supporting a hypothesis that pRNA synthesis initiation is sensitive to nucleotide triphosphate levels linking pRNA synthesis timing to outgrowth. The influence of nucleotide levels on pRNA synthesis efficiency has been noted in vitro (35, 64, 74, 78, 80). However, other studies have detected pRNA from *Bs*6S-1RNA (and *Bs*6S-2 RNA) in both exponential and earlier stationary phase (83), although timing in stationary phase, strains and growth conditions varied between different studies. Changes in pRNA length at different stages of growth also have been observed (70), suggesting the potential for additional levels of regulation of pRNA synthesis, such as in pRNA synthesis elongation and release rates in response to nucleotide levels. Certainly further work to address these different observations and the potential connections between pRNA synthesis, 6S RNA stability and accumulation profiles, and the impact of pRNA length distribution need to be addressed experimentally in all organisms. More quantitative information about pRNA synthesis efficiency throughout growth is needed, and an examination of whether additional cellular signals or factors regulate pRNA synthesis is

required before the contribution of pRNA synthesis in regulating 6S RNA levels throughout growth is fully understood.

The impact of pRNA synthesis on the ability of cells to restart growth upon exit from stationary phase (i.e. outgrowth) was investigated in *E. coli* using mutant *Ec*6S RNAs that retain the ability to bind $E\sigma^{70}$ but lack the ability to be released from $E\sigma^{70}$ through pRNA synthesis (35, 79). In one study it was found that cells expressing a mutant 6S RNA were delayed in restarting growth, suggesting pRNA-synthesis mediated release of $E\sigma^{70}$ from *Ec*6S RNA is required for efficient restart of growth after stationary phase (35). Toxic effects of expressing this type of mutant RNA also were observed, although the extent of toxicity was dependent on expression levels (35, 79). However, the non-releasing 6S RNA mutant regulated transcription similarly to wild type 6S RNA in stationary phase, both in specificity of promoters sensitive and insensitive to 6S RNA and the extent of regulation (35). Therefore, is has been suggested that pRNA synthesis is one mechanism to regulate 6S RNA accumulation profiles and to facilitate concerted release of $E\sigma^{70}$ during outgrowth, but does not otherwise influence mechanisms of 6S RNA action in regulating transcription. Although it is enticing to question whether pRNA itself has a function as an sRNA in addition to the role of its synthesis on regulating 6S RNA levels, currently there is no evidence to support an independent function, and no mutant phenotypes have been revealed in cells expressing mutant *Ec*6S RNAs that direct synthesis of pRNAs with different sequences (33). In *B. subtilis*, a requirement for pRNA synthesis-mediated release of $E\sigma^{A}$ to promote efficient outgrowth has also been demonstrated (35).

6S RNA - FUTURE QUESTIONS

Many questions about 6S RNA function in *E. coli* and to a lesser extent in *B. subtilis*, have been addressed at some level. However, even in these well studied organisms questions remain, including how specific changes in gene expression contribute to phenotypes associated with loss of 6S RNA function and what specific mechanisms mediate promoter-specific regulation of transcription. Even more questions await investigation in other bacteria and include: 1. How many genes are regulated by 6S RNA and by what mechanism(s); 2. How diverse 6S RNA structures are recognized by their cognate RNA polymerases and which interactions are conserved or species specific; 3. What are the details underpinning the relationship between accumulation profile and physiological impact; 4. What other factors contribute to regulation of 6S RNA activity beyond pRNA synthesis; and 5. Are there broader impacts of pRNA synthesis beyond the studied release mechanism.

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References

1. Storz G, Vogel J, Wassarman KM. Regulation by Small RNAs in Bacteria: Expanding Frontiers. Mol Cell. 2011; 43:880–891. [PubMed: 21925377]

2. Svensson, SL., Sharma, CM. Virulence Mechanisms of Bacterial Pathogens. Fifth. American Society of Microbiology; 2016. Small RNAs in Bacterial Virulence and Communication; p. 169-212.

3. Holmqvist E, Wagner EGH. Impact of bacterial sRNAs in stress responses. Biochem Soc Trans. 2017; 45:1203–1212. [PubMed: 29101308]

4. Hess WR, Berghoff BA, Wilde A, Steglich C, Klug G. Riboregulators and the role of Hfq in photosynthetic bacteria. RNA Biol. 2014; 11:413–26. [PubMed: 24651049]

5. Cavanagh AT, Wassarman KM. 6S RNA, a Global Regulator of Transcription in Escherichia coli, Bacillus subtilis, and Beyond. Annu Rev Microbiol. 2014; 68:45–60. [PubMed: 24742053]

6. Rediger A, Geißen R, Steuten B, Heilmann B, Wagner R, Axmann IM. 6S RNA - An old issue became blue-green. Microbiol (United Kingdom). 2012; 158:2480–2491.

7. Burenina OY, Elkina DA, Hartmann RK, Oretskaya TS, Kubareva EA. Small Noncoding 6S RNAs of Bacteria. Biochemistry. 2015; 80:1429–46. [PubMed: 26615434]

8. Steuten B, Hoch PG, Damm K, Schneider S, Köhler K, Wagner R, Hartmann RK. Regulation of transcription by 6S RNAs insights from the *Escherichia coli* and *Bacillus subtilis* model systems. RNA Biol. 2014; 11:508–21. [PubMed: 24786589]

9. Hindley J. Fractionation of ^{32}P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. J Mol Biol. 1967; 30:125–136. [PubMed: 4865141]

10. Brownlee GG. Sequence of 6S RNA of *E. coli*. Nat New Biol. 1971; 229:147–149. [PubMed: 4929322]

11. Wassarman KM, Storz G. 6S RNA regulates *E. coli* RNA polymerase activity. Cell. 2000; 101:613–623. [PubMed: 10892648]

12. Feklístov A, Sharon BD, Darst SA, Gross CA. Bacterial Sigma Factors: A Historical, Structural, and Genomic Perspective. Annu Rev Microbiol. 2014; 68:357–376. [PubMed: 25002089]

13. Browning DF, Busby SJW. Local and global regulation of transcription initiation in bacteria. Nat Rev Microbiol. 2016; 14:638–650. [PubMed: 27498839]

14. Trotochaud AE, Wassarman KM. A highly conserved 6S RNA structure is required for regulation of transcription. Nat Struct Mol Biol. 2005; 12:313–319. [PubMed: 15793584]

15. Trotochaud AE, Wassarman KM. 6S RNA function enhances long-term cell survival. J Bacteriol. 2004; 186:4978–4985. [PubMed: 15262935]

16. Trotochaud AE, Wassarman KM. 6S RNA regulation of *pspF* transcription leads to altered cell survival at high pH. J Bacteriol. 2006; 188:3936–3943. [PubMed: 16707685]

17. Kim KS, Lee Y. Regulation of 6S RNA biogenesis by switching utilization of both sigma factors and endoribonucleases. Nucleic Acids Res. 2004; 32:6057–6068. [PubMed: 15550566]

18. Chae H, Han K, Kim KS, Park H, Lee J, Lee Y. Rho-dependent termination of *ssrS* (6S RNA) transcription in *Escherichia coli*: Implication for 3′ processing of 6S RNA and expression of downstream *ygfA* (putative 5-formyl-tetrahydrofolate cyclo-ligase). J Biol Chem. 2011; 286:114–122. [PubMed: 21036909]

19. Lee JY, Park H, Bak G, Kim KS, Lee Y. Regulation of transcription from two *ssrS* promoters in 6S RNA biogenesis. Mol Cells. 2013; 36:227–234. [PubMed: 23864284]

20. Hsu LM, Zagorski J, Wang Z, Fournier MJ. E*scherichia coli* 6S RNA gene is part of a dual-function transcription unit. J Bacteriol. 1985; 161:1162–1170. [PubMed: 2579060]

21. Neußer T, Gildehaus N, Wurm R, Wagner R. Studies on the expression of 6S RNA from *E. coli*: Involvement of regulators important for stress and growth adaptation. Biol Chem. 2008; 389:285–297. [PubMed: 18177266]

22. Chen H, Dutta T, Deutscher MP. Growth phase-dependent variation of RNase BN/Z affects small RNAs. J Biol Chem. 2016; 291:26435–26442. [PubMed: 27875308]

23. Fadouloglou VE, Lin HTV, Tria G, Hernández H, Robinson CV, Svergun DI, Luisi BF. Maturation of 6S regulatory RNA to a highly elongated structure. FEBS J. 2015; 282:4548–4564. [PubMed: 26367381]

24. Li Z, Pandit S, Deutscher MP. 3' exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*. Proc Natl Acad Sci U S A. 1998; 95:2856–61. [PubMed: 9501180]

25. Barrick JE, Sudarsan N, Weinberg Z, Ruzzo WL, Breaker RR. 6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter. RNA. 2005; 11:774–84. [PubMed: 15811922]
26. Wehner S, Damm K, Hartmann RK, Marz M. Dissemination of 6S RNA among bacteria. RNA Biol. 2014; 11:1467–1478. [PubMed: 25483037]
27. Gottesman S, Storz G. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. Cold Spring Harb Perspect Biol. 2011; 3:a003798–a003798. [PubMed: 20980440]
28. Himeno H, Kurita D, Muto A. Mechanism of trans-translation revealed by in vitro studies. Front Microbiol. 2014
29. Faucher SP, Friedlander G, Livny J, Margalit H, Shuman HA. Legionella pneumophila 6S RNA optimizes intracellular multiplication. Proc Natl Acad Sci U S A. 2010; 107:7533–8. [PubMed: 20368425]
30. Faucher SP, Shuman HA. Small regulatory RNA and *Legionella pneumophila*. Front Microbiol. 2011; 2:98. [PubMed: 21833335]
31. Weissenmayer BA, Prendergast JGD, Lohan AJ, Loftus BJ. Sequencing illustrates the transcriptional response of *Legionella pneumophila* during infection and identifies seventy novel small non-coding RNAs. PLoS One. 2011; 6:e17570. [PubMed: 21408607]
32. Lechner M, Nickel AI, Wehner S, Riege K, Wieseke N, Beckmann BM, Hartmann RK, Marz M. Genomewide comparison and novel ncRNAs of Aquificales. BMC Genomics. 2014; 15:522. [PubMed: 24965762]
33. Cavanagh AT, Wassarman KM. unpublished data.
34. Cavanagh AT, Wassarman KM. 6S-1 RNA function leads to a delay in sporulation in *Bacillus subtilis*. J Bacteriol. 2013; 195:2079–2086. [PubMed: 23457253]
35. Cavanagh AT, Sperger JM, Wassarman KM. Regulation of 6S RNA by pRNA synthesis is required for efficient recovery from stationary phase in *E. coli* and *B. subtilis*. Nucleic Acids Res. 2012; 40:2234–2246. [PubMed: 22102588]
36. Hoch PG, Burenina OY, Weber MHW, Elkina DA, Nesterchuk MV, Sergiev PV, Hartmann RK, Kubareva EA. Phenotypic characterization and complementation analysis of *Bacillus subtilis* 6S RNA single and double deletion mutants. Biochimie. 2015; 117:87–99. [PubMed: 25576829]
37. Cavanagh AT, Chandrangsu P, Wassarman KM. 6S RNA regulation of *relA* alters ppGpp levels in early stationary phase. Microbiology. 2010; 156:3791–3800. [PubMed: 20829285]
38. Cavanagh AT, Klocko AD, Liu X, Wassarman KM. Promoter specificity for 6S RNA regulation of transcription is determined by core promoter sequences and competition for region 4.2 of σ70. Mol Microbiol. 2008; 67:1242–1256. [PubMed: 18208528]
39. Suzuma S, Asari S, Bunai K, Yoshino K, Ando Y, Kakeshita H, Fujita M, Nakamura K, Yamane K. Identification and characterization of novel small RNAs in the *asps-yrvM* intergenic region of the *Bacillus subtilis* genome. Microbiology. 2002; 148:2591–8. [PubMed: 12177353]
40. Ando Y, Asari S, Suzuma S, Yamane K, Nakamura K. Expression of a small RNA, BS203 RNA, from the *yocI-yocJ* intergenic region of *Bacillus subtilis* genome. FEMS Microbiol Lett. 2002; 207:29–33. [PubMed: 11886746]
41. Mikulík K, Bobek J, Zídková J, Felsberg J. 6S RNA modulates growth and antibiotic production in *Streptomyces coelicolor*. Appl Microbiol Biotechnol. 2014; 98:7185–7197. [PubMed: 24859522]
42. Axmann IM, Holtzendorff J, Voß B, Kensche P, Hess WR. Two distinct types of 6S RNA in Prochlorococcus. Gene. 2007; 406:69–78. [PubMed: 17640832]
43. Heilmann B, Hakkila K, Georg J, Tyystjärvi T, Hess WR, Axmann IM, Dienst D. 6S RNA plays a role in recovery from nitrogen depletion in *Synechocystis sp*. PCC 6803. BMC Microbiol. 2017; 17:229. [PubMed: 29216826]
44. Watanabe T, Sugiura M, Sugita M. A novel small stable RNA, 6Sa RNA, from the cyanobacterium *Synechococcus sp*. strain PCC6301. FEBS Lett. 1997; 416:302–306. [PubMed: 9373174]
45. Voss B, Hölscher M, Baumgarth B, Kalbfleisch A, Kaya C, Hess WR, Becker A, Evguenieva-Hackenberg E. Expression of small RNAs in *Rhizobiales* and protection of a small RNA and its degradation products by Hfq in *Sinorhizobium meliloti*. Biochem Biophys Res Commun. 2009; 390:331–336. [PubMed: 19800865]

46. Madhugiri R, Pessi G, Voss B, Hahn J, Sharma CM, Reinhardt R, Vogel J, Hess WR, Fischer HM, Evguenieva-Hackenberg E. Small RNAs of the *Bradyrhizobium*/*Rhodopseudomonas* lineage and their analysis. RNA Biol. 2012; 9:47–58. [PubMed: 22258152]
47. Darby AC, Armstrong SD, Bah GS, Kaur G, Hughes MA, Kay SM, Koldkjær P, Rainbow L, Radford AD, Blaxter ML, Tanya VN, Trees AJ, Cordaux R, Wastling JM, Makepeace BL. Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. Genome Res. 2012; 22:2467–2477. [PubMed: 22919073]
48. Elkina D, Weber L, Lechner M, Burenina O, Weisert A, Kubareva E, Hartmann RK, Klug G. 6S RNA in *Rhodobacter sphaeroides*: 6S RNA and pRNA transcript levels peak in late exponential phase and gene deletion causes a high salt stress phenotype. RNA Biol. 2017; 14:1627–1637. [PubMed: 28692405]
49. Berghoff BA, Glaeser J, Sharma CM, Vogel J, Klug G. Photooxidative stress-induced and abundant small RNAs in *Rhodobacter sphaeroides*. Mol Microbiol. 2009; 74:1497–1512. [PubMed: 19906181]
50. Ghosh SS, Dureja C, Khatri I, Subramanian S, Raychaudhuri S, Ghosh SS. Identification of novel small RNAs in *Burkholderia cenocepacia* KC-01 expressed under iron limitation and oxidative stress conditions. Microbiology. 2017; 163:1924–1936.
51. Yan Y, Su S, Meng X, Ji X, Qu Y, Liu Z, Wang X, Cui Y, Deng Z, Zhou D, Jiang W, Yang R, Han Y. Determination of sRNA Expressions by RNA-seq in *Yersinia pestis* Grown In Vitro and during Infection. PLoS One. 2013; 8:e74495. [PubMed: 24040259]
52. Schroeder CLC, Narra HP, Sahni A, Rojas M, Khanipov K, Patel J, Shah R, Fofanov Y, Sahni SK. Identification and Characterization of Novel Small RNAs in *Rickettsia prowazekii*. Front Microbiol. 2016; 7:859. [PubMed: 27375581]
53. Warrier I, Hicks LD, Battisti JM, Raghavan R, Minnick MF. Identification of novel small RNAs and characterization of the 6S RNA of *Coxiella burnetii*. PLoS One. 2014; 9:e100147. [PubMed: 24949863]
54. Ren J, Sang Y, Qin R, Cui Z, Yao Y-F. 6S RNA is involved in acid resistance and invasion of epithelial cells in *Salmonella enterica serovar Typhimurium*. Future Microbiol. 2017; 12:1045–1057. [PubMed: 28796533]
55. Jones AJ, Venkataramanan KP, Papoutsakis T. Overexpression of two stress- responsive, small, non-coding RNAs, 6S and tmRNA, imparts butanol tolerance in *Clostridium acetobutylicum*. FEMS Microbiol Lett. 2016; 363:fnw063. [PubMed: 26989157]
56. Landt SG, Abeliuk E, McGrath PT, Lesley JA, McAdams HH, Shapiro L. Small non-coding RNAs in *Caulobacter crescentus*. Mol Microbiol. 2008; 68:600–614. [PubMed: 18373523]
57. Soutourina OA, Monot M, Boudry P, Saujet L, Pichon C, Sismeiro O, Semenova E, Severinov K, Le Bouguenec C, Coppée JY, Dupuy B, Martin-Verstraete I. Genome-Wide Identification of Regulatory RNAs in the Human Pathogen *Clostridium difficile*. PLoS Genet. 2013; 9:e1003493. [PubMed: 23675309]
58. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiß S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R, Stadler PF, Vogel J. The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature. 2010; 464:250–255. [PubMed: 20164839]
59. Vogel DW, Hartmann RK, Struck JC, Ulbrich N, Erdmann VA. The sequence of the 6S RNA gene of *Pseudomonas aeruginosa*. Nucleic Acids Res. 1987; 15:4583–4591. [PubMed: 2438656]
60. Pánek J, Krásný L, Bobek J, Ježková E, Korelusová J, Vohradský J. The suboptimal structures find the optimal RNAs: Homology search for bacterial non-coding RNAs using suboptimal RNA structures. Nucleic Acids Res. 2011; 39:3418–3426. [PubMed: 21193488]
61. Hnilicová J, Jirát Matějčková J, Šiková M, Pospíšil J, Halada P, Pánek J, Krásný L. Ms1, a novel sRNA interacting with the RNA polymerase core in mycobacteria. Nucleic Acids Res. 2014; 42:11763–11776. [PubMed: 25217589]
62. Chen J, Wassarman KM, Feng S, Leon K, Feklistov A, Winkelman JT, Li Z, Walz T, Campbell EA, Darst SA. 6S RNA Mimics B-Form DNA to Regulate *Escherichia coli* RNA Polymerase. Mol Cell. 2017; 68:388–397.e6. [PubMed: 28988932]

63. Shephard L, Dobson N, Unrau PJ. Binding and release of the 6S transcriptional control RNA. RNA. 2010; 16:885–892. [PubMed: 20354151]
64. Wassarman KM, Saecker RM. Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. Science (80-). 2006; 314:1601–1603.
65. Steuten B, Setny P, Zacharias M, Wagner R. Mapping the spatial neighborhood of the regulatory 6S RNA bound to *Escherichia coli* RNA polymerase holoenzyme. J Mol Biol. 2013; 425:3649–3661. [PubMed: 23867276]
66. Bae B, Feklistov A, Lass-Napiorkowska A, Landick R, Darst SA. Structure of a bacterial RNA polymerase holoenzyme open promoter complex. Elife. 2015; 4
67. Feklistov A, Darst SA. Structural Basis for Promoter −10 Element Recognition by the Bacterial RNA Polymerase σ Subunit. Cell. 2011; 147:1257–1269. [PubMed: 22136875]
68. Saecker RM, Record MT, Dehaseth PL. Mechanism of bacterial transcription initiation: RNA polymerase - Promoter binding, isomerization to initiation-competent open complexes, and initiation of RNA synthesis. J Mol Biol. 2011; 412:754–771. [PubMed: 21371479]
69. Klocko AD, Wassarman KM. 6S RNA binding to Eσ^{70} requires a positively charged surface of σ^{70} region 4.2. Mol Microbiol. 2009; 73:152–164. [PubMed: 19538447]
70. Beckmann BM, Burenina OY, Hoch PG, Kubareva EA, Sharma CM, Hartmann RK. In vivo and in vitro analysis of 6S RNA-templated short transcripts in *Bacillus subtilis*. RNA Biol. 2011; 8:839–849. [PubMed: 21881410]
71. Köhler K, Duchardt-Ferner E, Lechner M, Damm K, Hoch PG, Salas M, Hartmann RK. Structural and mechanistic characterization of 6S RNA from the hyperthermophilic bacterium *Aquifex aeolicus*. Biochimie. 2015; 117:72–86. [PubMed: 25771336]
72. Willkomm DK, Minnerup J, Hüttenhofer A, Hartmann RK. Experimental RNomics in *Aquifex aeolicus*: Identification of small non-coding RNAs and the putative 6S RNA homolog. Nucleic Acids Res. 2005; 33:1949–1960. [PubMed: 15814812]
73. Cabrera-Ostertag IJ, Cavanagh AT, Wassarman KM. Initiating nucleotide identity determines efficiency of RNA synthesis from 6S RNA templates in *Bacillus subtilis* but not *Escherichia coli*. Nucleic Acids Res. 2013; 41:7501–7511. [PubMed: 23761441]
74. Burenina OY, Hoch PG, Damm K, Salas M, Zatsepin TS, Lechner M, Oretskaya TS, Kubareva EA, Hartmann RK. Mechanistic comparison of *Bacillus subtilis* 6S-1 and 6S-2 RNAs--commonalities and differences. RNA. 2014; 20:348–359. [PubMed: 24464747]
75. Barker MM, Gaal T, Gourse RL. Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. J Mol Biol. 2001; 305:689–702. [PubMed: 11162085]
76. Gildehaus N, Neußer T, Wurm R, Wagner R. Studies on the function of the riboregulator 6S RNA from *E. coli*: RNA polymerase binding, inhibition of in vitro transcription and synthesis of RNA-directed de novo transcripts. Nucleic Acids Res. 2007; 35:1885–1896. [PubMed: 17332013]
77. Neußer T, Polen T, Geissen R, Wagner R. Depletion of the non-coding regulatory 6S RNA in *E. coli* causes a surprising reduction in the expression of the translation machinery. BMC Genomics. 2010; 11:165. [PubMed: 20222947]
78. Wurm R, Neußer T, Wagner R. 6S RNA-dependent inhibition of RNA polymerase is released by RNA-dependent synthesis of small de novo products. Biol Chem. 2010; 391:187–196. [PubMed: 20030589]
79. Oviedo Ovando M, Shephard L, Unrau PJ. In vitro characterization of 6S RNA release-defective mutants uncovers features of pRNA-dependent release from RNA polymerase in *E. coli*. RNA. 2014; 20:670–680. [PubMed: 24681966]
80. Beckmann BM, Hoch PG, Marz M, Willkomm DK, Salas M, Hartmann RK. A pRNA- induced structural rearrangement triggers 6S-1 RNA release from RNA polymerase in *Bacillus subtilis*. EMBO J. 2012; 31:1727–1738. [PubMed: 22333917]
81. Steuten B, Wagner R. A conformational switch is responsible for the reversal of the 6S RNA-dependent RNA polymerase inhibition in *Escherichia coli*. Biological Chemistry. 2012:1513–1522. [PubMed: 23667906]
82. Panchapakesan SSS, Unrau PJ. E. coli 6S RNA release from RNA polymerase requires σ^{70} ejection by scrunching and is orchestrated by a conserved RNA hairpin. RNA. 2012; 1:2251–2259.

83. Hoch PG, Schlereth J, Lechner M, Hartmann RK. *Bacillus subtilis* 6S-2 RNA serves as a template for short transcripts in vivo. RNA (New York, NY). 2016; 22:614–622.

84. Hubin EA, Lilic M, Darst SA, Campbell EA. Structural insights into the mycobacteria transcription initiation complex from analysis of X-ray crystal structures. Nat Commun. 2017; 8

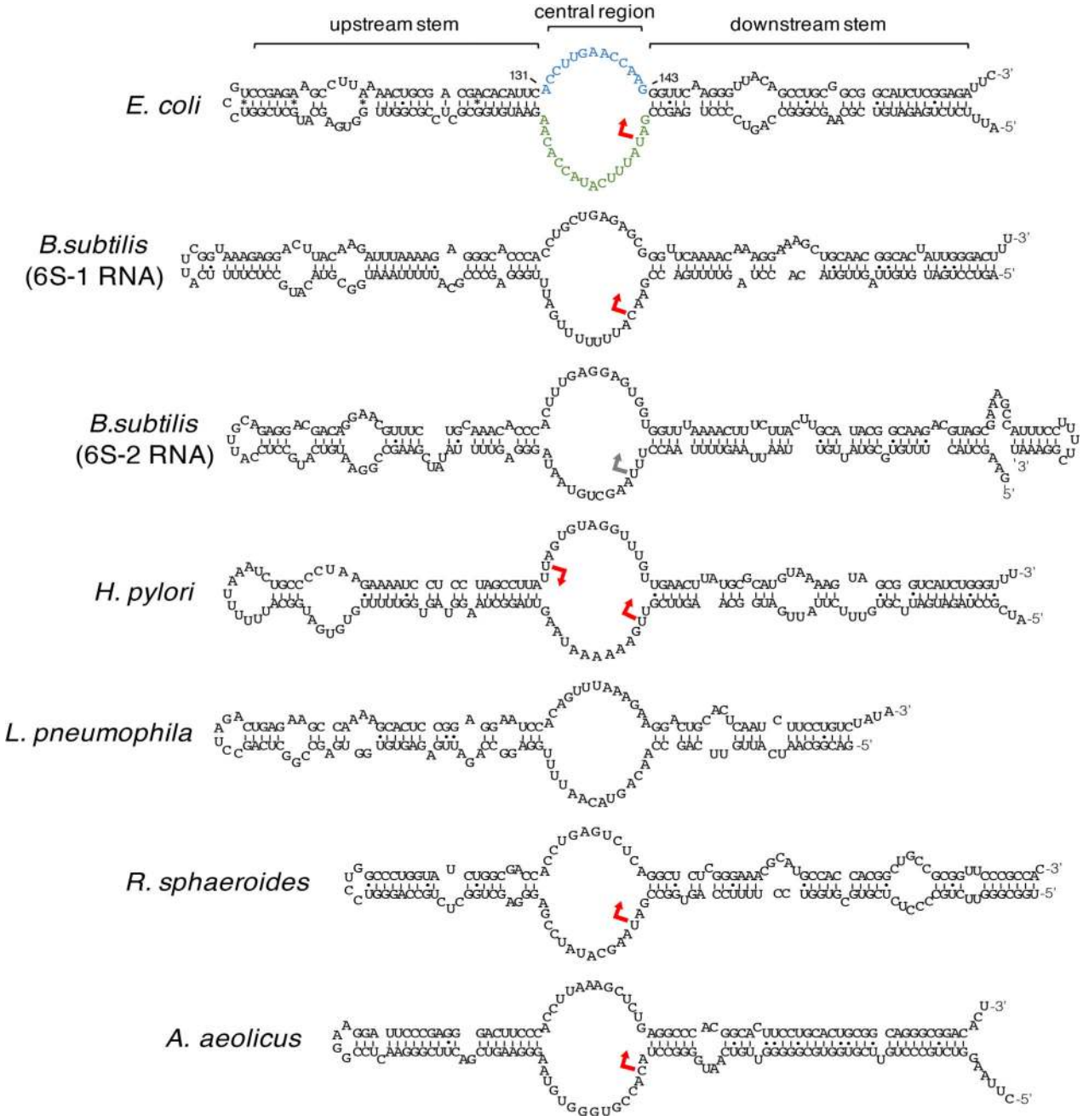

Figure 1.

E. coli 6S RNA is shown in a secondary structure observed from the cryo-EM 6S RNA: Eσ^{70} structure (upstream stem and central region) or predicted from secondary structure analysis (downstream stem) (14, 62). For reference, the upstream stem, central region and downstream stem are indicated. The template central region is in green; the site of initiation for pRNA synthesis is indicated by red arrow; the nontemplate central region is in blue. 6S RNAs from *B. subtilis* (*Bs*6S-1 and *Bs*6S-2), *H. pylori, L. pneumophila* (*Lp*6S RNA), *R. sphaeroides*, and *A. aeolicus*, are shown in secondary structures expected when in complex with RNA polymerase, with sites of pRNA synthesis initiation indicated by arrows (14, 25,

30, 49, 58, 71, 72). pRNA synthesis has not been examined for Lp6S RNA. Note, some 6S RNAs have been demonstrated or predicted to have basepairing within the central region in isolated RNA (Bs6S-1, Bs6S-2 RNA, Hp6S RNA) (25, 58, 74, 80), but binding studies support that the central region is fully single stranded when bound to RNA polymerase (14, 35, 64, 73). Other alternative structures that contribute to 6S RNA release from RNA polymerase during pRNA synthesis also have been observed (74, 80–82).

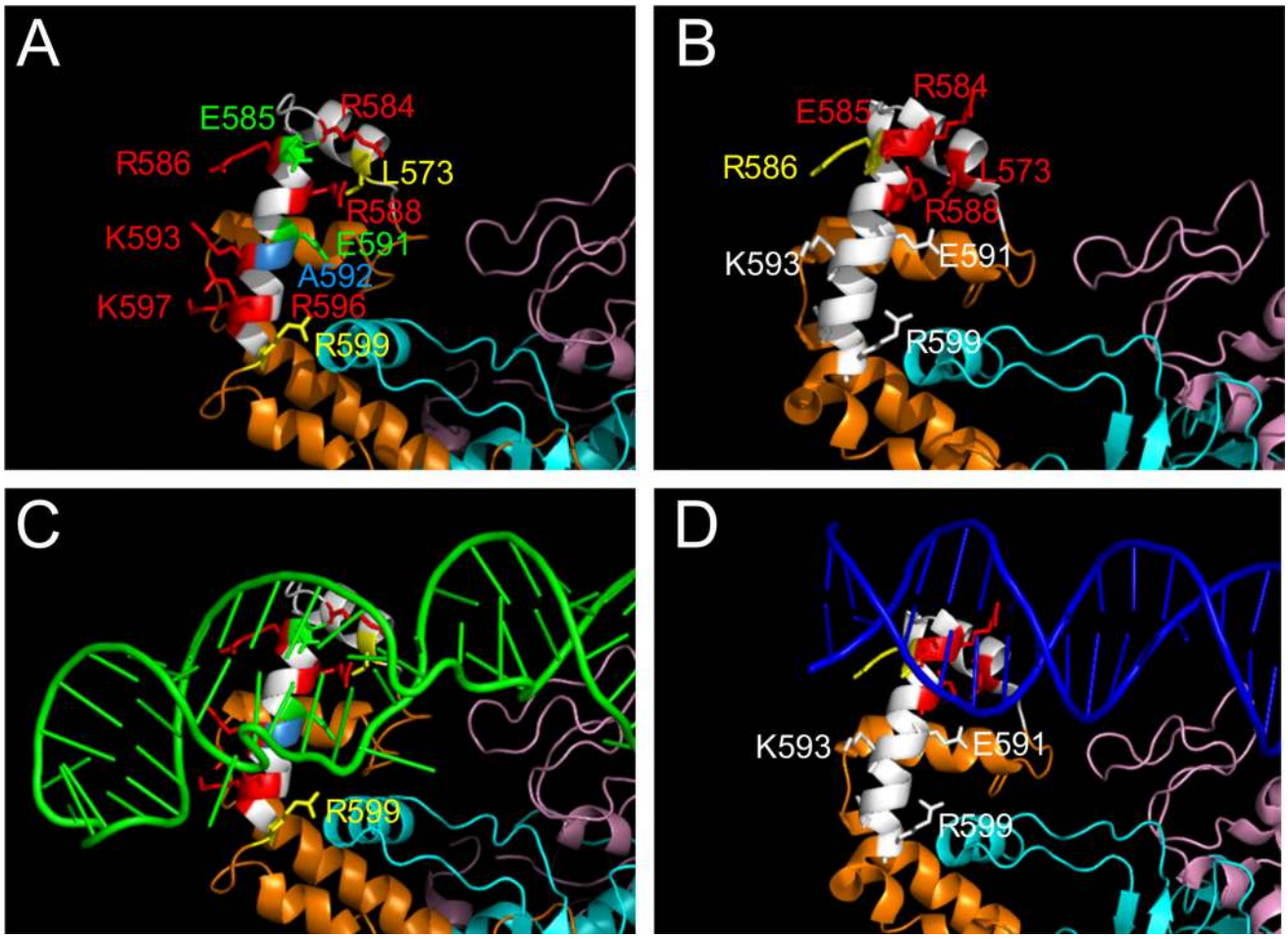

Figure 2.

Comparison of 6S RNA and promoter DNA interactions with region 4.2 of σ^{70}. Holoenzyme structures complexed with RNA (PDB ID 5VT0 (62)) (A and C) and promoter DNA (PDB ID 5VI5 (84))(B and D) are centered on region 4.2 of σ^{70} and shown without (A and B) or with (C and D) the nucleic acids visible. The cryoEM structure with 6S RNA is *E. coli* holoenzyme and the crystal structure with DNA in an open complex is *Mycobacterium smegmatis* holoenzyme and thus there are small variations in structure due to sequence changes, although region 4.2 of σ^{70} is very highly conserved. Residues within region 4.2 of σ^{70} are labelled (*E. coli* numbering) with color coding based on impact of alanine substitution on binding to RNA (A) or DNA (B) (69): Red, strong decrease; yellow, moderate decrease; green, increase. A592 (blue) is a position where substitution of a positive or negative residue strongly influences 6S RNA binding with little effect on DNA binding (69). Residues labelled in white (B and D) are locations where alanine substitution did not influence DNA binding, but are included to assist with comparison of the two structures. Other coloring: 6S RNA, green; Promoter DNA, blue; β subunit, cyan; β' subunit, pink, σ^{70} (outside of region 4.2) orange.