Bacillus subtilis 6S-2 RNA serves as a template for short transcripts in vivo

PHILIPP G. HOCH,1 JULIA SCHLERETH,1 MARCUS LECHNER, and ROLAND K. HARTMANN
Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, 35037 Marburg, Germany

ABSTRACT
The global transcriptional regulator 6S RNA is abundant in a broad range of bacteria. The RNA competes with DNA promoters for binding to the housekeeping RNA polymerase (RNAP) holoenzyme. When bound to RNAP, 6S RNA serves as a transcription template for RNAP in an RNA-dependent RNA polymerization reaction. The resulting short RNA transcripts (so-called product RNAs = pRNAs) can induce a stable structural rearrangement of 6S RNA when reaching a certain length. This rearrangement leads to the release of RNAP and thus the recovery of transcription at DNA promoters. While most bacteria express a single 6S RNA, some harbor a second 6S RNA homolog (termed 6S-2 RNA in Bacillus subtilis). Bacillus subtilis 6S-2 RNA was recently shown to exhibit essentially all hallmark features of a bona fide 6S RNA in vitro, but evidence for the synthesis of 6S-2 RNA-derived pRNAs in vivo has been lacking so far. This raised the question of whether the block of RNAP by 6S-2 RNA might be lifted by a mechanism other than pRNA synthesis. However, here we demonstrate that 6S-2 RNA is able to serve as a template for pRNA synthesis in vivo. We verify this finding by using three independent approaches including a novel primer extension assay. Thus, we demonstrate the first example of an organism that expresses two distinct 6S RNAs that both exhibit all mechanistic features defined for this type of regulatory RNA.

Keywords: 6S RNA; 6S-1 RNA; bsrA; 6S-2 RNA; bsrB; pRNA; product RNA; RNA-seq; Northern blot; primer extension

INTRODUCTION
6S RNA is a small noncoding RNA involved in transcriptional regulation in bacteria (Wassarman and Storz 2000; Wehner et al. 2014). It binds to the housekeeping RNA polymerase (RNAP) holoenzyme by mimicking an open promoter structure (Wassarman 2002; Barrick et al. 2005). Escherichia coli 6S RNA reaches its highest cellular levels when cells enter stationary growth, where the RNA is thought to contribute to the economical utilization of nutrients and metabolites (Cavanagh and Wassarman 2013; for review, see Steuten et al. 2014). Most bacteria have a single 6S RNA, such as E. coli, where the function of 6S RNA as a transcriptional regulator was first described (Wassarman and Storz 2000). In contrast, some species were reported to harbor two or even more 6S RNA homologs (Wehner et al. 2014), among them Bacillus subtilis expressing 6S-1 (bsrA) and 6S-2 (bsrB) RNA (Ando et al. 2002; Suzuma et al. 2002; Barrick et al. 2005; Trotochaud and Wassarman 2005). At present, the regulatory role of the paralogous 6S-2 RNA is poorly understood. While 6S-1 RNA levels peak upon entry into stationary phase, 6S-2 RNA was reported to reach highest steady-state levels between early and mid-exponential phase. Its levels were described to either decrease toward stationary phase (Ando et al. 2002; Barrick et al. 2005; Beckmann et al. 2011) or to remain largely constant over the entire growth period (Trotochaud and Wassarman 2005; Cavanagh et al. 2012), possibly depending on the strain background (Steuten et al. 2014).

While bound to 6S RNA, RNAP is able to use 6S RNA as a template for transcription of so-called product RNAs (pRNAs) in an RNA-dependent RNA polymerization reaction (Wassarman and Saecker 2006). For 6S-1 RNA, thought to be the canonical 6S RNA in B. subtilis, RNA-seq of stationary phase cells combined with pRNA in vitro transcription analyses identified pRNAs with a length of 8–9 nt to be abundant; pRNAs shorter than 8 nt are detectable in vitro as well (Beckmann et al. 2011, 2012; Burenina et al. 2014), but their abundance in vivo could not be assessed owing to uncertain assignment of such short reads to the 6S-1 RNA locus. Upon nutritional upshift when stationary cells reenter exponential growth, the total number of pRNA transcripts and

© 2016 Hoch et al. This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
particularly the fraction of longer pRNAs (≥14 nt) were found to increase (Beckmann et al. 2011, 2012). While shorter pRNAs (approximately <10 nt) are thought to be abortive transcripts that dissociate from 6S-1 RNA during idling cycles of abortive transcription (Beckmann et al. 2012), longer pRNAs remain stably bound and are able to cotranscriptionally induce a rearrangement of the 6S-1 RNA structure. The resulting structural change of 6S-1 RNA then triggers the release of RNAP from its sequestration (Wassarman and Saecher 2006; Beckmann et al. 2012). Subsequently the released and now accessible 6S-1 RNAP:pRNA hybrid is assumed to be degraded, possibly initiated by endonucleolytic cleavage in the apical loop of 6S-1 RNA (Beckmann et al. 2011).

_Bacillus subtilis_ 6S-2 RNA has been studied as well in recent years and was shown to possess all hallmark features of a bona fide 6S RNA in vitro, including the ability to serve as a template for pRNA transcription (Supplemental Fig. S1; Buremina et al. 2014). However, previous 454 RNA-seq and Northern blot analyses failed to prove the presence of 6S-2 RNA-derived pRNAs in RNA fractions extracted from _B. subtilis_ cells (Beckmann et al. 2011; Cavanagh et al. 2012). This may be attributed to the fact that _B. subtilis_ RNAP initiates 6S-2 pRNA synthesis with ATP (Beckmann et al. 2011), which is less efficient than 6S-1 pRNA initiation with GTP (Cabrera-Ostertag et al. 2013). These findings fostered the idea that 6S-2 RNA might be displaced from RNAP by serving as a template for pRNA synthesis analogous to 6S-1 RNA. However, regulation of the δ⁵-RNAP holoenzyme by 6S-2 RNA seems to be less dynamic than the enzyme’s functional interplay with 6S-1 RNA.

**RESULTS**

### 6S-2 pRNAs are detectable by Illumina-based RNA-seq

So far, 6S-2 RNA-derived pRNAs could not be detected in _B. subtilis_, neither by 454 RNA-seq nor by Northern blotting (Beckmann et al. 2011; Cavanagh et al. 2012). However, here we show that 6S-2 RNA-derived pRNAs are observable in libraries enriched for small primary transcripts (<50 nt; Table 1) when using Illumina-based RNA-seq. We also included _B. subtilis_ 6S RNA deletion strains in this analysis to verify the specific nature of pRNA reads. The parental _B. subtilis_ PY79 strain is termed wild type (wt), and the respective deletion strains are abbreviated as ΔbsrA (deletion of the 6S-1 RNA gene), ΔbsrB (deletion of the 6S-2 RNA gene),

| TABLE 1. Overview of the pRNA length species identified by RNA-seq in wild type (wt) and deletion/complementation strains ΔbsrA (ΔA), ΔbsrB (ΔB), ΔbsrAΔbsrB (ΔAB), ΔbsrAΔbsrBΔbsrA (ΔAB+A), and ΔbsrAΔbsrBΔbsrAΔbsrB (ΔAB+B) for 6S-1 RNA-derived pRNAs (upper part) and 6S-2 RNA-derived pRNAs (lower part). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| [nt] | /unif044 | 6S-1 pRNA | 6S-2 pRNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 22-23 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔB) | 6S-1 pRNA (ΔAB) | 6S-2 pRNA (ΔAB+A) | 6S-1 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 20-21 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 14-15 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 12-13 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 10-11 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 8-9 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 6-7 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 4-5 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|
| 2-3 | 6S-1 pRNA (ΔA) | 6S-2 pRNA (ΔAB) | 6S-1 pRNA (ΔAB+A) | 6S-2 pRNA (ΔAB+B) |
|---|---|---|---|---|---|

Note that the numbers of total reads in the last two rows are rounded, while the exact read numbers were used to calculate the numbers above bars in Figure 1; M, million; nt, nucleotide(s); ³-terminal A residues could not be unequivocally assigned to the respective pRNAs because poly(A)-tailing was used for library construction. (Exponential) RNA extracted from cells at OD₆₀₀ = 0.9; (stationary) RNA extracted from cells at OD₆₀₀ = 7 (before death phase).
ΔbsrAB (ΔbsrA/ΔbsrB, deletion of both 6S RNA genes), ΔbsrAB+A (bsrA reintegrated into the ΔbsrAB strain at the amyE locus under control of the native 6S-1 RNA gene promoter), and ΔbsrAB+B (bsrB reintegrated into the ΔbsrAB strain at the amyE locus under control of the native 6S-2 RNA gene promoter) (Hoch et al. 2015). 6S-2 RNA-derived pRNA reads were exclusively detected in strains expressing 6S-2 RNA (wt, ΔbsrA and ΔbsrAB+B); but not in a deletion of the bsrB gene (ΔbsrB and ΔbsrAB+; Table 1; Fig. 1A). This finding verified that the detected reads indeed represent genuine 6S-2 pRNAs. Based on the library from exponentially growing wt cells (Table 1, first column on the left), it appears that roughly equal numbers of 6S-1 and 6S-2 pRNAs are synthesized in this growth phase. Moreover, 6S-2 pRNAs were much more abundant in libraries of cellular RNA prepared from exponentially growing cells relative to RNA preparations from stationary phase cells. This is in line with 6S-2 RNA levels being higher in exponential than stationary phase and with the presence of low levels of the rivaling 6S-1 RNA during exponential growth (Ando et al. 2002; Barrick et al. 2005; Beckmann et al. 2011).

With respect to 6S-1 pRNA reads, substantial read numbers were also linked to the presence of the bsrA gene encoding 6S-1 RNA (Table 1). In line with our previous 454 RNA-seq analysis (Beckmann et al. 2011), pRNAs ≤12 nt prevailed and more reads were identified in libraries from stationary relative to exponential phase cells, consistent with 6S-1 RNA levels peaking toward stationary phase (Ando et al. 2002; Barrick et al. 2005; Beckmann et al. 2011). Interestingly, the fraction of 6S-1 RNA pRNAs shows very long transcripts (including runoffs in the case of 6S-2 pRNAs) was higher for 6S-2 than 6S-1 RNA, and this was even more pronounced for stationary cells. The lack of any 6S-1 pRNA runoffs (40-mers) may not necessarily indicate their lack of synthesis, as they might not have entered the cDNA libraries owing to resisting dissociation of 6S-1 RNA:pRNA hybrids because of increased duplex stability (~43% G/C of 6S-1 runoffs relative to ~29% G/C of 6S-2 runoffs).

Compared with the wt strain, more 6S-1 pRNA reads were detected in libraries from strains lacking 6S-2 RNA (illustrated in Fig. 1B). This difference gains even more weight when considering that the wt libraries had the highest total numbers of reads (Table 1). A similar but less pronounced and not fully consistent trend was seen for 6S-2 pRNA reads in bsrA deletion strains relative to the wt strain (Table 1; Fig. 1B; see Discussion).

6S-2 pRNAs are detectable by Northern blotting

In a preceding study (Beckmann et al. 2010) we succeeded in visualizing very small RNA species (~14 nt) by Northern blotting, combining (i) the highly efficient EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] crosslinking for immobilization of short RNAs on nylon membranes (Pall et al. 2007), (ii) 5’-digoxigenin-labeled

---

**FIGURE 1.** (A) cDNA reads mapping antisense to 6S-1 RNA (left) and 6S-2 RNA (right) visualized using the Integrated Genome Browser (1GB). (Red) No reads expected because the corresponding 6S RNA gene is deleted; (blue): reads expected due to the presence of the corresponding 6S RNA gene. 3’-terminal A residues could not be unequivocally assigned to the respective pRNAs because poly(A)-tagging was used for library construction; since these A residues were nevertheless included in the mapping, we have marked those positional coverages in light blue. RNAs extracted from the following strains were analyzed: wt, the parental _B. subtilis_ PY79 strain; ΔbsrA; ΔbsrB; ΔbsrAB (ΔbsrA/ΔbsrB); ΔbsrAB+A, bsrA reintegrated into the ΔbsrAB strain at the amyE locus under control of the native 6S-1 RNA gene promoter; ΔbsrAB+B, bsrB reintegrated into the ΔbsrAB strain at the amyE locus under control of the native 6S-2 RNA gene promoter; exp., RNA extracted from exponentially growing cells at OD_{600} = 0.9; stat., RNA extracted from stationary cells at OD_{600} = 7 (before death phase). For more details, see text. Canonical pRNAs were found in all strains expressing the respective 6S RNA, but not (or at least in negligible amounts) whenever the gene was deleted, indicating that these are actually 6S RNA-templated pRNA transcripts. (B) Normalized read levels (per 10 million reads ≥ 7 nt) of 6S-1 and 6S-2 RNA-derived pRNAs in exponential and stationary growth phase on a logarithmic scale. Example calculation to illustrate how we arrived at the read numbers: for “wt exponential, 6S-1 pRNAs” in Table 1, we had 21,086,349 sequence reads after filtering, which were rounded to 21.0 M in Table 1. We then divided the number of 6S-1 pRNA reads (2303) by 21,086,349 and multiplied with 10 M to normalize the pRNA reads to the total number of reads: 2303/21,086,349 × 10,000,000 = 1092.18 (rounded to 1092) reads per 10 M reads.
probes containing DNA and a few locked nucleic acid (LNA) (Vester and Wengel 2004) residues—which increase affinity for complementary sequences—and (iii) native PAA gel systems to avoid EDC reaction with the amino groups of the urea denaturant instead of the primary amino groups of the membrane. Although 6S-1 pRNAs were detected with this technique (Beckmann et al. 2010), a detection of 6S-2 pRNAs was unsuccessful so far (RK Hartmann, unpubl.). With the following modifications we were able to detect 6S-2 pRNAs (Fig. 2): We used RNA/LNA mixmer probes with a high LNA content (up to 10 out of 14 nt), a double digoxigenin label at the 5′- and 3′-end (to increase signal intensity), and applied the TRIzol RNA extraction method to enrich for small RNAs (Damm et al. 2015). In Northern blots of the type shown in Figure 2B, we detected signals for 6S-2 pRNAs in the range of ∼14–25 nt for RNA preparations from wt and ΔbsrA bacteria, but not for those originating from ΔbsrB and ΔbsrAB bacteria lacking the 6S-2 RNA gene. This indicates that the signals in lanes 1 and 2 of Figure 2B in the size range below ∼25 nt indeed represent genuine 6S-2 pRNAs. The main pRNA signals in lanes 1 and 2 in Figure 2B represent a higher length range compared with the pRNA length distribution in Table 1 (6S-2 pRNA reads for the wt and ΔbsrA strain in exponential phase). An explanation could be the decreasing Northern blot detection efficiency at decreasing pRNA length.

Detection of 6S-2 pRNAs by a primer extension assay

For detection of 6S-2 pRNAs in total cellular RNA, we developed a novel primer extension-based approach. The central idea was to detect 6S-2 pRNAs via their ability to function as RNA primers in a primer extension reaction (see scheme in Fig. 3A). For this purpose, we designed a DNA oligonucleotide complementary to 6S-2 pRNAs in its 3′-portion (the 3′-terminal DNA nucleotide matching the initiating pRNA nucleotide). All pRNA length variants up to the 41-nt runoff pRNA transcript could potentially anneal to this oligonucleotide. After addition of radiolabeled dNTPs and Avian Myeloblastosis Virus (AMV) reverse transcriptase, which

![Figure 2](https://www.rnajournal.org/617)

**FIGURE 2.** Detection of 6S-2 pRNAs by Northern blotting. (A) Secondary structure of *B. subtilis* 6S-2 RNA (Trotochaud and Wassarman 2005). The pRNA initiation site is indicated by the red arrow and the maximal (runoff) pRNA sequence by small blue letters along the 6S-2 RNA sequence. 6S RNA helix P2, disrupted at the beginning of pRNA synthesis, is marked in purple. CB, central bulge. The two RNA/LNA mixmer probes labeled with digoxigenin (DIG) at the 5′- and 3′-ends are depicted as red letters on top of the pRNA sequence; capital letters indicate LNA residues and lower case letters RNA residues of the probes. (B) Representative Northern blot experiment for the detection of 6S-2 pRNAs in total RNAs prepared by the TRIzol procedure from *B. subtilis* PY79 strains grown to mid-exponential phase (OD600 = 0.7). (Lanes 1–4) Total RNAs prepared from wild type (wt), ΔbsrA, ΔbsrB, and ΔbsrAB cells, respectively; (lane 5) a chemically synthesized 6S-1 pRNA 14-mer loaded as negative control; (lanes 6,7) chemically synthesized RNAs representing the first 14 nt (lane 6) or nt 17–39 (lane 7) of 6S-2 pRNAs, used as positive controls. The membrane was simultaneously incubated with both probes depicted at the top of A.
has the ability to extend an RNA primer annealed to a DNA template strand, all pRNA length species that hybridized to the template strand should be elongated until the polymerase falls off the template DNA at its 5'-end. Thereby, the highly diverse 6S-2 pRNA length species are channeled into one single elongation product which can be detected by autoradiography after denaturing PAGE.

Using this assay, four different total cellular RNA extracts were screened for the presence of 6S-2 pRNAs, including RNA isolated from the wt, ΔbsrA, ΔbsrB, and ΔbsrAB strains. A distinct primer extension product was exclusively observed with RNA fractions from the wt and ΔbsrA strains (Fig. 3B, lanes 1–4). The extension product had the same gel mobility (72 nt) as in lane 6, where we added a synthetic 6S-2 pRNA 14-mer, indicating that endogenous pRNAs indeed annealed to the 3'-portion of the DNA template oligonucleotide as illustrated in Figure 3A. As control, a synthetic 14-meric 6S-1 pRNA was used as RNA component. Since no extension product was detectable here (Figure 3B, lane 6), unspecific primer annealing could be ruled out.

To further support our conclusion that cellular 6S-2 pRNA primary transcripts with 5'-triphosphate ends are the priming RNA species, we performed the same experiments as shown in Figure 3B, but using cellular RNA extracts pretreated with terminator 5'-phosphate-dependent exonuclease (TEX) before primer extension. TEX specifically degrades RNAs carrying 5'-monophosphate ends (Sharma et al. 2010) and thus enriches for primary transcripts. This primer extension experiment (Fig. 3C) showed a similar result as the one in Figure 3B, yet even with a somewhat improved signal-to-background ratio. In conclusion, the primer extension assay provided the third evidence in support of 6S-2 pRNA synthesis in B. subtilis cells.

DISCUSSION

6S-2 RNA is a template for pRNA synthesis in exponentially growing cells

Using Illumina-based RNA-seq, we were able to identify substantial amounts of 6S-2 pRNA reads in RNA preparations from exponentially growing B. subtilis cells (Table 1; Fig. 1). Detection of these pRNA reads depended on the presence of the respective 6S-2 RNA gene, supporting their identity as genuine pRNA reads. This finding, additionally supported by two other independent approaches (Figs. 2, 3), clarifies a central question in the field, i.e., how B. subtilis σA-RNAP can escape from sequestration by 6S-2 RNA. Evidently, RNAP does so by 6S-2 RNA-directed pRNA transcription, which complements previous in vitro results demonstrating that all functional hallmarks of 6S RNAs also apply to 6S-2 RNA: The RNA (i) serves as a template for pRNA transcription, (ii) binds with similar affinity as 6S-1 RNA to σA-RNAP, (iii) is capable of competitively inhibiting transcription from DNA promoters, and (iv)
forms 6S RNA:pRNA complexes that (v) disrupt binding to $\sigma^A$-RNAP (Burenina et al. 2014).

Only very low numbers of 6S-2 pRNA reads were detected in RNA libraries from stationary cells. A reverse correlation was observed for 6S-1 pRNA reads that are more abundant in stationary phase libraries (Table 1; Fig. 1). These findings are basically in line with the differential expression profiles of the 6S-1 and 6S-2 template RNAs: 6S-2 RNA peaks in exponentially growing cells and tends to thin out toward stationary phase, whereas 6S-1 RNA accumulates in stationary phase, but has low expression levels during early exponential growth (Beckmann et al. 2011). We infer from the data in Table 1 and Figure 1 that more RNAP molecules form a complex with 6S-2 RNA in early exponential phase than in stationary phase where the levels of the competing 6S-1 RNA are particularly high.

The detection of only weak 6S-2 pRNA Northern blot signals in Figure 2B is not surprising, for the following reason: We previously obtained very weak Northern blot signals for 6S-1 pRNAs in late exponential/early stationary phase (OD$_{600} =$ 4–5; Beckmann et al. 2011), where 6S-1 RNA levels are much higher than in the RNA fractions used in Figure 2B (mid-exponential phase; OD$_{600} = 0.7$). Since roughly equal numbers of 6S-1 and 6S-2 pRNA reads were detected in RNA preparations from mid-exponential growth phase (Table 1, exponential phase, wt cells), we would not only expect very weak signals for 6S-1 pRNAs but also for 6S-2 pRNAs.

Based on Northern blots using T7 transcripts of 6S RNAs as quantitative standards, we recently estimated 6S-2 RNA levels to be roughly threefold higher than 6S-1 RNA levels during exponential phase (OD$_{600} =$ ~0.9; Hoch et al. 2015). In view of the RNA-seq data (Table 1) suggesting roughly equal numbers for 6S-1 and 6S-2 pRNAs for wt cells during exponential phase, this may be taken as evidence that $\sigma^A$-RNAP less actively synthesizes pRNAs on 6S-2 RNA compared with 6S-1 RNA. This would be in keeping with the finding that initiation of 6S-1 pRNA transcription with GTP is more efficient than initiation of 6S-2 pRNAs with ATP (Cabrera-Ostertag et al. 2013). One should note that all considerations on 6S-1 and 6S-2 pRNA synthesis discussed here are based on the detection of pRNA steady-state levels. At present, it is unclear whether 6S-1 and 6S-2 pRNA are degraded at different rates.

The current picture is that 6S-1 and 6S-2 pRNA levels are low but measurable in exponential phase. 6S-1 pRNA levels increase and 6S-2 pRNA levels decrease when cells enter stationary phase (Table 1; Fig. 1). 6S-1 pRNA levels accumulate during extended stationary phase (death phase when optical density decreases; Beckmann et al. 2011), where 6S-2 pRNA levels are low. During early outgrowth from stationary phase, there is a burst of 6S-1 pRNA synthesis (Beckmann et al. 2011; Cavanagh et al. 2012) that largely exceeds 6S-2 pRNA synthesis at any of the conditions analyzed, in line with the notion that 6S-1 RNA is more intensely utilized as transcription template by _B. subtilis_ $\sigma^A$-RNAP than 6S-2 RNA. In view of the burst of 6S-1 pRNA synthesis during outgrowth, 6S-1 RNA may also be considered as a more dynamic regulator of RNAP function than 6S-2 RNA, at least as long as no specific conditions (e.g., stresses) are known at which 6S-2 RNA might give rise to a similar burst of pRNA synthesis. It also appears that the regulatory function of 6S-1 RNA is specifically in growth adaptation, while 6S-2 RNA may impact on other cellular functions yet to be pinpointed. Another issue is whether 6S-1 and 6S-2 RNAs have differential capacities to bind to RNAP holoenzymes with other sigma factors than $\sigma^A$. Such a capacity could be largely excluded for _E. coli_ 6S RNA where specific 6S RNA binding was exclusively observed with $\sigma^{70}$-RNAP, but neither with $\sigma^S$ or $\sigma^{32}$ holoenzymes nor with core RNAP or $\sigma^{70}$ alone (Trototchaud and Wassarman 2005). However, this specificity issue needs to be investigated for the _B. subtilis_ system.

While a 6S-2 RNA deletion resulted in no measurable growth phenotype under standard laboratory conditions (Cavanagh et al. 2012), or only in a minor phenotype under alkaline stress conditions (Hoch et al. 2015), this does not necessarily imply that 6S-2 RNA is functionally silent. We observed that deletion of the 6S-2 RNA gene leads to an up-regulation of numerous proteins involved in metabolism and stress responses, and for exponentially growing cells, this effect was stronger for a 6S-2 than a 6S-1 RNA deletion (Hoch et al. 2015). Several of the identified proteins were also up-regulated in the 6S-1 RNA deletion strain (in stationary phase), suggesting that there is functional overlap between the two 6S RNAs.

**Differences in 6S-1 and 6S-2 pRNA length patterns and mechanistic implication**

6S-1 pRNAs are more G/C-rich than 6S-2 pRNAs. As a consequence, 6S RNA:pRNA hybrid structures are estimated to be stable enough to cause RNAP release when 6S-1 pRNAs have a length of $\geq 13$ nt, whereas 6S-2 pRNAs have to be $\sim$20 nt in length to attain the same hybrid stability (Burenina et al. 2014). This may imply that essentially only the longest 6S-2 pRNAs that are close in length to 6S-1 RNA may also be considered as a more dynamic regulator of RNAP function than 6S-2 RNA, at least as long as no specific conditions (e.g., stresses) are known at which 6S-2 RNA might give rise to a similar burst of pRNA synthesis. It also appears that the regulatory function of 6S-1 RNA is specifically in growth adaptation, while 6S-2 RNA may impact on other cellular functions yet to be pinpointed. Another issue is whether 6S-1 and 6S-2 RNAs have differential capacities to bind to RNAP holoenzymes with other sigma factors than $\sigma^A$. Such a capacity could be largely excluded for _E. coli_ 6S RNA where specific 6S RNA binding was exclusively observed with $\sigma^{70}$-RNAP, but neither with $\sigma^S$ or $\sigma^{32}$ holoenzymes nor with core RNAP or $\sigma^{70}$ alone (Trototchaud and Wassarman 2005). However, this specificity issue needs to be investigated for the _B. subtilis_ system.

While a 6S-2 RNA deletion resulted in no measurable growth phenotype under standard laboratory conditions (Cavanagh et al. 2012), or only in a minor phenotype under alkaline stress conditions (Hoch et al. 2015), this does not necessarily imply that 6S-2 RNA is functionally silent. We observed that deletion of the 6S-2 RNA gene leads to an up-regulation of numerous proteins involved in metabolism and stress responses, and for exponentially growing cells, this effect was stronger for a 6S-2 than a 6S-1 RNA deletion (Hoch et al. 2015). Several of the identified proteins were also up-regulated in the 6S-1 RNA deletion strain (in stationary phase), suggesting that there is functional overlap between the two 6S RNAs.
region upon pRNA synthesis give rise to longer pRNAs. For example, a *B. subtilis* mutant 6S-1 RNA with a destabilized hairpin in the 3′-CB and thus increased conformational flexibility, had a lower affinity for σ^A^RNAP and yielded a pRNA length pattern shifted to longer transcripts relative to the wt 6S-1 RNA (Beckmann et al. 2012). Likewise, in the *Escherichia coli* system, a 6S RNA mutant unable to form the extended hairpin in the 3′-CB upon pRNA synthesis yielded pRNAs of 14 and 18–28 nt in length compared to 9- and 13-mers obtained with the wt 6S RNA (Panchapakesan and Umrau 2012). Finally, for *B. subtilis* 6S-2 RNA, formation of a central bulge collapse (CBC) helix upon pRNA-induced disruption of helix P2 is less stable and thus less favored than in the case of 6S-1 RNA (Beckmann et al. 2012).

**Potential interplay of 6S-1 and 6S-2 RNA**

The RNA-seq results indicate that the expression of 6S-1 pRNAs increases in the absence of 6S-2 RNA (Fig. 1B, left, strains ΔbssB and ΔbsrBΔ+A). In exponential phase, 18- to 35-fold more 6S-1 pRNA reads were detected than in the wt strain (blue bars in Fig. 1B, left part). For RNA samples from stationary phase cells this difference further increased to 24- to 92-fold even (red bars in Fig. 1B, left part). This observation suggested an influence of the presence of 6S-2 RNA on the utilization of 6S-1 RNA as template for pRNA transcription. We considered the possibility that the less efficient pRNA transcription on 6S-2 RNA, resulting in more effective sequestration of RNAP by this RNA, may normally restrict 6S-1 RNA access to RNAP and thus 6S-1 RNA utilization as a template for pRNA transcription. To validate this trend seen in the RNA-seq data, we performed 6S-1 pRNA-specific Northern blot analyses using total RNA extracted from the wt and mutant strains. However, with about 10 independent RNA extracts prepared from wt and ΔbsrB bacteria in parallel, either using the hot phenol or TRIzol method (Damm et al. 2015), no consistent results were obtained. With RNA prepared by the hot phenol method (as done in the RNA-seq experiment), we could see higher pRNA levels for the ΔbsrB relative to the wt library in most experiments, but not all. This was also observed for total RNAs prepared by the TRIzol method enriching for small RNAs, but here only in less than half of the preparations. On the other hand, in neither RNA preparation we saw a more intense 6S-1 pRNA signal for RNA prepared from the wt compared with the ΔbsrB strain. One possibility is that the transcriptomes of ΔbsrB cell populations are less stable than those of the wt strain and often (but not always) undergo a shift manifesting as increased 6S-1 pRNA levels.

**Concluding remarks**

The majority of bacteria encode a single 6S RNA which, according to present knowledge, acts as a growth phase-dependent global regulator of the housekeeping RNA polymerase holoenzyme. Surprisingly, a minority of bacteria, mainly those belonging to the firmicutes but also some others like the γ-proteobacterium *Legionella pneumophila*, express at least two different 6S RNAs (for review, see Wehner et al. 2014; Burenina et al. 2015). In the case of *B. subtilis*—the major model system for the study of bacteria expressing two 6S RNA paralogs—the available data suggest differential as well as overlapping roles of 6S-1 and 6S-2 RNAs (Cavanagh et al. 2012, 2013; Cabrera-Ostertag et al. 2013; Hoch et al. 2015). Further investigations are required to better understand the biological reasons why a second 6S RNA paralog with a different expression profile has evolved in *B. subtilis* and related bacteria. This concerns the question whether 6S-2 RNA may have specialized functions under so far unidentified environmental conditions or if the RNA may affect physiological conditions that are relevant to the survival of real wild-type strains rather than the so-called laboratory “wild-type” strains, such as *B. subtilis* 168 or PY79. Regardless of such possibilities, we have added here an important mechanistic piece of the jigsaw by demonstrating the ability of 6S-2 RNA to direct pRNA synthesis in vivo as a prerequisite to release σ^A^RNAP from its transcription block. This feature of 6S-2 RNA qualifies the RNA as an authentic 6S RNA and disfavors possible scenarios according to which 6S-2 RNA simply sequesters σ^A^RNAP as a static inhibitor that may only be removed from RNAP by competing RNAP ligands or by RNases attacking the RNA when bound to the RNAP holoenzyme. The capacity of 6S-2 RNA to direct pRNA synthesis in vivo as a means to release RNAP equips the second 6S RNA paralog with an important dynamic functional feature specific to this type of riboregulator.

**MATERIALS AND METHODS**

**Strains and cell growth**

For total RNA isolation, exponential phase cells were prepared as follows. Single colonies were picked from LB agar plates (containing the appropriate antibiotic for selection of mutant strains) to inoculate 3 mL of lysogeny broth (LB) (Bertani 1951). Cultures were grown overnight at 37°C, 220 rpm, in a shaking (warm air) incubator (GFL 3033). Of note, 1 mL of these overnight cultures was used to inoculate 300 mL LB medium in a 1000-mL baffled Erlenmeyer flask. Cell growth was performed at 37°C at 180 rpm in a water bath shaker (Infors AG, Infors HT, Aquatron). Growth was monitored until an OD600 between 0.6 and 0.9 (exponential growth phase) was reached. Cells were harvested by centrifugation at 4°C and 4000 rpm for 15–20 min and the cell pellets were snap frozen in liquid nitrogen and stored at ~80°C until further use. For the construction and phenotype of the *B. subtilis* PY79 6S RNA deletion strains, see Hoch et al. (2015).

**Isolation of total RNA from Bacillus subtilis cells**

Cellular total RNA used for deep sequencing (RNA-seq) was isolated using the hot phenol method (Mattatall and Sanderson 1996;
Beckmann et al. 2010). Cellular total RNA for Northern blotting and primer extension was prepared using the TRIzol (Ambion) method. For this purpose, pelleted B. subtilis cells were resuspended in an appropriate amount of TRIzol reagent (for a pellet deriving from 50 mL LB culture, 4 mL TRIzol were used) by vortexing and incubated for 5 min on ice, followed by the procedure described in Damm et al. (2015). The purity of the RNA preparation was determined by UV spectroscopy. If the 260/280 nm absorbance ratio of the RNA (dissolved in 20–100 µL ddH2O) was below 1.7, the RNA was precipitated again using isopropanol. The quality of the RNA preparation was analyzed by 5% denaturing (8 M urea) PAGE. For RNA quality, see Supplemental Figure S2.

RNA-seq procedure
Total cellular RNA was first enriched for RNAs smaller than 200 nt using the mirVana miRNA isolation Kit (Ambion) according to the protocol supplied by the manufacturer. Then the library was enriched for primary transcripts by treatment with terminator 5′-phosphate-dependent exonuclease (TEX; Biozym/Epicentre), which specifically degrades RNAs carrying a 5′-monophosphate, but leaves primary transcripts carrying a triphosphate at their 5′-end intact. Subsequently, a 50-nt internal size marker was added to the RNA, followed by preparative 10% denaturing PAGE. RNAs with a length below 50 nt were extracted from the gel by electro-elution, precipitated with ethanol and dissolved in RNase-free water. RNAs were subsequently treated with Tobacco Acid Pyrophosphatase (TAP, Epicentre) to convert 5′-triphosphates to 5′-monophosphates for linker ligation. Thereafter, RNA samples were poly(A)-tailed using poly(A) polymerase, and the 5′-adapter oligonucleotide was ligated to the 5′-monophosphate end. Next, first-strand cDNA synthesis was performed using an oligo(dT) primer and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV reverse transcriptase). The generated cDNA was amplified by PCR with 17–20 cycles until a DNA yield of ~20–30 ng/µL was reached. The PCR reaction mixtures containing the cDNA libraries were purified using the Agencourt AMPure XP Kit (Beckmann Coulter Genomics) and pooled. Library construction was carried out at vertis Biotechnologie AG (Freising, Germany). The sequencing reaction was conducted using an Illumina sequencer. Sequencing reads were cropped at the first poly(A) stretch (at least six consecutive A residues) and assigned to the respective 6S-1 or 6S-2 pRNA length species whenever the sequence matched perfectly. Given the very short length of the primary transcripts carrying a triphosphate at their 5′-end intact.

For elongation of pRNAs by AMV reverse transcriptase, a chemically synthesized DNA oligonucleotide (5′-GACGCAGTGACACCAATG

Enrichment for primary transcripts prior to primer extension
For removal of processed and/or degraded RNAs, the cellular RNA extracts were treated with terminator 5′-phosphate-dependent exonuclease (TEX; Biozym/Epicentre). TEX specifically degrades RNAs carrying a 5′-monophosphate, while RNAs with a 5′-triphosphate, a 5′-cap structure, or a 5′-hydroxyl group are not affected. The TEX treatment was performed according to the protocol recommended by the supplier (Epicentre), with minor changes. In brief, 2 µL of Terminator 10× reaction buffer A (Biozym/Epicentre) were added to 10 µg of total cellular RNA in a 0.2 µL tube and supplemented with ddH2O to a volume of 15 µL. Then 5 µL of TEX (1 U/µL) were added and the reaction mixture was incubated for 90 min at 30°C in a thermocycler with a heated lid (50°C) to avoid condensation. Thereafter, the reaction mixture was adjusted to a volume of 200 µL with ddH2O, transferred to a 1.5-ml tube, and the reaction was terminated by addition of 1 volume of acidic phenol. The mixture was vortexed thoroughly and centrifuged for 5 min at maximum speed in a tabletop centrifuge (20,000g) for phase separation. The aqueous phase was transferred to a new tube and the RNA was precipitated for at least 30 min with 1 volume of isopropanol at −20°C. After centrifugation at 17,000g at 4°C for at least 30 min, the supernatant was discarded and the RNA pellet was washed with 75% ice-cold ethanol. The RNA pellet was dried for 5 min in a vacuum centrifuge at 35°C and resuspended in 5 µL ddH2O.

Primer extension using 6S-2 RNA-derived pRNAs as primers
For elongation of pRNAs by AMV reverse transcriptase, a chemically synthesized DNA oligonucleotide (5′-GACGCAGTGACACCAATG...
TACTAGTAGGGTCgaagctactttgtgcgtattgttaatt; small letters indicate complementarity to 6S-2 pRNAs, underlined nucleotides indicate the annealing region of the 6S-2 pRNA 14-mer, whose 3’-terminal nucleotide matched the 5’-terminal 6S-2 pRNA nucleotide, such that all 6S-2 pRNA 3’-length variants were able to anneal to the DNA template in the same register. A template DNA oligonucleotide with three mutations in the 3’-terminal part (...taagAttTaccAtt-3’) was used as a mismatch control. For 6S-2 pRNA extension, 2.3 µg total cellular RNA prepared by the TRIzol method were mixed with the DNA template (f.c. 0.1 µM), α-32P-dNTPs (f.c. 83 nM each, 3000 Ci/mmol; Hartmann Analytic), RNA were used: a 6S-1 pRNA 14-mer (5′-GUUCCGUCAAAA CU-3′) or a 6S-2 pRNA 14-mer (5′-AAAGGUTAAACLU-3′) (both from Integrated DNA Technologies). Samples were finally mixed with 1 volume of 2× denaturing loading buffer (see above) and heated to 95°C for 3 min. For the analysis of extension products, samples were subjected to denaturing 15% PAGE and visualized by autoradiography for 0.5–2 h using a Fuji FLA-300 R Phosphorimagertm (Fujifilm).

SUPPLEMENTAL MATERIAL
Supplemental material is available for this article.

ACKNOWLEDGMENTS
This project was funded by the Deutsche Forschungsgemeinschaft (DFG) (SPP 1258 and IRTG 1384) to R.K.H.

Received December 9, 2015; accepted January 14, 2016.

REFERENCES
Ando Y, Asari S, Suzuma S, Yamane K, Nakamura K. 2002. Expression of a small RNA, BS203 RNA, from the ycf1-ycf2 intergenic region of Bacillus subtilis genome. FEMS Microbial Lett 207: 29–33.
Barrick JE, Sudarsan N, Weinberg Z, Ruzzo WL, Breaker RR. 2005. 6S RNA is a widespread regulator of eubacterial RNA polymerase that triggers 6S-1 RNA release from RNA polymerase in Bacillus subtilis. RNA Biol 11: 774–784.

Bockmann BM, Grünweller A, Weber MH, Hartmann RK. 2010. Northern blot detection of endogenous small RNAs (~14 nt) in bacterial total RNA extracts. Nucleic Acids Res 38: e147.

Bockmann BM, Bureinina OY, Hoch PG, Kubareva EA, Sharma CM, Hartmann RK. 2011. In vivo and in vitro analysis of 6S RNA-templated short transcripts in Bacillus subtilis. RNA Biol 8: 839–849.

Bockmann BM, Hoch PG, Marz M, Willkomm DK, Salas M, Hartmann RK. 2012. A pRNA-induced structural rearrangement triggers 6S-1 RNA release from RNA polymerase in Bacillus subtilis. EMBO J 31: 1727–1738.

Bertani G. 1951. Studies on lysogeny. I. The mode of phage liberation by lysogenic Escherichia coli. J Bacteriol 62: 293–300.

Bureinina OY, Hoch PG, Damm K, Salas M, Zatsepin TS, Lechner M, Oretskaya TS, Kubareva EA, Hartmann RK. 2014. Mechanistic comparison of Bacillus subtilis 6S-1 and 6S-2 RNAs—commonalities and differences. RNA 20: 348–359.

Bureinina OY, Elkina DA, Hartmann RK, Oretskaya TS, Kubareva EA. 2015. Small noncoding 6S RNAs of bacteria. Biochemistry (Mosc) 80: 1429–1446.

Cabrera-Ostertag II, Cavanagh AT, Wassarman KM. 2013. Initiating nucleotide identity determines efficiency of RNA synthesis from 6S RNA templates in Bacillus subtilis but not Escherichia coli. Nucleic Acids Res 41: 7901–7911.

Cavanagh AT, Wassarman KM. 2013. 6S-1 RNA function leads to a delay in sporulation in Bacillus subtilis. J Bacteriol 195: 2079–2086.

Cavanagh AT, Spurger JM, Wassarman KM. 2012. Regulation of 6S RNA by pRNA synthesis is required for efficient recovery from stationary phase in E. coli and B. subtilis. Nucleic Acids Res 40: 2234–2246.

Damm K, Bach S, Müller KMH, Klug G, Bureinina OY, Kubareva EA, Grünweller A, Hartmann RK. 2015. The impact of RNA isolation protocols on RNA detection by northern blotting. Methods Mol Biol 1296: 29–38.

Hoch PG, Bureinina OY, Weber MHW, Elkina DA, Nesterchuk PVS, Hartmann RK, Kubareva EA. 2015. Phenotypic characterization and complementation analysis of Bacillus subtilis 6S RNA single and double deletion mutants. Biochimie 117: 87–99.

Mattatall NR, Sanderson KE. 1996. Salmonella typhimurium LT2 possesses three distinct 23S RNA intervening sequences. J Bacteriol 178: 2272–2278.

Pall GS, Godony-Servat C, Byrne J, Ritchie L, Hamilton A. 2007. Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35: e60.

Panchapakesan SS, Unrau PJ. 2012. E. coli 6S RNA release from RNA polymerase requires σ70 ejection by scrunching and is orchestrated by a conserved RNA hairpin. RNA 18: 2251–2259.

Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R, et al. 2010. The primary transcriptome of the major human pathogen Helicobacter pylori. Nature 464: 250–255.

Steuten B, Hoch PG, Damm K, Schneider S, Köhler K, Wagner R, Hartmann RK. 2014. Regulation of transcription by 6S RNAs: insights from the Escherichia coli and Bacillus subtilis model systems. RNA Biol 11: 1–14.

Suzuma S, Asari S, Bunai K, Yoshino K, Ando Y, Kakeshita H, Fujita M, Nakamura K, Yamane K. 2002. Identification and characterization of novel small RNAs in the aspS-yrvM intergenic region of Bacillus subtilis genome. Microbiology 148: 2591–2598.

Trotchaud AE, Wassarman KM. 2005. A highly conserved 6S RNA structure is required for regulation of transcription. Nat Struct Mol Biol 12: 313–319.

Vester B, Wengel J. 2004. LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA. Biochemistry 43: 13233–13241.

Wassarman KM. 2002. Small RNAs in bacteria: diverse regulators of gene expression in response to environmental changes. Cell 109: 141–144.

Wassarman KM, Saecker RM. 2006. Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. Science 314: 1601–1603.

Wassarman KM, Storz G. 2000. 6S RNA regulates E. coli RNA polymerase activity. Cell 101: 613–623.

Wehner S, Damm K, Hartmann RK, Marz M. 2014. Dissemination of 6S RNA among bacteria. RNA Biol 11: 1467–1478.