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Regulation of 6S RNA by pRNA synthesis is required for efficient recovery from stationary phase in *E. coli* and *B. subtilis*

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ABSTRACT

6S RNAs function through interaction with housekeeping forms of RNA polymerase holoenzyme ($E\sigma^{70}$ in *Escherichia coli*, $E\sigma^{A}$ in *Bacillus subtilis*). *Escherichia coli* 6S RNA accumulates to high levels during stationary phase, and has been shown to be released from $E\sigma^{70}$ during exit from stationary phase by a process in which 6S RNA serves as a template for $E\sigma^{70}$ to generate product RNAs (pRNAs). Here, we demonstrate that not only does pRNA synthesis occur, but it is an important mechanism for regulation of 6S RNA function that is required for cells to exit stationary phase efficiently in both *E. coli* and *B. subtilis*. *Bacillus subtilis* has two 6S RNAs, 6S-1 and 6S-2. Intriguingly, 6S-2 RNA does not direct pRNA synthesis under physiological conditions and its non-release from $E\sigma^{A}$ prevents efficient outgrowth in cells lacking 6S-1 RNA. The behavioral differences in the two *B. subtilis* RNAs clearly demonstrate that they act independently, revealing a higher than anticipated diversity in 6S RNA function globally. Overexpression of a pRNA-synthesis-defective 6S RNA in *E. coli* leads to decreased cell viability, suggesting pRNA synthesis-mediated regulation of 6S RNA function is important at other times of growth as well.

INTRODUCTION

6S RNA is a small, non-coding RNA that was first identified in *Escherichia coli*, where many of the functional studies have been performed (1,2). The *E. coli* 6S RNA (Ec6S RNA) accumulates throughout growth, reaching maximal levels many hours after the transition into stationary phase (3,4). The RNA binds tightly to the σ^{70}-containing form of RNA polymerase ($E\sigma^{70}$), but does not bind specifically to the core enzyme (α_2, β, β' and ω), free σ^{70}, or to alternative holoenzymes (e.g. $E\sigma^{S}$) (4,5).

Over the last decade, several key features important for 6S RNA and $E\sigma^{70}$ interactions have been identified. The 6S RNA is largely double-stranded with a central single-stranded region that is reminiscent of the conformation of DNA in the open complex during transcription initiation (Figure 1; 5,6). This structure directs 6S RNA binding to $E\sigma^{70}$ in the active site in a manner similar to the interactions between promoter DNA and $E\sigma^{70}$. 6S RNA blocks the ability of $E\sigma^{70}$ to bind to DNA, leading to downregulation of transcription at many σ^{70}-dependent promoters, although other σ^{70}-dependent promoters are insensitive to 6S RNA even during late stationary phase when the vast majority of $E\sigma^{70}$ is found in a complex with 6S RNA (7–11). σ^{70}-dependent promoters are recognized through two sequence hexamers, the −10 element and the −35 element, which are recognized primarily by σ^{70} region 2.4 and 4.2, respectively (12). In contrast to how 6S RNA binds in the active site of $E\sigma^{70}$, the 'upstream' region of 6S RNA does not mimic a −35 element and many residues within region 4.2 of σ^{70} contribute differentially to 6S RNA or DNA binding (13). Interestingly, promoters with weak −35 elements are sensitive to 6S RNA regulation (10) suggesting region 4.2 of σ^{70} may be a primary site for competition between 6S RNA and promoter binding.

6S RNAs and genes encoding putative 6S RNAs have been identified in many diverse organisms using biochemical, bioinformatic and sequencing approaches (5,6,14–17). The primary sequence of these RNAs is not well conserved, but instead the overall secondary structure is conserved, in agreement with studies in *E. coli* demonstrating the requirement for secondary structure in $E\sigma^{70}$ binding (5,18). Intriguingly, some species have two 6S RNAs, including *Bacillus subtilis* and *Legionella pneumophila* (5,6,15,17). The *B. subtilis* 6S RNAs (Bs6S RNAs) were first sequenced as abundant RNAs of unknown function called Bs190 (encoded by *bsrA*) and Bs203 (encoded by *bsrB*) (19,20), which later were identified as 6S RNAs based on their

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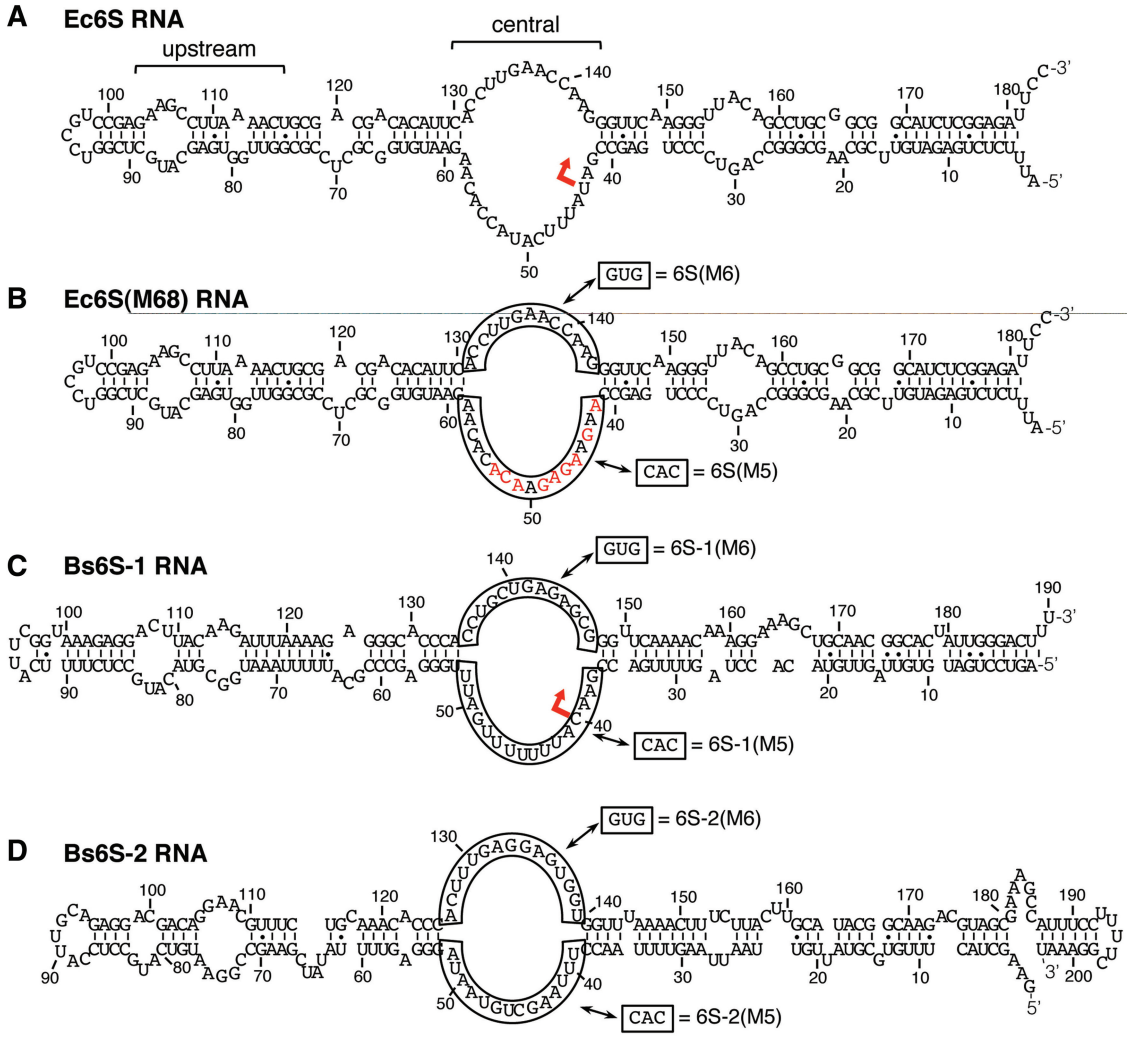

Figure 1. *Escherichia coli* and *Bacillus subtilis* 6S RNAs. Schematics of Ec6S RNA (**A**), 6S(M68) RNA (**B**) and *B. subtilis* 6S-1 RNA (**C**) and 6S-2 RNA (**D**) in secondary structures supported by phylogenetic and experimental analyses (5,6). The 'central' and 'upstream' regions are indicated by brackets in (A) and refer to the large single-stranded region required for Ec6S RNA interaction with $E\sigma^{70}$ and the region expected to interact with region 4.2 of σ^{70} within $E\sigma^{70}$, respectively (5,13). The template position where pRNA synthesis initiates in Ec6S RNA (A) and Bs6S-1 RNA (C) are indicated by a red arrow (8,22). For M5 and M6 variants, the boxed regions were replaced with CAC or GUG, respectively, which generates mutant RNAs that are unable to bind to RNA polymerase (ref. 5, Supplementary Figure S2).

co-immunoprecipitation with RNA polymerase or based on secondary structure similarity to the Ec6S RNA (Figure 1) (5,6). These *B. subtilis* RNAs and their genes have been referred to by multiple names; here we use the nomenclature Bs6S-1 RNA encoded by *bsrA* (also called Bs190 RNA, 6SB RNA and *ssrSB*) and Bs6S-2 RNA encoded by *bsrB* (also called Bs203 RNA, 6SA RNA and *ssrSA*) in agreement with the first assigned 6S RNA nomenclature and original gene designations (5,19,20). The Bs6S-1 and Bs6S-2 RNAs each bind the housekeeping form of RNA polymerase ($E\sigma^{A}$ in *B. subtilis*) (5) analogous to Ec6S RNA binding to $E\sigma^{70}$ in *E. coli*. Bs6S-1 and Bs6S-2 RNAs are not conserved at the primary sequence level, and are expressed differentially through growth (5,6,19,20). Specifically, Bs6S-1 RNA accumulates during stationary phase similarly to Ec6S RNA in *E. coli*. Bs6S-2 RNA levels remain fairly constant through growth, although a small increase in late exponential phase may occur. These differences have raised questions about whether these two RNAs provide independent or redundant functionality *in vivo*.

Ec6S RNA efficiently binds the vast majority of $E\sigma^{70}$ in stationary phase (40), which raised questions about how $E\sigma^{70}$ was released from 6S RNA-dependent regulation upon exit from stationary phase (outgrowth). Remarkably, it was found that $E\sigma^{70}$ uses 6S RNA as a template for RNA synthesis to produce a product RNA (pRNA) (8,9,21). Similarly, the Bs6S-1 RNA has been shown to direct pRNA synthesis in *B. subtilis* during outgrowth (22). However, less is known about the behavior of Bs6S-2 RNA.

We set out to characterize Bs6S-1 and Bs6S-2 RNAs in *B. subtilis* and to identify mutant phenotypes for cells lacking these RNAs. We show here that cells expressing 6S-2 RNA in the absence of 6S-1 RNA are delayed in their ability to restart growth, clearly indicating that 6S-1 and

6S-2 RNAs have independent biological functions. We demonstrate that 6S-2 RNA is not used as a template for pRNA synthesis *in vivo* or *in vitro*, suggesting the delay in outgrowth is a result of sequestration of $E\sigma^A$ during outgrowth. Furthermore, introduction of a mutant 6S RNA [6S(M68) RNA] that does not serve as a template for pRNA synthesis in *E. coli* cells also leads to a delay in outgrowth, indicating that pRNA synthesis is an important mechanism to regulate 6S RNA function during outgrowth in both *E. coli* and *B. subtilis*. In addition, overexpression of this mutant RNA in *E. coli* leads to decreased viability of stationary phase cells, suggesting pRNA synthesis may be an important mechanism to facilitate the dynamics and exchange of 6S RNA and $E\sigma^{70}$ complexes throughout growth.

MATERIALS AND METHODS

Strains

Bacillus subtilis and *E. coli* strains are listed in Table 1. *Bacillus subtilis* was grown in 2× YT medium (23); 25 μg/ml kanamycin was included in medium for growth of cells containing pSP plasmid derivatives. *Escherichia coli* was grown in Lennox broth (LB) (23); 25 μg/ml chloramphenicol (Cm) was included in medium for growth of cells containing pKK plasmid derivatives.

Bacillus subtilis ΔbsrA and *ΔbsrB* alleles have the chromosomal *bsrA* or *bsrB* genes precisely removed and replaced by a spectinomycin-resistance cassette (KW587 or KW589) or a tetracycline-resistance cassette (KW588). See Supplementary Methods for details. Briefly, the drug-resistance cassette flanked by sequences homologous to regions 5′ and 3′ of *bsrA* or *bsrB* were PCR amplified and transformed into *B. subtilis* 168 using the two-step transformation protocol (24) followed by selection for antibiotic resistance on plates containing the appropriate antibiotic. The double mutant (*ΔbsrA::tet ΔbsrB::spec*) was generated by transformation of PCR-amplified DNA of the tetracycline cassette with *bsrA* flanking sequences into *ΔbsrB::spec* cells. Note that *ΔbsrA*::spec and *ΔbsrA*::tet grew indistinguishably from each other in all assays here. All alleles were confirmed by sequencing the junctions in chromosomal DNA. The Ec6S RNA null allele *ssrS1* (25) was used for all studies here.

Table 1. Bacterial strains

Name	Genotype	Reference
KW72	Laboratory wild-type strain *E. coli* K12	4
GS075	KW72, *ssrS1* [Amp^R]	4
RLG3499	MG1655 *pyrE+ lacI lacZ* [VH1000]	41
KW372	RLG3499 λ*rsd*P2(−149+91)-*lacZ*	7
KW373	KW372 *ssrS1*	7
RLG3760	RLG3499 λ*bolA*1(−54+16)-*lacZ*	41
KW378	RLG3760 *ssrS1*	7
KW462	RLG3499 λ*lacUV5(−35weak)* (−48+4)-*lacZ*	10
KW463	KW462 *ssrS1*	10
KW586	*Bacillus subtilis* 168; 1A1	Bacillus Genetic Stock Center[a]
KW587	KW586*ΔbsrA*::*spec*	This work
KW588	KW586*ΔbsrA*::*tet*	This work
KW589	KW586*ΔbsrB*::*spec*	This work
KW590	KW586*ΔbsrA::tet ΔbsrB*::*spec*	This work
KW591	MH5636, *rpoC*-His_{10}	27

[a]Wild type *B. subtilis* 168 cells were obtained from the Bacillus Genetic Stock Center http://www.bgsc.org/.

Plasmids

pSP-Bs6S-1 or pSP-Bs6S-2 and derivatives were used for expression of Bs6S-1 RNA or Bs6S-2 RNA in *B. subtilis* cells, and contained *bsrA* or *bsrB* under control of the Pspac promoter in pDG148 (26). See Supplementary Methods for details of cloning. Note that all experiments here used uninduced Bs6S-1 RNA and Bs6S-2 RNA, which resulted in RNA levels very similar to endogenously expressed RNAs (Supplementary Figure S2). pKK-6S, pKK-6S+Y or pKK-Cm (empty vector) (4) were used for expression studies of 6S RNA in *E. coli*, which similarly relied on uninduced expression from the Ptac promoter. pKK-6S results in ∼2-fold higher 6S RNA expression than from the chromosomal *ssrS* gene, and pKK-6S+Y results in ∼10-fold increased 6S RNA levels relative to endogenous (4).

pCR-Bs6S-1, pCR-Bs6S-2 and pCR-Bs5S plasmids (5) were used to direct *in vitro* transcription of full-length RNA probes to Bs6S-1, Bs6S-2 and Bs5S RNAs. pBS-6S-upstream was used to generate an RNA probe complementary to nucleotides 59–130 of Ec6S RNA, which are not altered in 6S(M68) RNA, and therefore allowed equivalent detection of *E. coli* wild-type and M68 RNAs. See Supplementary Methods for details of cloning. pBS-5S was used to generate a full-length RNA probe to *E. coli* 5S rRNA (5).

pT3-Bs6S-1, pT3-Bs6S-2, pT3-6S and variants were used for synthesis of RNAs for *in vitro* experiments. pT3-Bs6S-1 and pT3-Bs6S-2 plasmids were generated by cloning PCR-amplified *bsrA* or *bsrB* sequences with a T3 promoter using a TOPO TA cloning kit (Invitrogen). pT3-6S (5) was used to generate Ec6S RNA.

pET-Bsσ^A was used to produce native σ^A without any additional sequence or tags. To make pET-Bsσ^A, *B. subtilis sigA* was PCR amplified from genomic DNA and cloned into pET-101D-TOPO using the Champion pET100 Directional TOPO Expression Kit (Invitrogen).

Various mutations (e.g. M5, M6 and M68) were introduced into necessary plasmids by Quikchange II site-directed mutagenesis (Stratagene). See Supplementary Table S1 for all oligonucleotide sequences used in cloning, for probes and for markers. All relevant regions of all plasmids were confirmed by sequencing.

Outgrowth

For outgrowth experiments, 195 μl of medium (2× YT or LB with appropriate antibiotic when plasmids were present) was inoculated with 5 μl of diluted stationary phase cells (grown for 18 h at 37°C, diluted to $OD_{600} = 0.3$ in 1× M9 salts) in a 96-well microtiter plate (flat bottomed, polystyrene from Corning). The final dilution represents ∼1:500 dilution of the original

stationary phase culture. Each experiment examined at least three independent stationary phase cultures, and outgrowth from each culture was tested in two independent wells of the microtiter plate. Growth at 37°C was monitored as OD_{595} in an absorbance microplate reader (Biotek Instruments, ELx808); readings were taken every 15 min. *Bacillus subtilis* growth was at 'medium' shaking while *E. coli* growth was at 'fast' shaking as each species had the most consistent day to day growth at these shaking speeds. Similar phenotypes were observed for *ΔbsrA* cells compared to wild-type *B. subtilis* cells and for *E. coli* cells containing pKK-6S(M68) compared to pKK-6S were grown in culture tubes and optical densities were taken over time manually (A.T.C. and J.M.S. unpublished results). In addition, under growth conditions here for *B. subtilis* (2× YT, ≤24 hours) very few spores were observed for any of the strains examined (wild-type, *ΔbsrA*, *ΔbsrB* and *ΔbsrAΔbsrB*).

To check that the OD_{600} readings were representative of viable cell numbers in the starting cultures, the diluted stationary phase cells (the initial 0.3 OD dilutions) were serially diluted in 1× M9 salts and 10 µl of each dilution was spotted onto LB agar plates. The extent of growth was assessed in each spot after overnight incubation at 30°C. With the exception of *E. coli* cells containing pKK-6S+Y(M68) (Figure 8), 18 h overnight cultures gave similar OD_{600} readings (within 15% of wild-type) and normalized dilutions used for outgrowth assays had viable cell counts within ~2-fold for each experiment. For cells containing plasmids, growth was compared on plates with and without antibiotic to test for plasmid loss. No significant plasmid loss was observed in most experiments; results from experiments showing any detectable plasmid loss were discarded.

To monitor growth of *E. coli* cells containing pKK-6S+Y(M68), single colonies were streaked onto LB + Cm agar plates and grown overnight at 30°C. Cells collected by scraping from plates were resuspended in 1× M9 salts, and cell densities were determined by OD_{600}. Growth in culture was initiated by dilution into LB + Cm to a final $OD_{600} = 0.1$, and cell densities were measured over time by serial dilution and plating as described above. At least three independent cultures per strain were used for each experiment, and all experiments were repeated at least three times.

6S RNA: RNA polymerase binding and release assays

Reconstitution of 6S RNA:RNA polymerase complexes was done as previously described (8). Briefly, *in vitro* transcribed ^{32}P-labeled 6S RNA, 6S-1 RNA, 6S-2 RNA or derivatives 200 nM were incubated for 2–5 min at room temperature in 1× HM (20 mM Hepes, 5 mM $MgCl_2$) to allow folding. Binding reactions with 20 nM folded RNA, 20 nM active *E. coli* $E\sigma^{70}$ (Epicentre) or *B. subtilis* $E\sigma^A$ (see below) and 20 mM Hepes, pH 7.5, 120 mM KCl, 0.5 mM $MgCl_2$, 5% glycerol and 1 mM DTT were incubated for 15 min at 37°C. Heparin was added to 100 µg/ml, samples were incubated for 2 min at room temperature, an equal volume of load buffer (50% glycerol, 0.5× TBE, 0.1% Bromophenol Blue and 0.1% Xylene Cyanol) was added, samples were loaded onto native gels (5% polyacrylamide, 0.5× TBE and 5% glycerol) and gels were run at 200 V for 2 h at room temperature. To monitor complexes after pRNA synthesis, NTPs (0.05–0.1 mM final) and $MgCl_2$ (2.5 mM final) were added after formation of RNA:RNA polymerase complexes, and reactions were incubated an additional 10 min at 37°C prior to heparin challenge and native gel electrophoresis.

His-tagged *B. subtilis* RNA polymerase was purified using Ni-NTA agarose affinity chromatography from KW591 cells as previously described (27), followed by additional purification on HiTrap Heparin HP (GE Healthcare). *Bacillus subtilis* σ^A was purified from inclusion bodies after overproduction from pET-Bsσ^A in *E. coli* BL21star cells (Invitrogen), followed by refolding as previously described for *E. coli* σ^{70} (28,29). $E\sigma^A$ was reconstituted by incubation of His-tagged RNA polymerase with 10-fold molar excess of σ^A in storage buffer (50 mM Tris pH 8, 0.5 mM EDTA, 0.1 M NaCl, 50% glycerol and 0.1 mM DTT) for 30 min at room temperature. The active concentration of *E. coli* $E\sigma^{70}$ or *B. subtilis* $E\sigma^A$ was estimated from the minimum protein concentration required to obtain maximum binding to 10 nM DNA with a consensus extended −10 promoter for *E. coli*, or to 10 nM 6S-2 RNA for *B. subtilis*, as described by Roe *et al.* (30).

For the experiment shown in Figure 7, 6S RNA:pRNA duplexes were formed by heating ^{32}P-labeled 6S RNA (200 nM) and pRNA (200 or 400 nM) in 1× HM for 2 min followed by slow cooling to 37°C over ~1.5 h. For comparison, 6S RNA and pRNA were independently heated and cooled to test 6S RNA alone, or to add pRNA after folding (e.g. 6S RNA + pRNA independently rather than as 6S RNA:pRNA duplex; Figure 7, lanes 5 and 10).

In vitro pRNA synthesis reactions

pRNA synthesis reactions were essentially as described previously (8). Briefly, unlabeled *in vitro* transcribed and gel-purified *E. coli*, *B. subtilis* or mutant 6S RNAs were incubated with $E\sigma^{70}$ or $E\sigma^A$ under conditions described above for the binding assay, except that RNA was at 80 nM, Eσ was at 40 nM active and reconstitution was allowed to proceed for 5 min at room temperature. Note that 5 min was sufficient for full binding of RNA to RNA polymerase at these concentrations (13). pRNA synthesis was initiated by addition of nucleotides (0.05–0.1 mM final concentration including α-^{32}P-CTP) and $MgCl_2$ (2.5 mM final concentration). After incubation for 15–20 min at 37°C, reactions were stopped by addition of loading dye (10 M Urea, 0.5× TBE, 0.1% Bromophenol Blue and 0.1% Xylene Cyanol). RNAs were separated on denaturing gels (20% polyacrylamide, 8 M Urea and 1× TBE) and visualized on a Typhoon phosphorimager (GE Life Sciences).

Northern analysis of 6S RNAs and pRNAs

To examine 6S RNA levels, total RNA was isolated from *E. coli* and *B. subtilis* cells using Trizol Reagent

(Invitrogen) and analyzed on 10% denaturing gels with RNA probes as previously described (4,5) with the following modifications. To increase *B. subtilis* lysis, lysozyme was increased to 10 μg/ml final concentration, and following addition of Trizol reagent, ~200 μl of glass beads (0.1 mm; Thomas Scientific) were added and samples were vortexed for 2 min at room temperature before proceeding.

To examine *in vivo*-generated pRNAs, total small RNA from *E. coli* or *B. subtilis* was isolated using miRVana RNA isolation kit (Applied Biosystems) according to manufacturer methods for small RNA isolation except that cells were vortexed with glass beads (0.1 mm, Thomas Scientific) for 2 min in lysis buffer to facilitate cell lysis. 0.2–0.5 μg small RNA was separated on a denaturing MOPS gel (15% polyacrylamide, 8 M Urea, 1× MOPS) (Lonza 10× MOPS: 0.2 M 3-(N-morpholino) propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA and 10 mM ethylene glycol tetraacetic acid), transferred to uncharged nylon membrane (Hybond NX; Amersham) by semi-dry transfer (20 V, 25 min in 1× MOPS buffer), and chemically crosslinked by treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as previously described (31,32). 5′-end labeled locked nucleic acid (LNA) oligonucleotides complementary to pRNAs were used for probes (see Supplementary Table S1 for sequences). Hybridization was in 5× SSC, 20 mM Na_2HPO_4, 7% sodium dodecyl sulfate (SDS), 2× Denhardts at 50°C as previously described (32).

β-Galactosidase assays

β-Galactosidase activity was measured at 30°C as previously described (7) and activity is expressed in units (ΔOD_{420} per min/OD_{600} unit) (33). Briefly, cultures inoculated from a single colony were grown at 30°C for 24 h, diluted 1:100 in fresh medium (OD_{600} = 0.04), grown for an additional 18 h to late stationary phase. Cells were lysed with SDS and chloroform prior to β-galactosidase assays. Three independent cultures per strain were used for each experiment, and all experiments were repeated at least three times.

RESULTS

Stationary phase *B. subtilis* cells expressing 6S-2 RNA in the absence of 6S-1 RNA are delayed in their ability to re-enter growth

Growth experiments using *B. subtilis* strains lacking 6S-1 RNA (Δ*bsrA*; KW587), 6S-2 RNA (Δ*bsrB*; KW589) or both (Δ*bsrA*Δ*bsrB*; KW590) revealed that cells lacking 6S-1 RNA were delayed in outgrowth from stationary phase. For example, when stationary phase cells were diluted ~1:500 into fresh medium, cells lacking 6S-1 RNA were delayed for entry into exponential phase by 50 min compared to wild-type cells or cells lacking 6S-2 RNA (Figure 2A). The delay appeared to result primarily from an increase in lag time; once the *ΔbsrA* cells entered exponential phase, they grew with a similar doubling time to wild-type cells and obtained a similar maximal cell density in stationary phase. Intriguingly, cells lacking

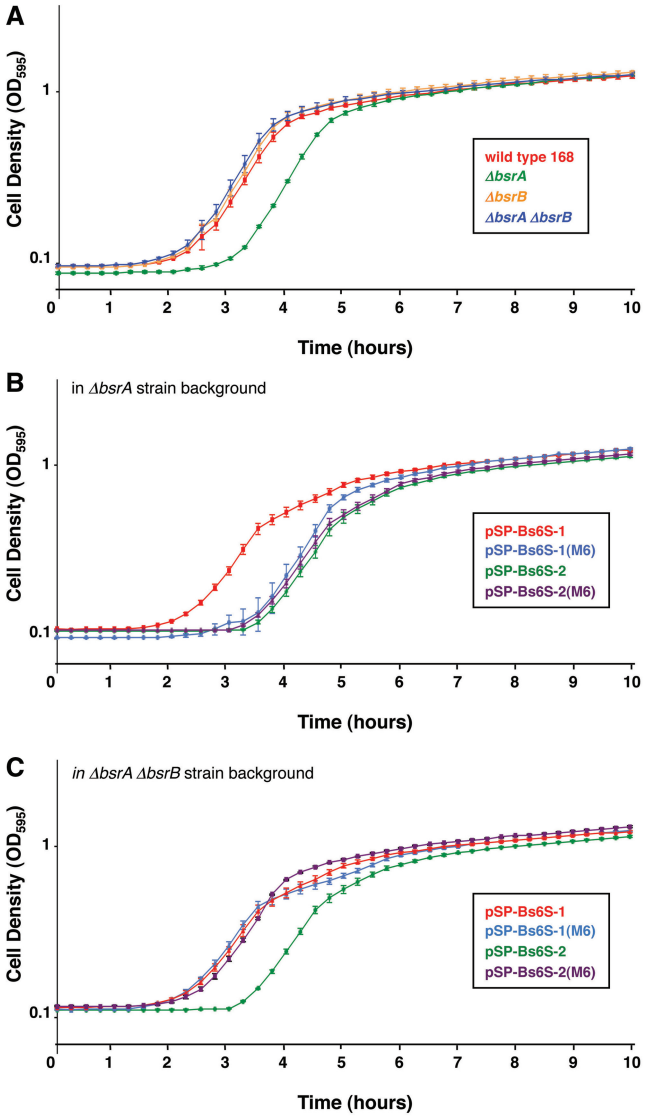

Figure 2. *Bacillus subtilis* cells expressing 6S-2 RNA but lacking 6S-1 RNA are delayed in outgrowth from stationary phase compared to wild-type cells. Growth of *B. subtilis* cells as monitored by optical density at 595 nm (OD_{595}) in an absorbance plate reader after stationary phase cells were diluted ~1:500 into 2× YT medium. Data shown are averages of three independent experiments with three biological replicates per experiment. Error bars correspond to ± standard deviations from the averages. (**A**) Comparing growth of *B. subtilis* wild-type 168 (red; KW586), *ΔbsrA* (green; KW587), *ΔbsrB* (orange; KW589) or *ΔbsrAΔbsrB* (blue; KW590) cells. (**B**) Comparing growth of *B. subtilis ΔbsrA* cells (KW587) containing plasmids pSP-Bs6S-1 (red), pSP-Bs6S-1(M6) (blue), pSP-Bs6S-2 (green) or pSP-Bs6S-2(M6) (purple). (**C**) Comparing growth of *B. subtilis ΔbsrAΔbsrB* cells (KW590) containing plasmids pSP-Bs6S-1 (red), pSP-Bs6S-1(M6) (blue), pSP-Bs6S-2 (green) or pSP-Bs6S-2(M6) (purple).

both 6S-1 and 6S-2 RNAs (Δ*bsrA*Δ*bsrB*) were not delayed in outgrowth, suggesting it is the presence of 6S-2 RNA in the absence of 6S-1 RNA that is required for the outgrowth delay.

Δ*bsrA* cells with a plasmid containing *bsrA* (pSP-Bs6S-1) grew indistinguishably from wild-type (Figure 2B). A plasmid expressing a Bs6S-1 RNA mutant that does not bind $E\sigma^A$ [Bs6S-1(M6) RNA from pSP-Bs6S-1(M6);

Figure 1; see Supplementary Figure S1 for binding] was not able to restore wild-type outgrowth behavior (Figure 2B), indicating the presence of active 6S-1 RNA is required for wild-type outgrowth behavior when 6S-2 RNA is expressed. Δ*bsrA* cells with a plasmid containing wild-type or an M6 mutant of *bsrB* [pSP-Bs6S-2 or pSP-Bs6S-2(M6)] also grew similar to *ΔbsrA* cells.

Introduction of a plasmid expressing 6S-2 RNA (pSP-Bs6S-2) into Δ*bsrA*Δ*bsrB* cells resulted in a delay in outgrowth (Figure 2C). However, expression of the inactive 6S-2(M6) RNA [from pSP-Bs6S-2(M6); see Supplementary Figure S1 for binding] in Δ*bsrA*Δ*bsrB* cells did not result in an outgrowth delay, supporting the hypothesis that 6S-2 RNA activity in the absence of 6S-1 RNA is responsible for the outgrowth delay. Expression of 6S-1 or 6S-1(M6) RNA in Δ*bsrA*Δ*bsrB* cells had no effect on growth.

We sought to test if overexpression of 6S-2 RNA in cells lacking 6S-1 RNA (Δ*bsrA*) or wild-type cells could exaggerate the outgrowth delay. However, we did not find conditions in which 6S-2 RNA levels were increased significantly over wild-type even in cells containing the pSP-Bs6S-2 plasmid (Supplementary Figure S2). In addition, there was not a large change in Bs6S-2 RNA levels in *ΔbsrA* cells compared to wild-type, although a small increase (≤2-fold) was sometimes observed (Supplementary Figures S2 and S6).

Bacillus subtilis 6S-2 RNA does not direct efficient pRNA synthesis *in vitro* or *in vivo*

Both Bs6S-1 and Bs6S-2 RNAs bind to $E\sigma^A$ efficiently *in vivo* and *in vitro* (5), which raised the question of what distinguishes 6S-1 and 6S-2 RNA functions. Analysis of RNAs in wild-type *B. subtilis* by deep sequencing failed to observe a 6S-2 RNA-directed pRNA ($pRNA_{6S-2}$), although 6S-1 RNA-directed pRNA ($pRNA_{6S-1}$) was readily detected (34), suggesting that 6S-2 RNA may not be used as a template for pRNA synthesis. Alternatively, it is possible that $pRNA_{6S-2}$ is unusually unstable relative to $pRNA_{6S-1}$, or that growth conditions examined were not optimal for observing synthesis of $pRNA_{6S-2}$. To fully assess the potential for 6S-2 RNA to direct pRNA synthesis, we turned to *in vitro* assays using purified *B. subtilis* $E\sigma^A$ and RNA to allow easy manipulation of RNA levels and eliminate potential effects from differential stability of generated pRNAs. Incubation of Bs6S-1 RNA:$E\sigma^A$ complexes with nucleotides resulted in production of readily detected $pRNA_{6S-1}$ products (Figure 3A), indicating Bs6S-1 RNA can direct pRNA synthesis in agreement with similar work by others and deep sequencing analysis (22,34). In contrast, incubation of Bs6S-2 RNA:$E\sigma^A$ complexes with nucleotides produced only minimal pRNA (Figure 3A), suggesting it is a poor template for pRNA synthesis.

Both Bs6S-1 and Bs6S-2 RNAs bound to $E\sigma^A$ efficiently *in vitro* (Figure 3B, lanes 2 and 5), in agreement with previous co-immunoprecipitation experiments that demonstrated that 6S-1 and 6S-2 RNAs bind to $E\sigma^A$ (5). Incubation of Bs6S-1 RNA:$E\sigma^A$ complexes with nucleotides under conditions to promote pRNA synthesis led to release of Bs6S-1 RNA from $E\sigma^A$ as visualized on native gels as a decrease in Bs6S-1 RNA:$E\sigma^A$ complexes and the appearance of Bs6S-1 RNA:pRNA (Figure 3B, compare lanes 2 and 3). In contrast, very little Bs6S-2 RNA was released under similar conditions (Figure 3B, compare lanes 5 and 6).

Next, we examined whether pRNA synthesis occurred in *B. subtilis* cells during outgrowth from stationary phase, the time we would expect maximum pRNA synthesis based on studies in *E. coli* (8,21). Indeed, $pRNA_{6S-1}$ was readily detected by northern analysis using locked nucleic acid probes to facilitate hybridization to very small RNAs (Figure 3C), which also has been observed by Beckmann *et al.* (22). In contrast, $pRNA_{6S-2}$ was not detected in agreement with the deep sequencing results (34). To confirm that the northern method used here would be able to detect $pRNA_{6S-2}$, we included a synthetic RNA corresponding to the predicted $pRNA_{6S-2}$, which was readily detected.

The orientation of Bs6S-2 RNA within $E\sigma^A$ has not been definitively demonstrated, and in *Helicobacter pylori*, pRNAs have been observed from both orientations of the Hp6S RNA within RNA polymerase (16). To test if Bs6S-2 RNA might direct synthesis of a pRNA in the opposite direction, northern analysis was done using a probe directed to a potential upstream pRNA, but no products were detected (Supplementary Figure S3), in agreement with lack of pRNA detection *in vitro* where any RNA product would be labeled. Note that there were no detectable upstream products from Bs6S-1 either, suggesting Bs6S-1 RNA is likely to bind $E\sigma^A$ with a specific orientation analogous to the Ec6S RNA (8,9).

6S(M68) RNA in *E. coli* mimics the behavior of Bs6S-2 RNA in *B. subtilis*

To address the role of pRNA synthesis in outgrowth generally, we next tested if we could recapitulate the observed delay in outgrowth in a divergent system, *E. coli*, in which we also had more detailed information available about the behavior and function of 6S RNA. Mature Bs6S-2 RNA did not accumulate when expressed in *E. coli* from pKK-Bs6S-2, which prevented testing the effects of this RNA in *E. coli*. We speculate that the small stem loop at the 3′-end of Bs6S-2 RNA might interfere with processing in *E. coli* as we observed a number of larger RNA species but minimal mature length Bs6S-2 RNA (J.M.S. and K.M.W., unpublished results). Therefore we tested a mutant Ec6S RNA [6S(M68) RNA] designed to mimic Bs6S-2 RNA behavior in *E. coli* (e.g. tight binding to RNA polymerase, unable to direct efficient pRNA synthesis) (Figure 1B). $E\sigma^{70}$ preferentially initiates transcription with purine nucleotides (35). Therefore, 6S(M68) RNA has several nucleotide substitutions in the region surrounding the wild-type start site of pRNA synthesis to prevent a purine start (e.g. U44G, U46A, U47G, U48A and C49G; note that changes shown here are in the 6S RNA that is the template strand, and will direct the complement in the pRNA). Other changes (e.g. U51A, A52C and C53A) were made to prevent potential

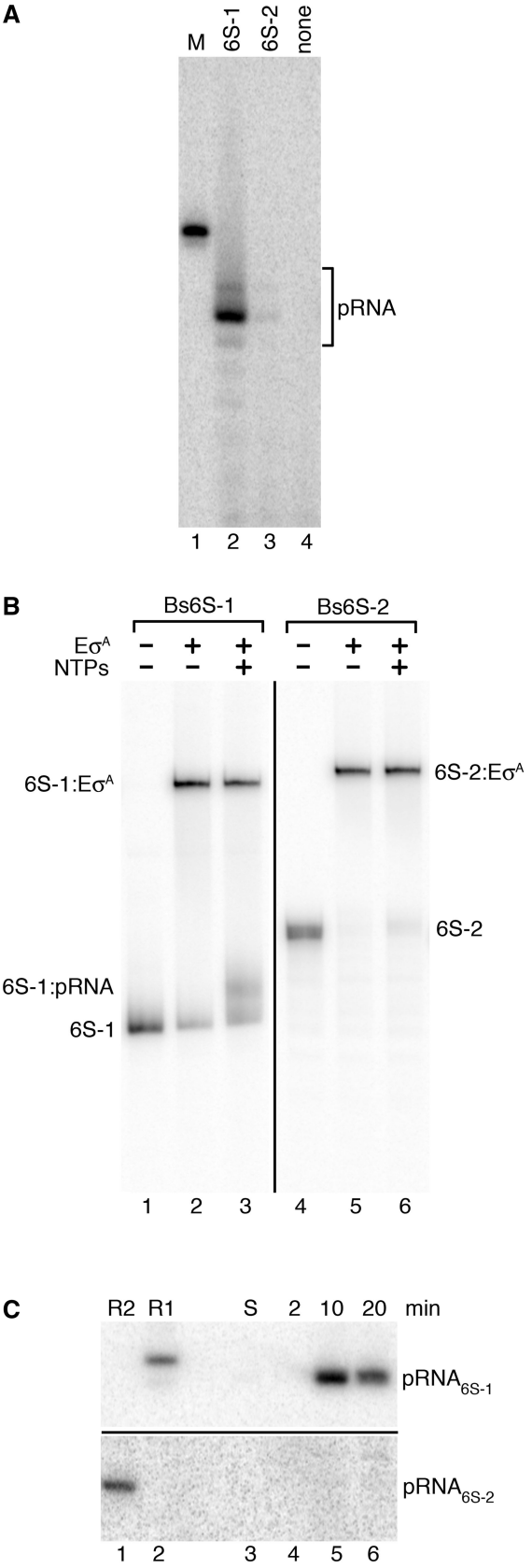

Figure 3. Characterization of Bs6S-1 and Bs6S-2 RNAs *in vitro* and *in vivo*. (**A**) pRNA generated *in vitro* from Bs6S-1:Eσ^A (lane 2) or Bs6S-2:Eσ^A (lane 3) complexes or with Eσ^A alone (lane 4) was visualized on a denaturing gel. Reactions contained 0.05 mM NTPs including α-^{32}P-CTP. Lane 1 is a 5′-end labeled oligonucleotide 19 nt in length for size comparison. (**B**) Bs6S-1 RNA (lanes 1–3) and Bs6S-2 RNA (lanes 4–6) association with Eσ^A was monitored by migration in native gels. *In vitro* reactions contained RNA alone (lanes 1, 4); RNA and Eσ^A (lanes 2, 5) or RNA, Eσ^A and NTPs (0.05 mM) (lanes 3, 6). The locations of free RNA, RNA:pRNA duplexes and RNA:Eσ^A migration are indicated. Note that Bs6S-1 RNA and Bs6S-2 RNA experiments were done in parallel, but run on separate gels as indicated by line between lanes 3 and 4. (**C**) Northern analysis of small RNA isolated from *B. subtilis* 168 cells (KW586) to examine *in vivo*-generated pRNAs in stationary phase (S; lane 3); or 2 (lane 4), 10 (lane 5) or 20 (lane 6) min after dilution of stationary phase cells into 2× YT medium. R1 (lane 2) and R2 (lane 1) contain synthetic RNAs corresponding to predicted $pRNA_{6S-1}$ and $pRNA_{6S-2}$, respectively, to test probing efficiency of LNA probes specific for $pRNA_{6S-1}$ (top panel) or $pRNA_{6S-2}$ (bottom panel).

alternative base pairing interactions within the central bulge that would most likely alter binding affinity for Eσ^{70} (5), as we desired a mutant RNA that retains high-affinity binding to Eσ^{70} to test defects in pRNA synthesis independently from any binding defects.

6S(M68) RNA was characterized in a series of *in vitro* and *in vivo* assays to test if it mimicked Bs6S-2 RNA behavior. *In vitro* binding with purified components demonstrated that 6S(M68) RNA bound to Eσ^{70} similar to wild-type Ec6S RNA *in vitro*, while the inactive 6S(M5) RNA did not (Figure 4A, compare lanes 2, 5 and 8). Incubation of wild-type Ec6S RNA:Eσ^{70} complexes with nucleotides under conditions that initiate pRNA synthesis has been shown to result in release of Ec6S RNA in a duplex with pRNA (8,21), which can be observed by native gel electrophoresis as a reduction in Ec6S RNA:Eσ^{70} complexes and the formation of Ec6S RNA:pRNA duplexes (Figure 4A, compare lanes 2 and 3). In contrast, incubation of the 6S(M68) RNA:Eσ^{70} complex with nucleotides did not lead to efficient release of 6S(M68) RNA:pRNA or reduction in 6S(M68) RNA:Eσ^{70} complexes (Figure 4A, lane 6). Direct analysis of *in vitro*-generated pRNA verified that 6S(M68) RNA is a poor template for pRNA synthesis (Figure 4B). A faint, smaller RNA was detected *in vitro* in the presence of 6S(M68) RNA that was not observed in the absence of RNA or with the inactive 6S(M6+M68) RNA, suggesting a low-level reaction can take place. However, any release of 6S(M68) RNA from Eσ^{70} is very low compared to wild-type 6S RNA (Figure 4A).

Finally, we tested if 6S(M68) RNA could direct pRNA synthesis *in vivo* by expressing 6S(M68) RNA from a plasmid [pKK−6S(M68)] in an *E. coli* background lacking an intact chromosomal 6S RNA gene (*ssrS1*; KW73). During outgrowth from stationary phase, only low levels of $pRNA_{EcM68}$ were detected compared to $pRNA_{Ec6S}$. Timing of the minimal $pRNA_{EcM68}$ production also was delayed as high levels of wild-type $pRNA_{Ec6S}$ were detected by 2 min of outgrowth but the low level of observed $pRNA_{EcM68}$ was higher at 10 min than 2 min (Figure 4C). Once again, we included a synthetic RNA with the sequence of the predicted $pRNA_{EcM68}$ as a marker to demonstrate that the probe used could detect the $pRNA_{EcM68}$ with similar efficiency to $pRNA_{Ec6S}$. The 6S(M68) RNA expressed from

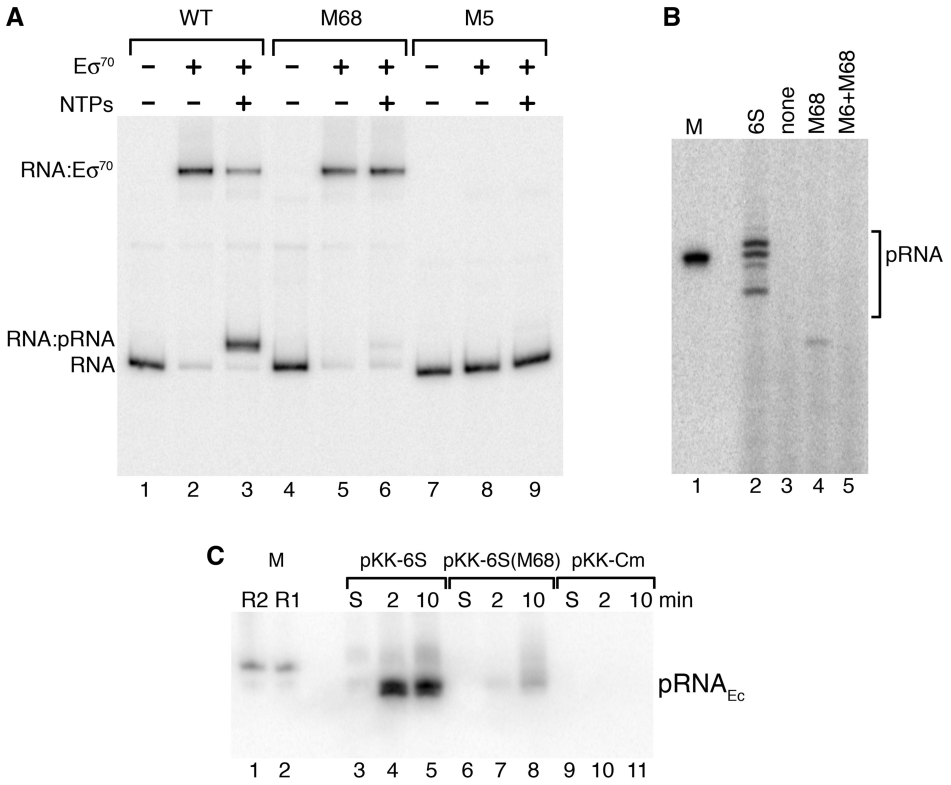

Figure 4. Characterization of *E. coli* 6S(M68) RNA *in vitro* and in *vivo*. (**A**) RNA association with Eσ^{70} was monitored by native gel electrophoresis. 6S (lanes 1–3); 6S(M68) (lanes 4–6); and 6S(M5) (lanes 7–9) RNAs were examined. *In vitro* reactions contained RNA alone (lanes 1, 4 and 7); RNA and Eσ^{70} (lanes 2, 5 and 8) or RNA, Eσ^{70} and NTPs (lanes 3, 6 and 9). The locations of free RNA, 6S RNA:pRNA duplexes and RNA:Eσ^{70} migration are indicated. (**B**) pRNA generated by Eσ^{70} *in vitro* from 6S RNA (lane 2); 6S(M68) RNA (lane 4); or 6S(M6+M68) RNA (lane 5) or Eσ^{70} alone (lane 3) was visualized on a denaturing gel. Lane 1 is a 5′-end labeled oligonucleotide 19 nt in length for size comparison. (**C**) Northern analysis of small RNA isolated from *ssrS1 E. coli* cells containing pKK-6S (lanes 3–5); pKK-6S(M68) (lanes 6–8), or an empty vector control (pKK-Cm, lanes 9–11) to examine *in vivo* generated pRNAs in stationary phase (S; 18 h) or after dilution of stationary phase cells into LB + Cm and continued incubation for 2 or 10 min as indicated. R1 (lane 2) and R2 (lane 1) contain synthetic RNAs corresponding to pRNA$_{Ec6S}$ and the predicted pRNA$_{EcM68}$, respectively, to test probing efficiency of the LNA probe.

pKK-6S(M68) accumulated to similar or slightly higher levels than Ec6S RNA from pKK-6S (Figure S4). 6S RNA expressed from pKK-6S in *ssrS1* cells is ~2-fold higher than endogenous 6S RNA in wild-type cells (4). All together, these data demonstrate that 6S(M68) RNA is not used efficiently for pRNA synthesis, and that the drastically reduced level of pRNA$_{EcM68}$ *in vivo* is not a result of decreased 6S(M68) RNA concentrations or a decreased binding to Eσ^{70}.

6S(M68) RNA regulates transcription similarly to 6S RNA in *E. coli*

Finally, we tested if 6S(M68) RNA was able to regulate transcription *in vivo* in stationary phase in a manner similar to Ec6S RNA by examining expression from previously characterized promoter-*lacZ* reporters (7,10). Indeed, the presence of 6S(M68) RNA downregulated transcription of the sensitive, σ^{70}-dependent *rsdP2* promoter similar to wild-type Ec6S RNA when compared to the inactive mutant 6S(M6) RNA or 6S(M6+M68) RNA (Figure 5). The presence of 6S(M68) RNA also resulted in increased transcription of an Ec6S RNA-sensitive σ^{S}-dependent promoter (e.g. *bolA*) in stationary phase, but had no effect on the 6S RNA-insensitive *lacUV5* promoter (Figure 5). These results demonstrate that the 6S(M68) RNA regulates transcription similar to wild-type Ec6S RNA in stationary phase.

pRNA synthesis-mediated release of Eσ^{70} from 6S RNA also is required for efficient outgrowth in *E. coli*

Having demonstrated that 6S(M68) RNA mimics Bs6S-2 RNA in its inability to direct efficient pRNA synthesis while retaining Eσ^{70} binding and transcription regulation activity, we next tested if the presence of 6S(M68) RNA in *E. coli* cells had an effect on outgrowth. Indeed, expression of 6S(M68) RNA from pKK-6S(M68) in cells lacking the endogenous RNA (*ssrS1*) exhibited a delay in outgrowth from stationary phase (Figure 6). In contrast, cells expressing wild-type Ec6S RNA (from pKK-6S), inactive 6S RNA mutants [from pKK-6S(M6) or pKK-6S(M68+M6)] or cells lacking any 6S RNA (with a vector control, pKK-Cm) were not delayed in outgrowth. All together, these results strongly support a conclusion that pRNA synthesis is an important step for regulation of 6S RNA activity, and is required for efficient transition out of stationary phase when an active 6S RNA is present.

6S RNA:pRNA duplexes are not able to bind to Eσ^{70}

To further address the role of pRNA synthesis, we sought to address the fate of the released 6S RNA:pRNA duplex by testing if Ec6S RNA:pRNA$_{Ec6S}$ could rebind to Eσ^{70}. For these experiments, Ec6S RNA:pRNA$_{Ec6S}$ duplexes were generated by annealing purified components ('Materials and Methods' section). The preformed Ec6S RNA:pRNA$_{Ec6S}$ duplexes were not able to bind to Eσ^{70} in contrast to Ec6S RNA, which bound efficiently as monitored by native gel electrophoresis (Figure 7, compare lanes 2 and 3 with 1). Note that Ec6S RNA:pRNA$_{Ec6S}$ duplex formation was not complete, and the level of Ec6S RNA:Eσ^{70} complexes formed corresponded to the level of free 6S RNA available after the annealing step (Figure 7, see lanes 7 and 8 lacking Eσ^{70}, compared to lanes 2 and 3 with Eσ^{70}). pRNA$_{Bs6S-1}$ was unable to bind to Ec6S RNA, as expected since these RNAs are not complementary, and also did not alter Ec6S RNA binding to Eσ^{70} (Figure 7, lanes 4 and 9). Moreover, free pRNA$_{Ec6S}$ did not interfere with 6S RNA binding to Eσ^{70} (Figure 7, lane 5), as tested by adding pRNA$_{Ec6S}$ to folded 6S RNA (e.g. without an annealing step), which did not result in 6S RNA:pRNA$_{Ec6S}$ duplexes (Figure 7, lane 10). These results suggest that 6S RNA within the 6S RNA:pRNA$_{Ec6S}$ has an altered structure that prevents reassociation with Eσ^{70}.

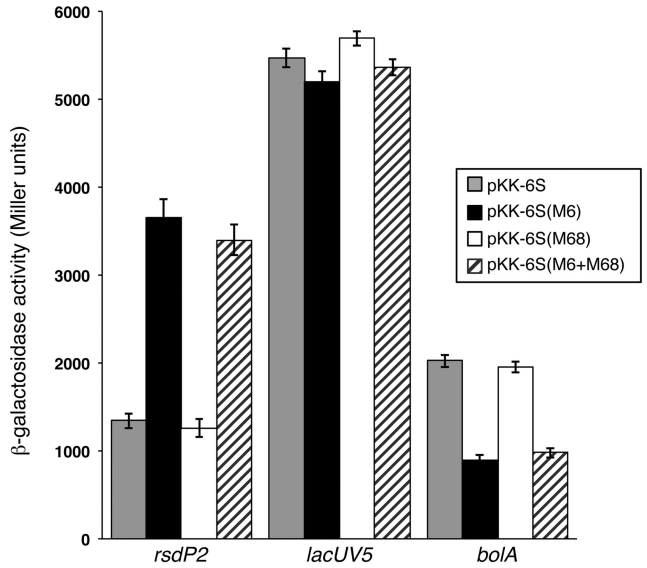

Figure 5. 6S(M68) RNA regulates transcription in *E. coli* similarly to 6S RNA. β-Galactosidase activity of promoter-*lacZ* reporter genes (*rsd*P2, *lacUV5* or *bolA*) in *E. coli ssrS1* cells expressing wild-type 6S RNA (gray bars); 6S(M6) RNA (black bars); 6S(M68) RNA (white bars); or 6S(M6+M68) RNA (hatched bars) from pKK-6S derived plasmids is shown. Data shown are averages of three independent experiments with three biological replicates per experiment. Error bars correspond to ± standard deviations from the averages.

Overexpression of 6S(M68) RNA leads to decreased viability of cells in stationary phase

Next, we asked if higher expression of 6S(M68) RNA could amplify the observed growth defects in *E. coli ssrS1* cells. Previous work determined that expression of Ec6S RNA from a plasmid (pKK-6S+Y) that includes the gene downstream of *ssrS* (*ygfA*) resulted in ~5-fold increased accumulation of Ec6S RNA compared to cells with pKK-6S (4). Although, it is not entirely clear why 6S RNA levels are higher from pKK-6S+Y than from pKK-6S, translation of *ygfA* is not required as mutations in the start site of translation of *ygfA* [e.g. pKK-6S+Y(M2)] have no effect on accumulation (4). The endogenously encoded mature 6S RNA is processed from a primary transcript in which the 3′-end occurs by rho-dependent termination within *ygfA* (36). We hypothesize that a rho-terminated transcript from pKK-6S+Y may be more efficiently processed than a rho-independent

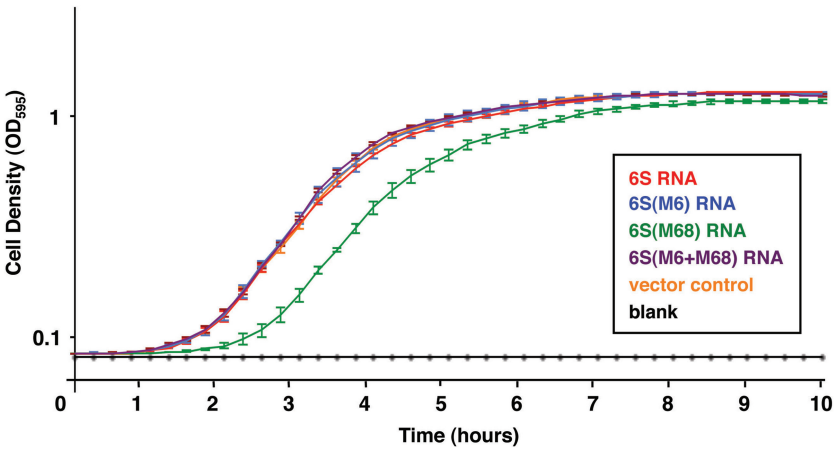

Figure 6. *Escherichia coli* cells expressing 6S(M68) RNA are delayed in outgrowth from stationary phase compared to wild-type. Growth of *E. coli* cells as monitored by optical density at 595 nm (OD_{595}) in an absorbance plate reader after stationary phase cells were diluted ~1:500 into LB + Cm medium. *E. coli ssrS1* cells expressing wild-type 6S RNA (red), 6S(M6) RNA (blue), 6S(M68) RNA (green) or 6S(M6+M68) RNA (purple) from pKK-6S derived plasmids as well as *ssrS1* cells containing the empty vector (pKK-Cm; orange) are shown relative to a blank (black). Data shown are averages of three independent experiments with three biological replicates per experiment. Error bars correspond to ± standard deviations from the averages.

terminated transcript containing a structured 3′-end as would be generated from pKK-6S.

We observed that cultures of cells containing pKK-6S+Y(M68) were severely defective in stationary phase. They did not reach cell densities similar to cells with pKK-6S+Y as measured by optical density, and when stationary phase cells with pKK-6S+Y(M68) were diluted into fresh medium they were unable to grow (data not shown). For simplicity here, we refer to the observed inability to grow as a decrease in viability, although it is also possible these cells have entered a viable but not culturable state. Interestingly, *ssrS1* cells transformed with pKK-6S+Y(M68) formed colonies on agar plates that were indistinguishable from pKK-6S+Y transformants. When cultures were initiated by scraping cells off of agar plates and resuspending into growth medium, *ssrS1* cells containing pKK-6S+Y or pKK-6S+Y(M68) grew similarly over the first 4 h of incubation (Figure 8), suggesting they have a similar growth rate during this time frame. The cells with pKK-6S+Y increased in viable cell counts up to 6 h and maintained a steady cell density through at least 24 h. In contrast, cells with pKK-6S+Y(M68) did not increase in viable cell numbers after 4 hours, but in fact decreased in viable cell numbers resulting in ~1000-fold reduction in viable cells by 24 h. Cells overexpressing the inactive 6S(M6+M68) grew like wild-type, and cells with a plasmid containing a mutation (M2) that prevented expression of *ygfA* from the plasmid [pKK-6S+Y(M2+M68)] grew similar to cells with pKK-6S+Y(M68) (Supplementary Figure S5) indicating it is the high expression of 6S(M68) RNA and not *ygfA* or the presence of the *ssrS*(M68) gene that is responsible for the mutant phenotype.

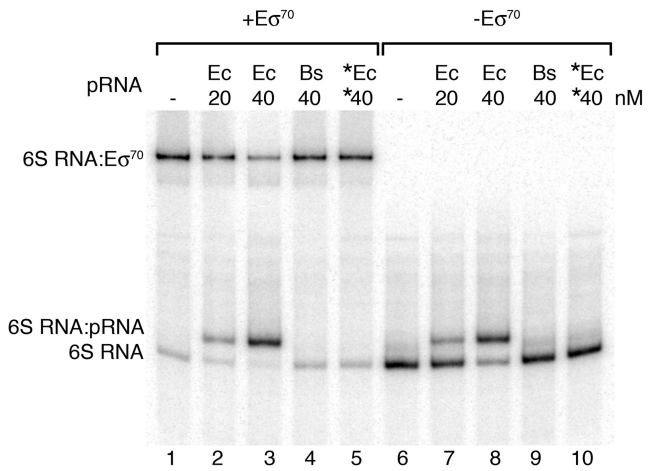

Figure 7. Ec6S RNA:pRNA duplexes are not able to rebind Eσ^{70}. RNA association with Eσ^{70} was monitored by native gel electrophoresis. ^{32}P-labeled Ec6S RNA alone (lanes 1 and 6), with $pRNA_{Ec6S}$ (Ec)(lanes 2, 3, 7 and 8) or $pRNA_{Bs6S-1}$ (Bs)(lanes 4 and 9) was tested. For lanes 5 and 10, $pRNA_{Ec6S}$ was added to prefolded 6S RNA (*Ec), which does not result in duplex formation as indicated by lack of 6S RNA:pRNA duplexes in the absence of Eσ^{70} (lane 10). 6S RNAs were incubated with 40 nM Eσ^{70} (lanes 1–5) or without RNA polymerase (lanes 6–10). 6S RNA was at 20 nM in the binding reaction, pRNA was at 20 nM (lanes 2 and 7) or 40 nM (lanes 3–5 and 8–10) as indicated. The locations of free 6S RNA, 6S RNA:pRNA duplexes and 6S RNA:Eσ^{70} migration are indicated.

DISCUSSION

We have discovered that 6S RNA activity is regulated by the process of pRNA synthesis, and that pRNA-synthesis mediated release of RNA polymerase from 6S RNA is

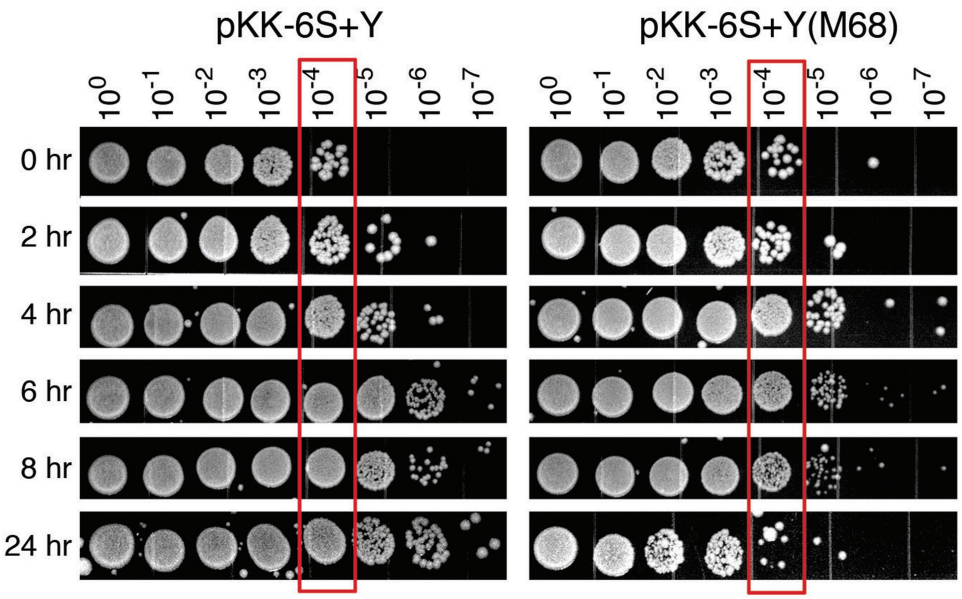

Figure 8. *Escherichia coli* cells overexpressing 6S(M68) RNA have decreased viability in stationary phase compared to cells overexpressing wild-type 6S RNA. Viability of *E. coli ssrS1* cells containing pKK-6S+Y (left panel) or pKK-6S+Y(M68) was monitored by spot titration from 0 to 24 h after growth in culture was initiated from a suspension of cells scraped off agar plates. Cultures were serially diluted 1:10 and 10 µl of each dilution was spotted onto LB plates. The red boxes surrounding 10^{-4} dilutions are to facilitate comparison. Experiments were done with at least three cultures per cell type. Results from matching cell types were very similar; a representative set is shown here.

required for efficient outgrowth in both *B. subtilis* and *E. coli*. In *B. subtilis*, cells expressing Bs6S-2 RNA, which does not serve as a template for pRNA synthesis *in vivo* or *in vitro* (Figure 3), in the absence of Bs6S-1 RNA were unable to efficiently exit stationary phase and restart growth upon nutrient upshift (Figure 2). These results demonstrate that Bs6S-1 RNA and Bs6S-2 RNA have functionally distinct roles and behaviors in *B. subtilis*, suggesting higher than anticipated diversity in regulation of RNA polymerase by 6S RNAs globally. pRNA synthesis has been shown to be a mechanism for release of 6S RNA from RNA polymerase during outgrowth in *E. coli* (8,21, Figure 4A), and Bs6S-1 RNA also was released from $E\sigma^A$ as a result of pRNA synthesis (Figure 3B). In contrast, Bs6S-2 RNA was not released from $E\sigma^A$ upon incubation with nucleotides (Figure 3B). Expression in *E. coli* of the M68 mutant RNA that likewise is unable to direct pRNA synthesis or $E\sigma^{70}$ release (Figure 4), also resulted in an outgrowth delay. Together, these results support conclusions that 6S RNA-mediated sequestration of the housekeeping holoenzyme is detrimental for efficient outgrowth, and that pRNA-synthesis is an important mechanism to relieve RNA polymerase from 6S RNA regulation at that time. Interestingly, overexpression of the 6S(M68) RNA leads to decreased viability suggesting pRNA synthesis, or potentially the pRNA product, is important at other times in addition to outgrowth (Figure 8).

pRNA synthesis: a mechanism to increase available RNA polymerase during outgrowth

We propose that one of the key features of the pRNA synthesis reaction during outgrowth is the rapid release of RNA polymerase from 6S RNA bound complexes formed in stationary phase. When stationary phase cells encounter an upshift in nutrients, a number of changes must occur before cells can start to divide and restart active growth. An important immediate response is increased transcription of many genes necessary to take advantage of the nutrient excess (37), such as high and immediate induction of transcription of the ribosomal RNA operons to allow new ribosome synthesis and an increase in translational capacity of the cell (38–40). An abundance of available RNA polymerase is presumably necessary to allow a full transcriptional response during outgrowth.

Here, we show that pRNA synthesis, which results in rapid release of RNA polymerase, is required for efficient outgrowth of *B. subtilis* and *E. coli* cells containing 6S RNAs that bind to RNA polymerase. In wild-type stationary phase *E. coli* cells, $E\sigma^{70}$ is essentially saturated with Ec6S RNA bound in its active site, competing for binding of promoter DNA by RNA polymerase (4,8). Upon nutrient upshift, pRNA synthesis leads to the rapid release of $E\sigma^{70}$ from Ec6S RNA, freeing RNA polymerase to increase binding at promoter DNA. Consistent with the model that it is the increase in available RNA polymerase levels that is required and not the actual pRNA product, cells lacking 6S RNA are not altered in their timing of outgrowth (Figure 6). In this model, one might anticipate that transcription will increase by mass action due to the increased available RNA polymerase, rather than by direct regulation of specific genes that are normally regulated by 6S RNA.

Alternatively, timing of outgrowth may be mediated by altered regulation of transcription of key genes in cells with wild-type 6S RNA or 6S(M68) RNA. However, 6S(M68) RNA-regulated transcription of tested promoters in stationary phase indistinguishably from wild-type Ec6S RNA (Figure 5), suggesting changes in which genes are regulated or to what extent they are regulated is unlikely to be responsible for the growth defect. However, the possibility remains that some promoters respond differentially to 6S(M68) RNA and 6S RNA and are not represented by the reporters tested here or at the time examined. In addition, 6S(M68) RNA also regulated a tested σ^S-dependent promoter previously shown to respond to Ec6S RNA (ref. 7; Figure 5) further demonstrating its ability to regulate transcription like Ec6S RNA.

pRNA synthesis: a mechanism to reduce 6S RNA levels during outgrowth

In addition to increasing the RNA polymerase off-rate from 6S RNA, a second key feature of pRNA synthesis is that 6S RNA is released in a duplex with the pRNA in a structure that is not able to rebind RNA polymerase (Figure 7). At a minimum, the inability of the Ec6S RNA:pRNA complex to rebind $E\sigma^{70}$ shows the timing of potential reassociation of Ec6S RNA with RNA polymerase that is dependent on how rapidly 6S RNA:pRNA duplexes melt. However, given the significant decrease of Ec6S and Bs6S-1 RNA levels during this time frame, we propose that the released 6S RNA is less stable than the RNA polymerase-bound fraction of 6S RNA. The 6S RNA:pRNA duplex is likely to be more accessible to cellular RNases when not bound in the main channel of RNA polymerase. Alternatively, it is possible that the duplex region of 6S RNA:pRNA could be targeted for specific degradation. In either case the outcome would be a decrease in 6S RNA pools available for binding to and regulation of RNA polymerase. Strikingly, Bs6S-2 RNA levels in *B. subtilis* remain fairly constant throughout growth, including during the transition from stationary phase to early exponential phase, further supporting the difference in behavior between RNAs that direct pRNA synthesis and those that do not, and the role of pRNA-synthesis in turnover of Ec6S and Bs6S-1 RNAs.

pRNA synthesis: a biological role beyond outgrowth?

Interestingly, *B. subtilis* cells expressing only Bs6S-2 RNA (Δ*bsrA*) and *E. coli* cells expressing near endogenous levels of 6S(M68) RNA eventually restart growth after a longer lag time. Once these cells enter exponential growth, they grow similarly to wild-type and obtain high cell densities in stationary phase (Figures 2A and 6). Therefore, we suggest it is the rapid removal of 6S RNA from RNA polymerase that is required for efficient outgrowth timing. In the absence of pRNA synthesis, 6S RNA will cycle on and off of RNA polymerase by

equilibrium dynamics, which may be facilitated by competition with promoters insensitive to 6S RNA. The 6S RNA off-rate via dissociation is expected to be considerably slower than that mediated through pRNA synthesis, based on the very high affinity of 6S RNA for RNA polymerase and the slow dissociation rates observed *in vitro* (13). In addition, increases in free RNA polymerase pools needed for outgrowth are likely to be slowed by the fact that simple 6S RNA dissociation from RNA polymerase leads to release of 6S RNA in a form competent for rebinding to RNA polymerase, leading to continued competition with promoter DNA. However, at some point, either the necessary cellular components are accumulated or active transcription outcompetes 6S RNA inhibition, leading to normal exponential growth. These results might suggest that pRNA synthesis is not as important for 6S RNA cycling on and off of RNA polymerase during exponential growth, although pRNA from wild-type Ec6S RNA and Bs6S-1 RNA are still readily detected at this time.

The observation that high overexpression of 6S(M68) RNA led to loss of cell viability in early stationary phase (Figure 8) suggests that pRNA synthesis is occurring during this time frame and may be an important mechanism to prevent overaccumulation of 6S RNA. It is intriguing that cells with high overexpression of 6S(M68) RNA grow indistinguishably from wild-type on agar plates, suggesting the possibility that the environment on plates does not require rapid changes in available RNA polymerase levels, is less sensitive to these changes or that other environmental factors influence 6S RNA regulation of transcription differentially on plates and in culture.

6S-1 RNA versus 6S-2 RNA in *B. subtilis*

One of the most intriguing questions is how the presence of Bs6S-1 RNA in *B. subtilis* alleviates the negative impact of Bs6S-2 RNA on outgrowth. Bs6S-2 RNA levels are not dramatically changed in cells lacking Bs6S-1 RNA (Supplementary Figure S2), and a similarly high fraction of Bs6S-2 RNA is bound to $E\sigma^A$ in both cell types in stationary phase (Supplementary Figure S6). One possibility is that the presence of Bs6S-1 RNA influences the competition between Bs6S-2 RNA and promoter DNA binding to RNA polymerase, thereby altering the kinetics of the RNA polymerase cycling enough to allow efficient outgrowth. Alternatively, the slight changes in Bs6S-2 RNA levels in *ΔbsrA* cells may be enough to mediate the growth defects. Attempts to overexpress Bs6S-2 RNA from plasmids have failed, even in a wild-type background, which might suggest Bs6S-2 RNA levels are tightly regulated. While it is clear that both RNAs bind to $E\sigma^A$ *in vivo* and *in vitro* (5, Figure 3B), it remains possible that Bs6S-1 RNA or Bs6S-2 RNA activity is not limited to $E\sigma^A$ as potential binding to alternative forms of RNA polymerase has not been tested. Finally, changes in Bs6S-1 RNA-regulated gene targets in *ΔbsrA* cells may lead to the observed phenotype, although it is unclear why such an effect would not be detrimental in *ΔbsrAΔbsrB* cells as well. More information regarding what promoters respond to Bs6S-1 and Bs6S-2 RNA in *B. subtilis* is needed to sort out these possibilities. In addition, mechanistic understanding about why Bs6S-2 RNA does not direct pRNA synthesis awaits further analysis.

Experiments presented here highlight the importance of pRNA synthesis as a mechanism to direct RNA polymerase toward active transcription during outgrowth. Perhaps surprisingly, it appears that pRNA synthesis continues to contribute to cycling of 6S RNA and RNA polymerase complexes at least into stationary phase, as demonstrated by loss of cell viability in cells overexpressing the negative M68 RNA. Alternatively, these later effects could result from lack of the pRNA itself if it has independent function from the role its synthesis plays in RNA polymerase release. In addition, the revealed difference in behavior of Bs6S-1 and Bs6S-2 RNAs in *B. subtilis* underscores a complexity in the use of RNA polymerase-binding RNAs not previously appreciated. Intriguingly, some bacteria appear to have only a 6S-2 RNA-like gene (5,6), raising interesting questions about how this RNA may influence growth profiles in divergent organisms. Future studies will be required to elucidate the mechanistic differences in these diverse 6S RNA functions as well as to provide a full understanding of how they impact cellular physiology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Methods, Supplementary Table 1, Supplementary Figures 1–6 and Supplementary References [42–44].

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