6S-1 RNA Function Leads to a Delay in Sporulation in *Bacillus subtilis*

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We have discovered that 6S-1 RNA (encoded by *bsrA*) is important for appropriate timing of sporulation in *Bacillus subtilis* in that cells lacking 6S-1 RNA sporulate earlier than wild-type cells. The time to generate a mature spore once the decision to sporulate has been made is unaffected by 6S-1 RNA, and, therefore, we propose that it is the timing of onset of sporulation that is altered. Interestingly, the presence of cells lacking 6S-1 RNA in coculture leads to all cell types exhibiting an early-sporulation phenotype. We propose that cells lacking 6S-1 RNA modify their environment in a manner that promotes early sporulation. In support of this model, resuspension of wild-type cells in conditioned medium from Δ *bsrA* cultures also resulted in early sporulation. Use of *Escherichia coli* growth as a reporter of the nutritional status of conditioned media suggested that *B. subtilis* cells lacking 6S-1 RNA reduce the nutrient content of their environment earlier than wild-type cells. Several pathways known to impact the timing of sporulation, such as the *skf*- and *sdp*-dependent cannibalism pathways, were eliminated as potential targets of 6S-1 RNA-mediated changes, suggesting that 6S-1 RNA activity defines a novel mechanism for altering the timing of onset of sporulation. In addition, 6S-2 RNA does not influence the timing of sporulation, providing further evidence of the independent influences of these two related RNAs on cell physiology.

Bacillus subtilis has exquisitely controlled responses to diverse environmental conditions that maximize flexibility in growth rate and survival. For example, in both exponential-growth and stationary phases, the growth rate can change rapidly with nutrient quality and availability, primarily as a result of changes in gene expression. However, upon persistent nutrient limitation *B. subtilis* can initiate more dramatic developmental programs such as competence (1, 2), matrix formation (3, 4), cannibalism (5, 6), and endospore formation (7, 8). These developmental processes allow increased individual cell survival during more extreme nutritional and environmental conditions that would be lethal to vegetative cells (9).

The decision for cells to enter the sporulation pathway, as well as the process of spore formation, is very carefully regulated (see references 7, 8, 10, and 11 for reviews). An endospore is a metabolically quiescent cell within a highly protective spore coat. The spore remains viable indefinitely and has very high resistance to environmental assaults and starvation. Sporulation can result from carbon, nitrogen, or, in some instances, phosphorus starvation, and how spores are formed and what regulates this developmental process have been characterized in great detail. For instance, the master regulator, Spo0A, directly and indirectly regulates hundreds of genes. The action of Spo0A, along with many other factors, including σ^H , leads to an asymmetric cell division that forms a large mother cell and a smaller prespore. Subsequent steps include migration of the membrane around the prespore in a process called engulfment and finally mother cell lysis to free the fully mature spore.

Despite the extreme resistance of spores to diverse environmental assaults, spore formation has its disadvantages as well. Sporulation is energy intensive and must commence while sufficient resources remain to complete the process. Once the decision to sporulate has been made, it takes ~7 h for a spore to form. During this time, the cell is unable to take advantage of any subsequent environmental changes (7, 12). In addition, for spores to reenter growth they must germinate first, a process that requires time before the robust growth necessary to ensure that continued

survival is attained (13, 14). Therefore, *B. subtilis* has evolved intricate mechanisms to promote spore formation when it is advantageous but also to prevent sporulation when other options for survival are sufficient.

6S RNA is a small noncoding RNA conserved in a broad range of bacteria. In *Escherichia coli*, it is important for long-term survival during nutrient-poor conditions and modulation of cellular responses to environmental stress (see reference 15 for a review). *E. coli* 6S RNA functions through interaction with the housekeeping form of RNA polymerase holoenzyme ($E\sigma^{70}$) (16, 17, 18). This RNA regulates transcription in a promoter-specific manner, mediating changes in expression of hundreds of genes during stationary phase (19, 20, 21). Stationary-phase cells exposed to an upshift in nutrients respond very rapidly by increasing transcription to promote an increase in gene products necessary to reenter rapid growth (22). Surprisingly, when cells experience an upshift of nutrients, such as during outgrowth, $E\sigma^{70}$ uses the 6S RNA as a template to produce a product RNA (pRNA) (18, 23). It is this pRNA synthesis reaction that leads to the release of $E\sigma^{70}$ from 6S RNA (23, 24), which is required for efficient outgrowth of stationary-phase *E. coli* cells (25).

Interestingly, *B. subtilis* has two 6S RNAs that were first sequenced as abundant RNAs of unknown function (26, 27) and later identified as 6S RNAs based on their coimmunoprecipitation with RNA polymerase and on their secondary structure similarity to the *E. coli* 6S RNA (17, 28). The *B. subtilis* 6S-1 and 6S-2 RNAs each bind to the housekeeping form of RNA polymerase ($E\sigma^A$),

Received 29 January 2013 Accepted 25 February 2013

Published ahead of print 1 March 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00050-13>.

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doi:10.1128/JB.00050-13

TABLE 1 *B. subtilis* strains

Strain	Description	Source
KW586	<i>Bacillus subtilis</i> 168 (wild type); 1A1	Bacillus Genetic Stock Center
KW587	$\Delta bsrA::spc$	25
KW588	$\Delta bsrA::tet$	25
KW589	$\Delta bsrB::spc$	25
KW590	$\Delta bsrA::tet \Delta bsrB::spc$	25
KW593	<i>amyE::cat</i>	This work
KW594	$\Delta bsrA::spc amyE::cat$	This work
KW595	<i>amyE::erm</i>	This work
KW596	$\Delta bsrA::spc amyE::erm$	This work
KW598	$\Delta skf::cat$	This work
KW599	$\Delta bsrA::spc \Delta skf::cat$	This work
KW600	$\Delta sdg::cat$	This work
KW601	$\Delta bsrA::spc \Delta sdg::cat$	This work
KW602	$\Delta sda::cat$	This work
KW603	$\Delta bsrA::spc \Delta sda::cat$	This work
KW604	$\Delta kipI::cat$	This work
KW605	$\Delta bsrA::spc \Delta kipI::cat$	This work

analogously to *E. coli* 6S RNA binding to σ^{70} (17). However, 6S-1 and 6S-2 RNAs have distinct characteristics as well. In contrast to their secondary structures, their primary sequences are not well conserved. The two RNAs are present at different levels through growth; 6S-1 RNA accumulates during stationary phase, while 6S-2 RNA levels remain fairly constant under tested conditions (17, 26, 27, 28). Interestingly, 6S-1 and 6S-2 RNAs also have distinct biochemical properties; 6S-1 RNA is an efficient template for pRNA synthesis, while 6S-2 RNA is not (25, 29, 30). Finally, it is clear that these two RNAs have independent biological functions as demonstrated by the behaviors of cells lacking either RNA. For example, stationary-phase cells lacking 6S-1 RNA are delayed in their ability to restart growth upon nutrient upshift, while cells lacking 6S-2 RNA are not (25).

Bacillus subtilis utilizes different pathways to respond to nutrient deprivation that are distinct from the long-term stationary phase of *E. coli*. Therefore, we sought to understand if and how the *B. subtilis* 6S RNAs were integrated into starvation response(s) in this divergent organism. Specifically, we tested whether either or both of the *B. subtilis* 6S RNAs had a role in influencing endospore formation. Here we demonstrate that 6S-1 RNA function is important for appropriate timing of the onset of endospore formation, as cells lacking 6S-1 RNA initiate sporulation at earlier times than wild-type *B. subtilis* cells. This change in timing is mediated by environmental conditions that result during growth of cells containing 6S-1 RNA. Interestingly, 6S-2 RNA is not required for wild-type levels and timing of sporulation, thereby providing additional support for the conclusion that 6S-1 and 6S-2 RNAs provide unique and nonredundant functions in *B. subtilis* cells.

MATERIALS AND METHODS

Strains and growth. *B. subtilis* strains (Table 1) were all generated in *B. subtilis* 168 and grown in 2×YT medium (31), Difco sporulation medium (DSM) (32), or S7 medium (32) as indicated. The onset of stationary phase was defined as the point during a growth curve when cell density reached a maximum level as measured by no further increase in optical density at 600 nm (OD₆₀₀) or in CFU counts (assayed by plating). For the decoyinine experiment (Fig. 1B), cells were grown to an OD₆₀₀ of 0.5 in S7 medium followed by the addition of decoyinine to 0.4 mg/ml as previ-

ously described (33, 34). *E. coli* K-12 (KW72) was grown in LB (Lennox broth) (31) or conditioned medium (CM) (see below), as indicated.

B. subtilis $\Delta bsrA$ and $\Delta bsrB$ alleles have the chromosomal *bsrA* or *bsrB* genes precisely replaced by a spectinomycin resistance cassette (KW587 or KW589) or a tetracycline resistance cassette (KW588) (25).

B. subtilis *amyE::cat* and *amyE::erm* alleles have antibiotic resistance cassettes inserted into the *amyE* locus. For construction of modified pDG1661 plasmids, antibiotic resistance cassettes were amplified and ligated into MfeI-BsiWI-digested pDG1661 (35), resulting in the *cat* or *erm* genes flanked by *amyE* upstream and downstream sequences. Plasmids were transformed into *bsrA*⁺ cells (KW586) and recombinants identified as those that were resistant to chloramphenicol or erythromycin and sensitive to spectinomycin. $\Delta bsrA::spc$ was introduced into these cells as needed by transformation and selection for spectinomycin resistance as previously described (25). For oligonucleotide sequences, see Table S1 in the supplemental material.

FIG 1 *B. subtilis* cells lacking 6S-1 RNA initiate sporulation earlier than wild-type cells. (A) Percentage of spores relative to total cells in cultures of wild-type *B. subtilis* 168 (KW586) or the $\Delta bsrA::spc$ strain (KW587) grown at 37°C in DSM over time. Time shown is hours after the onset of stationary phase (see Materials and Methods for determination of timing). (B) Percentage of spores in cultures of wild-type (KW586) or $\Delta bsrA::spc$ (KW587) cells after the addition of 0.4 mg/ml decoyinine to vegetative cell cultures grown in S7 medium. Time shown is hours after addition of decoyinine. For each, data shown are from one representative experiment with three biological replicates. Similar results were seen in three independent experiments. Error bars correspond to standard deviations from the averages. Values that were statistically different in wild-type and $\Delta bsrA$ cells as tested by Student's *t* test: *, *P* < 0.05; **, *P* < 0.005.

B. subtilis $\Delta skf::cat$, $\Delta sdp::cat$, $\Delta sda::cat$, and $\Delta kipI::cat$ alleles have the chromosomal *skf* or *sdp* operons or *sda* or *kipI* genes precisely replaced by a chloramphenicol resistance cassette. Strain construction was essentially as described previously (36). Specifically, modified pMAD plasmids were made that contained *cat* flanked by upstream and downstream regions for each gene of interest. These upstream and downstream regions, as well as the *cat* gene, were generated by PCR (for oligonucleotides, see Table S1 in the supplemental material), followed by sequential ligation into the BamHI and EcoRI sites in pMAD (37). Plasmids were transformed into *bsrA*⁺ cells (KW586) and recombinants identified as those that were resistant to chloramphenicol and sensitive to erythromycin. $\Delta bsrA::spc$ was introduced into these cells as needed by transformation and selection for spectinomycin resistance as previously described (25).

Quantitation of spores in cultures. Vegetative cells are sensitive to heat treatment, while spores are highly resistant, making it possible to assay the percentage of spores in a culture as heat-resistant cells (32). Basically, samples from the indicated times during growth were divided, and half of each sample was heated to 80°C for 20 min while the remainder was untreated. CFU counts of each half were determined by serial dilution in 1×M9 salts (31), plating onto 2×YT or LB, and counting colonies after 18 to 24 h at 37°C. The fraction of spores was determined by the formula (CFU/ml heated sample)/(CFU/ml untreated sample). At least three independent cultures per strain were used for each experiment, and all experiments were repeated at least three times.

qPCR. RNA was isolated from cells using the RNeasy minikit (Qiagen) as directed by the manufacturer except for additional cell lysis steps. Specifically, cells were resuspended in 100 μ l of 10 mM Tris, pH 8, 1 mM EDTA, 10 mg/ml lysozyme, vortexed with approximately equal volume of glass beads (~ 0.1 nm; Thomas Scientific) for 30 s, incubated for 10 min at room temperature, and centrifuged briefly to pellet the glass beads. The supernatant was transferred to a clean microcentrifuge tube before proceeding with the standard protocol for RNA isolation. cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) and 500 ng of total RNA. Quantitative PCR (qPCR) was done using a QuantiTect Sybr green PCR kit (Qiagen) in an ABI 7500 real-time PCR system using 0.1, 1, or 10 ng of cDNA and 0.3 μ M oligonucleotides. Data shown in Fig. 2 used 1 ng cDNA. Cycle thresholds (C_T) were determined by ABI 7500 SDS v1.4, and the data are expressed as $2^{(\Delta\Delta CT)}$ to represent the change in level over time relative to $t = 0$. For oligonucleotide sequences, see Table S1 in the supplemental material. At least two independent RNA preparations per time point were analyzed, and each RNA preparation was examined in triplicate.

Growth in coculture. Marked strains were grown independently overnight in 2×YT. The cell density of each culture was measured by OD₆₀₀ (and verified by plating), and equal numbers of each cell type were added to cocultures in DSM. The resulting dilution was $\sim 1:100$ into DSM. Cultures were grown at 37°C for times indicated, and the extent of sporulation was measured as the fraction of heat-resistant cells. Plating was done on LB with and without appropriate antibiotics to determine the total number of spores and the number from each genotype.

Conditioned medium experiments. For conditioned medium to test *B. subtilis* sporulation (Fig. 4), cells were grown overnight in 2×YT followed by 1:100 dilution into DSM and growth for 12 h after the onset of stationary phase (~ 20 -h total growth time in DSM). For CM generated to test nutritional status earlier in growth (Fig. 6), cells were grown for 8 h in DSM (i.e., approximately at the onset of stationary phase). To generate CM, cultures were centrifuged at $5,000 \times g$ for 10 min, and the supernatant was passed through a 0.22- μ m-pore-size filter to remove all cells. To test the behavior of *B. subtilis* in CM, vegetative cell cultures were generated by diluting overnight cultures 1:100 into fresh 2×YT followed by growth to an OD₆₀₀ approximately equal to 0.25. These vegetative cultures were diluted 1:10 in CM, and spore formation was monitored by plating. For *E. coli* growth, cultures were grown overnight in LB and diluted into CM or LB, and growth at 37°C was monitored as OD₅₉₅ in an absorbance microplate reader (ELx808; Biotek Instruments).

FIG 2 Genes involved in sporulation are induced earlier in $\Delta bsrA$ cells compared to wild-type cells. qPCR was used to measure relative mRNA levels of *spoIIIE* (A), *spoOA* (B), *kinA* (C), and *glnA* (D) from wild-type cells or $\Delta bsrA$ cells grown at 37°C in DSM. Time shown is hours after onset of stationary phase, and mRNA levels are given as the fold change relative to wild-type levels at $t = 0$. Data shown are from one representative experiment with three replicates. Similar results were seen in an experiment with independent RNA preparation. Error bars correspond to standard deviations from the averages. Values that were statistically different in wild-type and $\Delta bsrA$ cells as tested by Student's *t* test: *, $P < 0.05$; **, $P < 0.005$.

RESULTS

Cells lacking 6S-1 RNA have altered timing of sporulation. To test whether either 6S-1 RNA or 6S-2 RNA plays a role in sporulation, spore formation was monitored over time in cells lacking

6S-1 RNA ($\Delta bsrA$; KW587), 6S-2 RNA ($\Delta bsrB$; KW589), or both RNAs ($\Delta bsrA \Delta bsrB$; KW590) compared to wild-type cells (*B. subtilis* 168; KW586) (Fig. 1A; see Fig. S1 in the supplemental material). Specifically, cells were grown in Difco sporulation medium (DSM), and the fraction of spores in cultures was measured as the ratio of heat-resistant cells to total cells as determined by plating (see Materials and Methods). Cells lacking 6S-1 RNA ($\Delta bsrA$) had a significantly higher fraction of spores (i.e., heat-resistant cells) than wild-type cells at 16, 18, and 20 h after the onset of stationary phase (see Materials and Methods for determination of timing). For example, at 20 h, ~47% of cells in wild-type cultures were spores while ~61% of cells were spores in cells lacking 6S-1 RNA. Although this change is rather modest, statistical analysis indicates that there is a significant difference ($P < 0.005$). Interestingly, by 40 h all cultures had a similar fraction of spores (80 to 85%) suggesting that it was the timing of sporulation that was altered rather than the inherent ability to form spores. Cells lacking 6S-2 RNA ($\Delta bsrB$) sporulated with a timing similar to that of the wild type, while cells lacking both 6S-1 and 6S-2 RNAs ($\Delta bsrA \Delta bsrB$) sporulated earlier than wild-type cells in a manner similar to that of $\Delta bsrA$ cells (see Fig. S1 in the supplemental material).

Interestingly, germination timing and efficiency were very similar for wild-type and $\Delta bsrA$ cells (see Fig. S2 in the supplemental material), in contrast to observations that $\Delta bsrA$ cells are delayed in recovery from stationary phase (i.e., outgrowth) (25). Therefore, the observed changes in timing of sporulation were not influenced by changes in germination or by requiring germination for the spore-counting method used here.

We imagined two possible mechanisms that might contribute to the earlier appearance of spores in $\Delta bsrA$ cultures than in wild-type cultures: (i) $\Delta bsrA$ cells initiate sporulation at earlier times in growth than wild-type cells, or (ii) $\Delta bsrA$ cells go through the developmental program of sporulation faster once the decision to sporulate has been made.

To test the timing of spore formation once the decision to sporulate has been made (option ii), we added decoyinine to vegetative cultures and monitored the fraction of spores over time (Fig. 1B). Decoyinine is an inhibitor of GMP synthesis and has been shown to induce sporulation of vegetative cells (33, 34), thereby allowing us to monitor timing of spore formation independently from growth into stationary phase. Specifically, cells were grown in S7 medium to an OD_{600} of 0.5, decoyinine was added to 0.4 mg/ml, and the extent of spore formation was monitored over time by plating. We observed ~20% spores in wild-type cultures at 8 h after the addition of decoyinine, consistent with observations that the developmental program of sporulation takes ~7 h at 37°C (7). A similar level of sporulation was observed in $\Delta bsrA$ cells compared to wild-type cells at all times examined, suggesting that the time it takes to generate a spore once the decision to sporulate has been made is similar in both cell types and that the observed early-sporulation phenotype might result from changes in timing of the decision to sporulate.

The lack of 6S-1 RNA leads to changes in timing of accumulation of mRNAs encoding gene products necessary for sporulation. In *E. coli*, 6S RNA mediates both direct and indirect changes in transcription that result in altered physiological responses (20, 21). To address whether the presence of 6S-1 RNA influences the level and/or timing of transcription of genes involved in sporulation, we monitored the expression of several genes important for sporulation by qPCR on RNA collected from

wild-type and $\Delta bsrA$ cells grown in DSM at various time points (Fig. 2). Specifically, we examined *spoIIIE*, which encodes one of the first sporulation-specific proteins to be induced (8, 38), *spo0A*, encoding a master regulator that controls the initiation of several developmental programs, including endospore formation, matrix production, and competence (9), and *kinA*, whose product is primarily responsible for the phosphorylation of Spo0A (39). For comparison we also examined *glnA*, which encodes glutamine synthetase and is not expected to change during the timing examined here (40). mRNA levels are expressed as a comparison to the level in wild-type cells at the transition into stationary phase ($t = 0$), so when mRNA levels are unchanged the fold change will be 1. In all cases the level of mRNA was very similar (i.e., within error) in wild-type and $\Delta bsrA$ cells at $t = 0$. The sporulation-dependent increases after the transition for *spoIIIE*, *spo0A*, and *kinA* appeared earlier in $\Delta bsrA$ cells than in wild-type cells, while levels of *glnA* remained fairly constant in both wild-type and $\Delta bsrA$ cells. Although changes in *kinA* levels were more modest than those observed for *spoIIIE* and *spo0A*, statistical analysis suggests the observed differences at 1.5 and 2.0 h are significant ($P < 0.05$), and even modest changes in levels of KinA have been shown to have larger effects on sporulation (41, 42). These observations provide evidence at the molecular level that cells lacking 6S-1 RNA are altered in the timing of initiation of sporulation.

Changes in sporulation result from environmental changes mediated by cells lacking 6S-1 RNA. It is possible that 6S-1 RNA directly regulates transcription of a specific gene that is important for the timing of onset of sporulation, such as the changes observed for *kinA*, *spo0A*, and *spoIIIE* above (Fig. 2). Alternatively, such changes in expression could be a consequence of changes in the environment that lead to earlier initiation of sporulation. To start to distinguish between these possibilities, we tested sporulation timing in mixed cultures to test whether changes in timing of sporulation were dependent on intracellular signals (e.g., genotype dependent) or external signals (e.g., environment dependent).

For these assays, we followed cells in coculture to test the timing of sporulation when the environmental conditions experienced were the same. First we generated independently marked wild-type and $\Delta bsrA$ strains and tested whether the markers had any effects on timing of sporulation. Coculture of two strains containing 6S-1 RNA ($\Delta bsrA^+ amyE::cat$ [KW593] versus *bsrA*⁺ *amyE::erm* [KW595]) or coculture of the two $\Delta bsrA$ cells ($\Delta bsrA::spc amyE::cat$ [KW594] versus $\Delta bsrA::spc amyE::erm [KW596]) resulted in sporulation at a time dependent on the presence or absence of 6S-1 RNA, similarly to the unmarked strains (Fig. 1A and Fig. 3). Specifically, ~60% of cells lacking 6S-1 RNA were spores at 20 h while only ~41% of cells with 6S-1 RNA were spores, suggesting no impact of the drug resistance markers on sporulation. Coculture of wild-type cells and cells lacking 6S-1 RNA (*bsrA*⁺ *amyE::cat* versus $\Delta bsrA::spc amyE::erm$; *bsrA*⁺ *amyE::erm* versus $\Delta bsrA::spc amyE::cat$) led to earlier sporulation of both cell types in the coculture such that ~59% were spores at 20 h. These results suggest that the early-sporulation phenotype observed for $\Delta bsrA$ cells is dependent on an altered environment.$

Conditioned medium from $\Delta bsrA$ cultures is sufficient to mediate the early-sporulation phenotype. The coculture experiment suggested that the early-sporulation phenotype resulted from environmental changes in cultures containing cells lacking 6S-1 RNA. We next tested if the early-sporulation phenotype

FIG 3 Coculture of wild-type and Δ *bsrA* cells results in early sporulation. Percentage of spores in mixed cocultures of different strains carrying antibiotic resistance cassettes (*cat* or *erm*). The fraction of spores of each genotype after incubation at 37°C in DSM for 20 h after the onset of stationary phase was determined by plating on chloramphenicol or erythromycin. Testing whether the markers have an effect on sporulation timing is shown in the left panel. The right panel shows coculture of *bsrA*⁺ versus Δ *bsrA* cells in each configuration of markers (*cat* or *erm*; see bottom). Data shown are from one representative experiment with three biological replicates. Similar results were seen in three independent experiments. Error bars correspond to standard deviations from the averages. Statistically significant differences in percentage of spores as tested by Student's *t* test: *, *P* < 0.05.

could be observed in conditioned medium or if coculture was required. Conditioned medium from stationary-phase wild-type cells (CM_{wt} from KW586) or Δ *bsrA* cells (CM _{Δ *bsrA*} from KW587) was inoculated with vegetative cells (wild type or Δ *bsrA*), and the fraction of spores was measured after incubation for 8 h at 37°C (Fig. 4). Intriguingly, the fraction of spores was dependent on the genotype of the cells used to generate the conditioned medium but not the genotype of cells undergoing sporulation. Specifically, cultures with either wild-type or Δ *bsrA* cells had ~21% spores when grown in CM_{wt} but had a higher fraction of spores (~41%) when grown in CM _{Δ *bsrA*}.

One possible change in medium that has been linked to changes in expression of some sporulation-specific genes, and thus timing of sporulation, is pH (43). However, the pHs of CM_{wt} and CM _{Δ *bsrA*} were very similar, suggesting that pH is not responsible for observed differences. Treatment of CM with protease, DNase, or heat did not significantly change the timing of sporulation relative to that in untreated CM (data not shown).

6S-1 RNA does not act through known pathways that influence the timing of sporulation. Several genes have been shown to be important for the timing of sporulation, including those coding for spore-killing factor (*skf*) and spore-delaying protein (*sdp*) (6). We tested if the Δ *bsrA* phenotype required these operons by testing the sporulation behavior of various mutants (Fig. 5). Wild-type cells (KW586), Δ *bsrA* cells (KW587), Δ *skf* cells (KW598), Δ *sdp* cells (KW600), Δ *bsrA* Δ *skf* cells (KW599), and Δ *bsrA* Δ *sdp* cells (KW601) were grown in DSM, and the fraction of spores was

FIG 4 Δ *bsrA* cell growth leads to changes in the medium that lead to early sporulation. The fraction of spores from *bsrA*⁺ cells (KW586) or Δ *bsrA*::*spc* (KW587) cells was measured after growth at 37°C for 8 h in conditioned medium (CM). CM_{wt} or CM _{Δ *bsrA*} was made from cultures of KW586 or KW588 after growth in DSM to 12 h after the onset of stationary phase. Data shown are from one representative experiment with three biological replicates. Similar results were seen in three independent experiments. Error bars correspond to standard deviations from the averages. Statistically significant differences in percentage of spores between cells grown in CM_{wt} compared to CM _{Δ *bsrA*} as tested by Student's *t* test: *, *P* < 0.05.

measured 14 h after the onset of stationary phase. Note that this earlier time was chosen to maximize the ability to detect changes in Δ *skf* and Δ *sdp* cells. The difference between wild-type and Δ *bsrA* cultures was not as large as at 20 h (Fig. 1A) but remained

FIG 5 6S-1 RNA-dependent changes in timing of sporulation do not require the action of several tested regulatory genes. The percentage of spores in cultures of cells with or without 6S-1 RNA in the presence of other gene deletions (see bottom labeling) was measured after growth at 37°C in DSM to 14 h after the onset of stationary phase. Data shown are from one representative experiment with three biological replicates. Similar results were seen in three independent experiments. Error bars correspond to standard deviations from the averages. Values that were statistically different as tested by Student's *t* test: *, *P* < 0.05.

FIG 6 $\Delta bsrA$ cell growth leads to decreased nutrients in the medium compared to wild type. *E. coli* K-12 (KW72) cells were diluted into CM or LB, and *E. coli* cell density was monitored over time at 37°C by OD₅₉₅ in a plate reader. CM_{wt} or CM $\Delta bsrA$ was made from cultures of KW586 or KW587 after growth in DSM to the onset of stationary phase. Data shown are from one representative experiment with three biological replicates. Similar results were seen in three independent experiments. Error bars correspond to standard deviations from the average.

statistically significant ($P < 0.05$). Wild-type cultures had ~17% spores, while the fraction of spores was increased for $\Delta bsrA$, Δskf , and Δsdg single mutants (28%, 31%, and 34%, respectively) as expected. Interestingly, the double mutants had a significantly higher fraction of spores than any single mutant (47% and 50%). Thus, the 6S-1 RNA effects on the timing of sporulation were additive to either cannibalism pathway, suggesting that 6S-1 RNA is not acting on sporulation through these pathways.

Other genes implicated in timing of sporulation are *sda* (encoding an inhibitor of KinA and KinB) (44) and *kilI* (encoding an inhibitor of KinA) (45). Once again, the cultures with single mutants had a higher fraction of spores than wild-type cultures, as expected (Fig. 5). The double mutants with $\Delta bsrA$ were increased further, again suggesting that 6S-1 RNA does not act on sporulation through changes in expression or activity of these molecules.

Cells lacking 6S-1 RNA utilize nutrients less efficiently than wild-type cells. We postulated that cells lacking 6S-1 RNA might be inefficiently utilizing nutrients available in their media, which would result in earlier reduction of nutrients and earlier sporulation in response to this altered nutrient status. Alternatively, cells lacking 6S-1 RNA could be generating a “signal” to initiate sporulation earlier than the wild type that is independent of overall nutrient status. To help distinguish between these possibilities, we tested the nutritional status of conditioned medium by monitoring the growth of *E. coli*, which does not sporulate and therefore would not be expected to respond to sporulation-specific signals. *E. coli* cells (KW72) were diluted in CM_{wt} or CM $\Delta bsrA$ generated from *B. subtilis* cells (KM586 or KW587) at the onset of stationary phase, and *E. coli* growth was monitored over time at 37°C (Fig. 6). *E. coli* had a longer lag time and a lower maximal cell density in CM $\Delta bsrA$ than in CM_{wt}, consistent with the hypothesis that growing $\Delta bsrA$ *B. subtilis* cells decreases the nutritional status of their medium earlier than for wild-type *B. subtilis* cells.

DISCUSSION

We have discovered that 6S-1 RNA is important for appropriate timing of sporulation in *B. subtilis* in that spores are formed earlier in cultures of cells lacking 6S-1 RNA than in wild-type cells (Fig. 1A). Germination timing and efficiency, as well as the time to generate a mature spore once the decision to sporulate has been

made, are unchanged in cells with or without 6S-1 RNA (Fig. 1B; see Fig. S2 in the supplemental material). We also have shown that genes associated with sporulation are induced earlier in cells lacking 6S-1 RNA than in wild-type cells (Fig. 2). Together, these data support the hypothesis that it is the timing of onset of sporulation that is altered in cells lacking 6S-1 RNA ($\Delta bsrA$). Interestingly, the presence of $\Delta bsrA$ cells in coculture with wild-type cells leads to both cell types exhibiting an early-sporulation phenotype (Fig. 3), which led us to propose that cells lacking 6S-1 RNA modify their environment in a manner that promotes early sporulation. In support of this model, resuspension of wild-type cells in conditioned medium from $\Delta bsrA$ cultures also resulted in early sporulation (Fig. 4). The use of *E. coli* growth to monitor nutrient content in conditioned medium also suggests that there is a reduction in nutrients in CM $\Delta bsrA$ compared to CM_{wt} based on the increase in lag time and lower overall cell density obtained (Fig. 6). Therefore, we propose that the lack of 6S-1 RNA function in $\Delta bsrA$ cells leads to a general increase in nutrient utilization. In addition, we have shown that several pathways known to impact timing of sporulation are not required for 6S-1 RNA-mediated changes, suggesting that they are not targets of 6S-1 RNA regulation (Fig. 5). Interestingly, 6S-2 RNA does not influence the timing of sporulation, providing further evidence for independent functions for these two related RNAs in cell physiology.

Our data support conclusions that 6S-1 RNA activity leads to changes in the local environment, that these changes promote early sporulation, and that these environmental changes are dominant over the genotype of cells growing. The fact that conditioned medium recapitulates coculture indicates that cell-cell contact is not required. What specific changes exist between CM_{wt} and CM $\Delta bsrA$ are of great interest, and we postulate two nonexclusive models. (i) $\Delta bsrA$ cells utilize nutrients at a different rate than wild-type cells, leading to faster depletion of optimal nutrients and earlier onset of sporulation. (ii) $\Delta bsrA$ cells generate and secrete a sporulation “signal” into the medium at an earlier time, leading to early sporulation independently from nutrient status. Although we cannot eliminate the possibility that both models work together, the observation that CM_{wt} supports more growth of *E. coli* than CM $\Delta bsrA$ suggests that $\Delta bsrA$ cells sporulate earlier due to changes in nutrient status. We would not expect *E. coli* to respond to specific sporulation signals, and therefore the decreased growth is likely the result of an altered nutrient composition of the medium. Interestingly, treatment of CM with protease or DNase did not alter sporulation timing, suggesting that protein or DNA components are not responsible. Also, the early-sporulation phenotype was observed for $\Delta bsrA$ cells grown in chemically defined sporulation medium (32) and phosphate starvation medium (46), suggesting that the type of starvation leading to sporulation does not need to be specific (data not shown).

Cannibalism is a response to nutrient limitation in *Bacillus subtilis* that allows individual cells within a population to produce a toxin to kill neighboring cells as a source of nutrients (see reference 6 for review). Deletion of one or both operons known to be important for independent cannibalism responses (*skf* and *sdg*) results in an early-sporulation phenotype similar to the phenotype observed for $\Delta bsrA$ cells. However, neither *skf* or *sdg* is required for 6S-1 RNA-dependent changes in timing of sporulation. Therefore, we conclude that these cannibalism pathways are not the target of 6S-1 RNA regulation responsible for changes in sporulation in $\Delta bsrA$ cells.

spoIIE, *spoOA*, and *kinA* were all induced earlier in $\Delta bsrA$ cells than wild-type cells, suggesting that 6S-1 RNA functions earlier in decision pathways for sporulation than these gene products. Although Spo0A is regulated primarily at the level of phosphorylation, transcriptional regulation has been shown to play a role in increasing levels of Spo0A at the onset of sporulation (47, 48, 49), and 10- or 30-fold changes in Spo0A levels were observed by 2 h after the onset of stationary phase in wild-type and $\Delta bsrA$ cells, respectively. We suggest that the observed changes in timing of *spoOA* induction are likely to account for the changes observed in *spoIIE* induction. KinA is primarily responsible for phosphorylation of Spo0A (39). Although changes to *kinA* levels in $\Delta bsrA$ cells compared to wild-type cells are small and occur a bit later than observed *spoOA* changes, it has been shown that small changes in the level of KinA can lead to surprisingly large changes in sporulation levels (41, 42). We propose that at a minimum the differences in timing of induction of *kinA* lend additional support to altered timing in $\Delta bsrA$ cells. However, it might also serve to place 6S-1 RNA function upstream of the KinA phosphorelay system in the sporulation pathway. Considering that CM_{wt} and $CM_{\Delta bsrA}$ generated from cells at the onset of stationary phase supported different levels of growth of *E. coli*, this observation also suggests that changes occur prior to this time, which is consistent with the model that 6S-1 RNA is acting earlier and upstream of the KinA phosphorelay. However, the onset of stationary phase occurs with very similar timing in wild-type and $\Delta bsrA$ cells, suggesting that any changes to nutrient status are not large enough or early enough to alter the timing of this transition. In addition, Bs6S-1 RNA does not reach maximal levels until hours after the transition into stationary phase, consistent with minimal if any effect on exponential growth and the transition into stationary phase.

We have hypothesized that 6S RNA is important for appropriate resource management in *E. coli*, allowing cells to conserve energy by limiting the response to several environmental stresses (15, 50). The trade-off is the enhancement of optimal survival in long-term stationary phase (19). It is intriguing that 6S-1 RNA is likely to be involved in nutrient conservation in *B. subtilis* as well. Sporulation is highly regulated, in part to balance optimal survival with flexibility to changes in environmental conditions (7, 10). For example, if a large number of cells sporulate early, the population is compromised in its ability to respond quickly to an upshift in nutrient quality or quantity. However, if sporulation is delayed, there may not be sufficient resources necessary for completion of endospore formation once begun, thereby decreasing long-term survival rates from populations of cells. Therefore, we propose that *B. subtilis* 6S-1 RNA and *E. coli* 6S RNA both play a role in balancing resource utilization and cell survival.

We anticipate that 6S-1 RNA is regulating transcription in a manner similar to that of *E. coli* 6S RNA, although the specific gene targets and the number of gene targets for 6S-1 RNA regulation in *B. subtilis* remain to be unveiled. Even in *E. coli*, where many of the changes in transcription are known (19, 20, 21), the details of how 6S RNA-dependent regulation leads to altered long-term survival are not known. It is possible these 6S RNAs directly regulate transcription of one or a few critical genes that are important for managing nutrient utilization efficiency or responding to nutrient changes appropriately. For example, *relA* expression is regulated by 6S RNA in *E. coli* (51), and *RelA* activity is responsive to nutrient status and also mediates further transcriptional changes (via changes in ppGpp levels) to alter cell physiology appropriately

(52). Therefore, some of the 6S RNA-dependent changes in *E. coli* are likely to be dependent on *relA*. Alternatively, the absence of this type of 6S RNA (e.g., Bs6S-1 and Ec6S RNAs) may lead to an overall increase in energy use as a direct result of increasing transcription of a large set of genes. In *E. coli*, a global increase in transcription of hundreds of genes in stationary phase has been observed (20, 21).

Interestingly, although 6S-2 RNA in *B. subtilis* also interacts with the housekeeping RNA polymerase ($E\sigma^A$) (17), it does not appear to play a role in the timing of sporulation (see Fig. S1 in the supplemental material). *E. coli* 6S RNA and *B. subtilis* 6S-1 RNA share several characteristics that differ from those of 6S-2 RNA. For example, both Ec6S RNA and Bs6S-1 RNA accumulate to high levels in stationary phase and are released from RNA polymerase during outgrowth by the process of pRNA synthesis (16, 23, 25, 27, 53). 6S-2 RNA does not serve as a template for pRNA synthesis, and its levels remain more consistent through growth (17, 25, 26, 28). In addition to these biochemical distinctions, we now know that 6S-1 RNA and 6S-2 RNA do not have redundant physiological functions, as 6S-1 RNA influences timing of sporulation but 6S-2 RNA does not, although the mechanism determining these different behaviors remains to be uncovered.

While many questions about 6S RNA function globally in diverse bacteria still await further study, it is clear that 6S-1 RNA plays a role in timing of the onset of sporulation in *B. subtilis*. Sporulation is a carefully regulated process to balance cell survival under changing environmental conditions, and the work here adds another small RNA regulator to this complex process.

ACKNOWLEDGMENTS

We thank R. Gourse and W. Winkler for providing strains, plasmids, and protocols and the members of the Wassarman lab for helpful discussions and comments on the manuscript.

This work was supported by the National Institutes of Health (GM67955) and the Greater Milwaukee Foundation through a Shaw Scientist Award (K.M.W.).

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