Natural Variability in S-Adenosylmethionine (SAM)-Dependent Riboswitches: S-Box Elements in *Bacillus subtilis* Exhibit Differential Sensitivity to SAM In Vivo and In Vitro

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Riboswitches are regulatory systems in which changes in structural elements in the 5′ region of the nascent RNA transcript (the “leader region”) control expression of the downstream coding sequence in response to a regulatory signal in the absence of a trans-acting protein factor. The S-box riboswitch, found primarily in low-G+C gram-positive bacteria, is the paradigm for riboswitches that sense S-adenosylmethionine (SAM). Genes in the S-box family are involved in methionine metabolism, and their expression is induced in response to starvation for methionine. S-box genes exhibit conserved primary sequence and secondary structural elements in their leader regions. We previously demonstrated that SAM binds directly to S-box leader RNA, causing a structural rearrangement that results in premature termination of transcription at S-box leader region terminators. S-box genes have a variety of physiological roles, and natural variability in S-box structure and regulatory response could provide additional insight into the role of conserved S-box leader elements in SAM-directed transcription termination. In the current study, in vivo and in vitro assays were employed to analyze the differential regulation of S-box genes in response to SAM. A wide range of responses to SAM were observed for the 11 S-box-regulated transcriptional units in *Bacillus subtilis*, demonstrating that S-box riboswitches can be calibrated to different physiological requirements.

Several genetic systems (termed “riboswitches”) have been identified recently in which the nascent RNA transcript directly senses a regulatory signal (which can be a cellular metabolite, a small RNA, or a physical parameter, such as temperature) in the absence of an accessory protein or translating ribosome (11, 12, 17, 32, 36, 38, 39). Expression of the downstream coding region(s) is controlled by an RNA structural transition that occurs in the 5′ region of the transcript (the “leader region”) in response to the regulatory signal. Modulation of the leader RNA structure by the regulatory signal can control gene expression by premature termination of transcription, inhibition of translation initiation, or regulation of RNA processing (11, 12, 17, 19, 43). In general, riboswitches exhibit conserved sequence and structural features that confer physiologically relevant affinity and high specificity for the regulatory signal, which allows the cell to efficiently regulate gene expression in response to changes in the environment.

Three riboswitch classes (the S-box, S\textsubscript{MK}-box, and SAM-II riboswitches) that respond to the level of S-adenosylmethionine (SAM) in the cell have been identified in bacteria. The S-box system is found primarily in low-G+C gram-positive bacteria and regulates expression of many genes involved in biosynthesis and transport of methionine and SAM (8, 9, 10, 34, 38) (Fig. 1). S-box genes are characterized by the presence of a set of highly conserved primary sequence and secondary structural elements in the leader region (8) (Fig. 2A). S-box gene expression is usually regulated at the level of premature transcription termination. A rarer class of S-box elements is predicted to regulate gene expression at the level of translation initiation by a SAM-dependent RNA structural rearrangement that results in sequestration of the Shine-Dalgarno sequence (17). A second SAM-dependent riboswitch, the S\textsubscript{MK} box, is found in the leader region of *metK* (SAM synthetase) genes in lactic acid bacteria; S\textsubscript{MK}-box genes are regulated at the level of translation initiation by SAM-dependent inhibition of ribosome binding to the mRNA (6, 7). A third class of SAM-dependent riboswitches (SAM-II) was identified in alphaproteobacteria, but the mechanism of regulation has yet to be determined (4).

There are 11 S-box-regulated transcriptional units in *Bacillus subtilis* (8, 9, 10), and regulation of many of these genes has been characterized both in vivo and in vitro. Efficient termination of transcription during growth in the presence of methionine (when SAM levels are high) has been demonstrated for several *B. subtilis* S-box genes, while terminator readthrough is induced in response to starvation for methionine (when SAM levels are low) (3, 8, 18, 30). SAM promotes termination at S-box leader region terminators in a purified in vitro transcription system in the absence of any additional trans-acting factors (25). SAM binds specifically and directly to the helix 1-4 region of S-box leader RNAs, causing an RNA structural rearrangement that results in stabilization of the antiantiterminator. Stabilization of the antiantiterminator structure prevents formation of the antiterminator structure (which is generally predicted to be more stable than the terminator and is transcribed first) and allows premature termination of transcription (5, 25, 26, 45) (Fig. 2B). Leader region mutations that cause a loss of repression during growth in the presence of methionine also inhibit binding of SAM and SAM-directed transcription termination in vitro (25, 26). Overexpression of SAM synthetase decreases expression of S-box genes (2, 25), while a...
mutation in the \textit{B. subtilis} SAM synthetase gene that results in decreased SAM synthetase activity and a reduction in SAM pools causes derepression of S-box gene expression during growth in the presence of methionine (27).

The secondary structure model of the S-box leader region was derived from phylogenetic analysis of the arrangement of structural elements in the leader region and sequence covariation within the helical domains (8) (Fig. 2). Support for the model was provided by analysis of additional S-box leader regions (9, 10, 34, 44). An interaction between the loop of helix 2 and the junction region between helices 3 and 4 was predicted based on covariation to form a pseudoknot, and mutational analysis demonstrated that this interaction is required for SAM binding, SAM-directed transcription termination in vivo and in vitro, and SAM-induced structural rearrangements (26). A conserved element (the GA motif) within helix 2 fits the pattern of a kink-turn structural element and was predicted to facilitate formation of the pseudoknot (26, 44). Structural mapping by various methods revealed multiple changes throughout the S-box leader in response to SAM binding, including stabilization of the antiantiterminator (5, 25, 26, 45). The structural model (including the pseudoknot, the kink-turn, and the predicted importance of conserved sequence elements) was corroborated by the crystal structure of a \textit{Thermoanaerobacter tengcongensis} S-box leader RNA in complex with SAM (29). The crystal structure also revealed that SAM is embedded in a pocket formed by the pseudoknot in a complex four-way helical junction, the base of which is the antiantiterminator element.

Although a high level of conservation of primary sequence and secondary structural elements has been identified, differences in structural predictions have been observed in some S-box leader regions. Differences have also been detected in SAM-directed regulation of S-box gene expression, including variability in the amount of repression during growth in the presence of methionine and in the level of expression during starvation for methionine (2, 8, 30). Variability was also observed for different S-box leaders in the efficiency of termination in the absence of SAM and in the degree of response to SAM in vitro (25). Further analysis of different S-box leader RNAs could provide additional information about elements required for SAM-directed transcription termination and insight into the correlation between the physiological roles of S-box gene products and the variability in their SAM-dependent regulation. In this study, in vivo and in vitro assays were performed to examine how expression of individual S-box genes is differentially regulated in response to SAM and how individual S-box leader RNAs interact with SAM.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The \textit{B. subtilis} strains used in this study were BR151 (lys-3 metB10 trpC2), BR151MA (lys-3 trpC2), and ZB307A (SP\textsubscript{4}lc\textsubscript{2}del\textsubscript{2};Tnp917;psK10\Delta6) (47). \textit{B. subtilis} strains were grown on tryptose blood agar base medium (Difco), Spizizen minimal medium (1), or 2XYT broth (28). Strains containing lacZ fusions were grown in the presence of chloramphenicol (5 \textmu g ml\textsuperscript{-1}). All growth was at 37°C.

**Genetic techniques.** Transformation of \textit{B. subtilis} was carried out as described previously (16). Chromosomal DNA was prepared using the DNeasy tissue kit (Qiagen). Wizard columns (Promega) were used for plasmid preparations. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases and DNA-modifying enzymes were
FIG. 2. The S-box riboswitch. (A) *B. subtilis* yitJ leader secondary structural model. Numbering is relative to the predicted transcription start site (+1). The sequence is shown in the terminator conformation; red and blue residues illustrate the alternate pairing required for formation of the antiterminator, shown above the terminator. Asterisks indicate covarying residues that form a pseudoknot. Helices 1 to 5 are identified by boxed numbers; T, terminator; AT, antiterminator; AAT, antiantiterminator. (B) Model for regulation of S-box gene expression in response to SAM. The antiterminator structure (AT, red-blue) forms in the absence of SAM, allowing expression of the downstream coding region(s). Binding of SAM (represented by the asterisk) stabilizes the antiantiterminator structure (AAT), which sequesters sequences (red) required for formation of the antiterminator and frees sequences (blue) required for formation of the terminator helix (T), resulting in premature termination of transcription.
methionine, and samples were collected at 15-min intervals and assayed for β-galactosidase activity as described previously (28) using toluidine permethylation of the cells. β-Galactosidase assays were carried out in triplicate, and variation was <10%.

**Determination of SAM pools in vivo.** Strain BR151 containing a yitJ-lacZ fusion was grown in Spizizen minimal medium containing all required amino acids (50 μg ml⁻¹) until early exponential growth. Cells were harvested by centrifugation and resuspended in fresh Spizizen minimal medium in the absence of methionine, and cell samples were collected by filtration at the indicated time points and extracted with 1.5 ml of 0.5 M formic acid; the formic acid was removed by lyophilization as described by Ochi et al. (33). Cell extracts were tested in an in vitro transcription termination assay using a β-gal assay and compared to a SAM standard curve, as previously described (27). Samples were also harvested at each time point and assayed for β-galactosidase activity, as described above.

**In vitro transcription termination assays.** DNA templates were generated and single-round in vitro transcription assays using B. subtilis RNAp were carried out as previously described (25). The templates contained the B. subtilis phyG5 promoter fused to each S-box sequence such that the leader region started 15 to 25 bases before the 5'-end of helix 1 and transcription initiation was directed with the appropriate dinucleotide (APG for yadA and yutC and APc for all other templates). Templates were approximately 400 bp in length and included 50 to 150 bp downstream from the transcription terminator to allow resolution of terminated and readthrough products. Transcription elongation reactions were carried out in the presence of various concentrations of SAM, as indicated, and products were resolved by denaturing polyacrylamide gel electrophoresis and visualized and quantitated by PhosphorImager (Molecular Dynamics) analysis. Efficiency of termination was calculated as the amount of termination product divided by the sum of the readthrough and termination products. The half-maximal response was the SAM concentration that resulted in a termination efficiency at the midpoint between the termination efficiency in the absence of SAM and the maximum termination efficiency for each gene in the presence of SAM.

Free-energy predictions for terminators and antiterminators were determined using the mfold web server (23, 48).

**Determination of equilibrium dissociation constants and dissociation half-lives.** DNA templates for T7 RNAp transcription were generated by PCR using a primer containing a T7 RNAp promoter initiating at tandem G residues fused to position +14 for the yitJ leader and a similar position upstream of helix 1 for other genes. The endpoints of the PCR products corresponded to the position just 5' of the start of the terminator helix. T7 RNAp transcription was carried out using an Ampliscribe T7 transcription kit (Epicenter Biotechnologies). For determination of the equilibrium dissociation constants, RNA (0.1 μM) was heated to 65°C for 5 min in 1× transcription buffer (13) and then slowly cooled to 45°C before addition of [methyl-3H]SAM (15 Ci/mmol [555 GBq/mmol]; GE Healthcare) and incubation at 37°C for 30 min. Final concentrations of [3H]SAM varied from 10 nM to 10 μM. Samples were passed through Nanoprep 10K Omega filter membranes (Pall; preincubated with bovine serum albumin [BSA][10 mg ml⁻¹]; Sigma) for 15 min at room temperature and then washed four times with 100 μl 1× transcription buffer) and washed with 40 μl 1× transcription buffer. Material retained by the filter was mixed with Packard Bioscience Ultima Gold scintillation fluid and counted in a Packard Tri-Carb 2100TR liquid scintillation counter. Nonlinear regression analysis was performed using GraphPad Prism version 4.0 (GraphPad Software). The margin of error was ±5%.

The binding assay was modified for determination of complex stability. In each assay, an aliquot of RNA (1 μM) was denatured, slow cooled, and incubated with various concentrations of [3H]SAM (1.5 to 30 μM). After incubation at 37°C for 30 min, nonradioactive SAM was added as a competitor compound (at a concentration 100-fold greater than the [3H]SAM concentration added for each RNA); samples were removed at intervals and were passed through BSA-treated Nanoprep 10K filters and washed with 25 μl 1× transcription buffer. The amount of [3H]SAM retained by the filter was calculated, and nonlinear regression analysis was performed. The margin of error was ±5%.

**RESULTS**

Analysis of S-box transcripts during starvation for methionine. Northern blot analysis was carried out to monitor the presence of the terminated and readthrough transcripts of S-box genes in B. subtilis cells during starvation for methionine. RNA was extracted from B. subtilis cells harvested at...
0.5-h intervals during starvation for methionine, separated on denaturing agarose gels, and hybridized with an RNA probe complementary to the 5'-untranslated region of each S-box transcript (to detect both terminated and readthrough transcripts). The terminated transcripts were the major product for both metE and yusC in the cells harvested at the zero time point (Fig. 3A and C). A slight increase (~2-fold) in the amount of terminated product was observed for both metE and yusC after 0.5 h of starvation for methionine, but after 1 h of starvation the amount of terminated product decreased. Analysis of the readthrough transcript showed a ~2-fold increase in readthrough after 0.5 h of starvation, while 72-fold and 3.8-fold increases in the amount of readthrough product were observed for metE and yusC, respectively, after 1 h of starvation (Fig. 3A and C). A similar decrease in the terminated transcript and increase in the readthrough transcript were observed for the yitJ, ykrT, ykrW, yicI, yjgG, yjH, and yoaD genes, with the increase in readthrough transcript ranging from 17- to 130-fold (data not shown). No significant increase in the readthrough transcript during starvation for methionine was detected for cysH, while metK showed a transient increase after 0.5 h (data not shown).

Since the primary sequence of S-box leaders is highly conserved, a second set of RNA probes that hybridize in the coding region was designed to ensure specific detection of S-box readthrough transcripts. A similar pattern of increase in abundance of readthrough transcripts during starvation for methionine was observed using this set of probes (Fig. 3B and D; also data not shown).

Quantitation of S-box transcripts during growth in the presence or absence of methionine was also carried out using qRT-PCR. For each S-box gene examined, a standard curve was generated using a PCR product synthesized with the same primers. Reverse transcription was performed to synthesize cDNA from cysH, yitJ, ykrT, metE, ykrW, yusC, yoaD, and metK RNA. At each time point, the same amount of total RNA was used for reverse transcription, as determined by spectrophotometric analysis, and qRT-PCR was carried out with the cDNA products. No significant increase in the copy number of cysH RNA was detected during starvation for methionine. It was reported previously that the cysH operon in B. subtilis is regulated primarily at the level of transcription initiation in response to cysteine availability (22); the small differences observed in the RNA copy number of cysH (10 to 20%) were therefore attributed to the efficiency of RNA extraction, and the cysH transcript was used as the reference RNA.

The copy numbers of different S-box genes exhibited a 34-fold range in variability during growth in the presence of methionine and a 36-fold range under inducing conditions. The lowest initial RNA copy number was observed for yoaD, while the highest initial copy number was observed for metK (Table 1; Fig. 4). High expression of metK is not surprising, since SAM synthetase is required even when methionine is abundant. A 15- to 30-fold increase in the RNA copy number was observed for metE, yitJ, ykrT, and yoaD after 1 h of starvation for methionine, while 5-fold and 11-fold increases were observed for yusC and ykrW, respectively. After an additional 0.25 h of starvation, a 70- to 340-fold increase in the RNA copy number of metE, yitJ, ykrT, ykrW, and yoaD was detected, while a smaller increase was detected for yusC (Table 1). A sixfold increase in the metK RNA copy number was detected at 0.5 to 0.75 h, but after 1.25 h the RNA copy number decreased to the level detected prior to starvation. This result suggests that metK transcription is induced in response to a decrease in SAM levels in vivo but that some additional mechanism may be involved in regulation of this gene, resulting in reduced transcript levels later during starvation.

Expression of S-box gene-lacZ fusions in vivo. Efficient termination of transcription during growth in the presence of methionine and terminator readthrough in response to starvation for methionine have been demonstrated for B. subtilis yitJ, yjcl, ykrT, ykrW, yusC, yjgG, and yjH (3, 8, 18, 30; F. J. Grundy and T. M. Henkin, unpublished data). However, these studies were done in multiple laboratories, using various strain backgrounds and growth conditions. The effect of starvation for methionine on in vivo expression of S-box gene-lacZ fusions

![FIG. 3. Northern blot analysis. BR151 cells were grown in Spizizen minimal medium (1) containing methionine and resuspended in Spizizen minimal medium in the absence of methionine. Cells were harvested at 0.5-h intervals, and total RNA was extracted. (A) 5'-UTR probe for metE. (B) Coding region probe for metE. (C) 5'-UTR probe for yusC. (D) Coding region probe for yusC.](https://jb.asm.org/content/190/8/827)

<table>
<thead>
<tr>
<th>Gene</th>
<th>RNA copy no. (10^4)a</th>
<th>Increase (fold)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Met</td>
<td>Maximum</td>
<td>0.5 h</td>
</tr>
<tr>
<td>metE</td>
<td>0.81 ± 0.095</td>
<td>280 ± 48</td>
</tr>
<tr>
<td>yitJ</td>
<td>0.54 ± 0.11</td>
<td>77 ± 0.55</td>
</tr>
<tr>
<td>ykrT</td>
<td>0.23 ± 0.013</td>
<td>69 ± 11</td>
</tr>
<tr>
<td>ykrW</td>
<td>0.74 ± 0.10</td>
<td>54 ± 3.3</td>
</tr>
<tr>
<td>yoaD</td>
<td>0.065 ± 0.0083</td>
<td>7.8 ± 1.3</td>
</tr>
<tr>
<td>yusC</td>
<td>1.2 ± 0.22</td>
<td>15 ± 1.6</td>
</tr>
<tr>
<td>metK</td>
<td>2.2 ± 0.44</td>
<td>14 ± 1.1</td>
</tr>
</tbody>
</table>

*a Values are reported as the means ± the standard deviations for three assays. +Met, value for growth in the presence of methionine; Maximum, value after 0.5 h of methionine starvation for metK or after 1.25 h of starvation for methionine for all other S-box transcripts.

*b Increase in RNA copy no. after indicated period of starvation for methionine.
was therefore examined using identical conditions and more-frequent sampling (15-min intervals during the first 1.5 h of starvation) to more precisely compare induction of a variety of S-box genes.

Expression of the *metE*, *yitJ*, *ykrT*, *yxjW*, *yxjH*, and *yoaD-lacZ* transcriptional fusions was tightly repressed during growth in the presence of methionine (0.20 to 0.65 Miller units; Table 2). In contrast, the repressed level of expression for *yjcI*, *yxjG*, *yusC*, and *metK* was 1.0 to 4.6 Miller units. The highest level of expression during growth in the presence of methionine was observed for the *cysH* transcriptional fusion, which exhibited no change in expression during starvation; the *metK* fusion also exhibited no increase in expression under these starvation conditions. The S-box genes that responded to limitation for methionine varied in the range of expression observed, with a 250-fold range in induction ratios after 4 h. The most tightly repressed genes were generally shown to have the most dramatic induction, while less tightly repressed genes (e.g., *yxjG* and *yusC*) were induced to a lower degree and a few genes (*yjcI*, *yxjH*, and *yoaD*) exhibited an intermediate phenotype. All genes that responded to starvation for methionine reached the maximum level of expression after 4 h of starvation (Table 2; Fig. 5).

The S-box–*lacZ* transcriptional fusions also varied in the kinetics of their response to starvation for methionine. In the case of *metE*, an increase in β-galactosidase activity was detected after 1.25 h of starvation for methionine, followed by a rapid increase in expression that leveled off at approximately 720 Miller units (Fig. 5A; Table 2). In contrast, an increase in *yusC-lacZ* expression was detected after 0.75 h of starvation for methionine, followed by a more gradual increase in β-galactosidase activity that leveled off at approximately 35 Miller units (Table 2). An increase in expression to 10% of the maximum was set as the standard to determine the induction start. Induction of expression started earliest for the S-box genes that were not completely repressed in the presence of methionine (*yjcI*, *yxjG*, and *yusC*), while induction started after 1 to 1.25 h of starvation for the remaining fusions (Table 2). These results as a whole demonstrate that the level of repression of expression during growth in the presence of methionine, the kinetics of induction after starvation for methionine, and the maximum expression during growth in the absence of methionine are all variable for different S-box genes.

**TABLE 2. Expression of S-box gene–*lacZ* fusions in vivo**

<table>
<thead>
<tr>
<th>Gene</th>
<th>β-Galactosidase activity (MU)*</th>
<th>Induction ratio (4 h)</th>
<th>Induction startb (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Met</td>
<td>−Met</td>
<td></td>
</tr>
<tr>
<td><em>metE</em></td>
<td>0.61</td>
<td>720</td>
<td>1,200</td>
</tr>
<tr>
<td><em>yitJ</em></td>
<td>0.20</td>
<td>460</td>
<td>2,300</td>
</tr>
<tr>
<td><em>yjcI</em></td>
<td>4.0</td>
<td>37</td>
<td>9.3</td>
</tr>
<tr>
<td><em>yxjG</em></td>
<td>1.0</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td><em>yxjH</em></td>
<td>0.54</td>
<td>25</td>
<td>46</td>
</tr>
<tr>
<td><em>yoaD</em></td>
<td>0.41</td>
<td>61</td>
<td>150</td>
</tr>
<tr>
<td><em>yusC</em></td>
<td>2.3</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td><em>metK</em></td>
<td>4.6</td>
<td>4.6</td>
<td>1.0</td>
</tr>
<tr>
<td><em>cysH</em></td>
<td>110</td>
<td>110</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Cells were grown in minimal medium (1) containing methionine, harvested by centrifugation, and resuspended in minimal medium in the presence (+Met) or absence (−Met) of methionine. β-Galactosidase activity is expressed in Miller units (MU) (28) and is reported for samples taken 4 h after the cultures were split.

b Time at which an increase in β-galactosidase activity was first detected. NA, not applicable.
SAM-dependent transcription termination in vitro. Previous studies showed that all of the *B. subtilis* S-box genes (with the exception of *metK*) exhibit increased termination of transcription when SAM is added to an in vitro transcription assay (25). Those studies were carried out at a single high concentration of SAM. The SAM response was therefore titrated for each of the genes, and the termination efficiency was determined for each concentration of SAM. Since SAM-dependent termination is promoter independent (25), the *B. subtilis* gylQS promoter was used to initiate transcription for each gene so that transcription initiation would be consistent for each construct. Variability was observed both in the termination efficiency in the absence of SAM and in the SAM concentration required for a maximal response (Table 3); the maximal response was obtained at 160 μM SAM for most genes, with the exception of *yxjH* (620 μM) and *yoaD* and *cysH* (1.6 mM). The *metE*, *yitJ*, *yicI*, and *ykrT* genes exhibited the highest sensitivity to SAM (as indicated by the concentration of SAM required for half-maximal termination and the termination efficiency at 3 μM); this group generally corresponds to the genes that are most tightly repressed during growth in the presence of methionine. This set of genes also exhibited similar termination efficiencies in the absence of SAM and similar maximal efficiencies of termination, consistent with tight regulation in vivo. The *yoaD* and *yusC* genes, which required the highest concentrations of SAM to reach a half-maximal response in vitro, had similar termination efficiencies at 3 μM SAM and high termination in the absence of SAM, but *yoaD* required a much higher concentration of SAM to reach its maximal response. The *yusC* gene is also not tightly repressed in vivo (as indicated by lacZ fusion data and transcript measurements), whereas *yoaD* is tightly repressed in vivo. The remaining genes (which showed an intermediate sensitivity to SAM) exhibited a broad range of background termination (6 to 23%), termination at 3 μM SAM (41 to 75%), and maximal termination (63 to 92%).

Most S-box genes exhibited a termination frequency of 10 to 15% in the absence of SAM. The genes with the highest termination frequency without SAM (*yoaD* at 32% and *yusC* at 52%) exhibited the least-sensitive half-maximal response concentration; however, *yxjH* (with only a slightly lower termination frequency of 23% without SAM) had a much lower half-maximal response concentration (1.5 μM). Similarly, the *cysH* gene (which was not regulated in vivo under the conditions tested) showed an intermediate sensitivity to SAM in vitro, with a half-maximal response at 4 μM SAM, although the maximal response required very high SAM concentrations. Overall these results show that while genes that are tightly repressed in vivo during growth under conditions where SAM pools are large are also highly sensitive to SAM during transcription in vitro, there is a broad range (>100-fold) in the
response to SAM. It is important to note that the in vitro transcription experiments specifically monitor termination effects; differences in promoter activity and mRNA stability in vivo could further influence the regulatory response.

**Affinity of S-box leader RNAs for SAM.** T7 RNA polymerase-transcribed yitJ and ykrW leader RNAs were previously shown to specifically bind SAM in a binding assay that utilizes size exclusion filtration (25, 26). To assess the affinity of S-box leader RNAs for SAM, a protocol adapted from this assay was used to determine the equilibrium dissociation constant (K_d) for each S-box leader RNA–SAM complex. The K_d value was measured by incubation of S-box leader RNA with [3H]SAM and filtration through a BSA-treated Nanosep 10K filter to determine the amount of [3H]SAM retained by the filter.

**TABLE 3. Response of S-box genes to SAM in vitro**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcription termination in vitro</th>
<th>Value for SAM binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Termination efficiency (%)^a</td>
<td>K_d (μM)</td>
</tr>
<tr>
<td></td>
<td>−SAM</td>
<td>+SAM (3.0 μM)</td>
</tr>
<tr>
<td>metE</td>
<td>12</td>
<td>89</td>
</tr>
<tr>
<td>yitJ</td>
<td>14</td>
<td>92</td>
</tr>
<tr>
<td>yjcI</td>
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<td>89</td>
</tr>
<tr>
<td>ykrT</td>
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<td>89</td>
</tr>
<tr>
<td>ykrW</td>
<td>6.0</td>
<td>59</td>
</tr>
<tr>
<td>yxjG</td>
<td>15</td>
<td>43</td>
</tr>
<tr>
<td>yxjH</td>
<td>23</td>
<td>75</td>
</tr>
<tr>
<td>yoaD</td>
<td>32</td>
<td>44</td>
</tr>
<tr>
<td>yscC</td>
<td>52</td>
<td>53</td>
</tr>
<tr>
<td>cysH</td>
<td>15</td>
<td>41</td>
</tr>
</tbody>
</table>

^a Termination efficiency is the amount of the terminated product relative to the sum of the terminated and readthrough products.

^b Maximum termination is the highest termination efficiency obtained at the indicated SAM concentration: ×, 160 μM; ++, 620 μM; ++*, 1.6 mM.

^c The K_d value was measured by incubation of S-box leader RNA with [3H]SAM and filtration through a BSA-treated Nanosep 10K filter to determine the amount of [3H]SAM retained by the filter.

^d Nonradioactive SAM was added as a competitor compound to the preformed leader RNA/[3H]SAM complex, and samples were removed at intervals and filtered to determine the amount of [3H]SAM retained by the filter.

**DISCUSSION**

The gene products regulated by the S-box transcription termination control system in *B. subtilis* are involved in different steps of sulfur metabolism (3, 8, 9, 18, 30) (Fig. 1), and expression of these transcriptional units is induced during starvation for methionine in response to a drop in the intracellular concentration of the molecular effector, SAM. The goal of the current study was to investigate the differential regulation of expression of the 11 S-box transcriptional units in *B. subtilis* and to determine whether there is a correlation between the physiological roles of S-box genes and their sensitivity to SAM in vivo and in vitro.

Northern analysis revealed that the terminated transcript generally predominates during growth in the presence of methionine. Starvation for methionine resulted in a decrease in the amount of terminated transcript and an increase in the readthrough transcript (Fig. 3). The *metK* gene, encoding SAM synthetase, was unusual in that the maximal level of the readthrough transcript was detected at 0.5 h of starvation and the level of this transcript was greatly reduced at 1 h (data not shown). The *yxjG* and *yoaD* leader RNAs formed complexes that were somewhat less stable (t_{1/2} value of 1.8 and 1.6 min, respectively). Although the *yusC* leader RNA appears to have a weak affinity for SAM (as indicated by the calculated K_d value), it is capable of forming a relatively stable complex with SAM (t_{1/2} value of 4.3 min). In general, the S-box leader RNAs that exhibit the strongest affinity for SAM (*metE*, *yitJ*, *yjcI*, *ykrT*, and *ykrW*) form the most stable SAM/leader RNA complexes, while the leader RNAs that have a weaker affinity for SAM (*yxjG* and *yoaD*) form less-stable SAM/leader RNA complexes.
conditions under which the cysH S box can affect expression in vivo.

Quantitative RT-PCR showed an increase in all S-box transcripts (except cysH) by 0.5 h of starvation; levels of the metK transcript decreased after 1.0 h, while transcript levels for all other genes continued to rise (Table 1; Fig. 4). The 28-fold range in induction ratios after 1.5 h illustrates the variability of the responses of genes in this regulon. A similar pattern was observed using S-box gene–lacZ transcriptional fusions (Table 2; Fig. 5), with a 250-fold range in induction ratios after 4 h. Expression of metK was previously shown to decrease in B. subtilis during growth in the presence of methionine (2, 46); however, those studies examined steady-state growth of a methionine prototroph rather than transient starvation of a methionine auxotroph. Our failure to detect an increase in metK-lacZ expression despite the transient increase in transcript levels suggests that metK may be subject to an additional level of regulation that is responsible for both the lack of induction of the fusion and the disappearance of the transcript under conditions when other S-box genes are highly expressed. In lactic acid bacteria, metK is regulated at the translational level in response to SAM (6, 7). B. subtilis metK may be subject to translational control or effects on mRNA stability that could not be detected with the metK-lacZ transcriptional fusion under these growth conditions.

In all of the measurements of in vivo expression, the genes that are directly involved in methionine biosynthesis (metE, which encodes methionine synthase, yjcI [metIC], which encodes cystathionine γ-synthase and β-lyase, and yitJ, which encodes methylenetetrahydrofolate reductase) exhibited the tightest regulation, including the lowest expression level during growth in the presence of methionine and the greatest increase in expression during starvation; metE and yitJ also exhibited the longest delay before induction was detected. In contrast, the yusCBA (metNPQ) operon, which encodes an ABC-type methionine transporter (8, 9, 18), exhibited a higher level of expression during growth in the presence of methionine, a lower magnitude of induction, and rapid induction. These properties are consistent with a role in methionine transport, since it is more efficient for the cell to take up and utilize exogenous methionine than to induce the biosynthetic pathway. The ykrTS and ykrWXYZ operons encode genes involved in recycling of methylthioadenosine (a by-product of utilization of SAM in polyamine biosynthesis) to methionine (9, 30, 35). These genes exhibit a regulatory pattern similar to that of the genes in the central biosynthetic pathway. The ysjG and ysjH genes, which are in-tandem, separate transcriptional units, are highly similar to each other and encode products with low similarity to methionine synthase (metE); their physiological role is unknown since they are unable to replace metE (9). Expression of both of these genes was induced, but to a level lower than that of most other S-box genes. The yoaD gene is a gene of unknown function that encodes a product similar to phosphoglycerate dehydrogenase (serA) (8, 9). This gene exhibited a pattern of induction similar to that of genes known to be involved in methionine biosynthesis, with tight repression and a >100-fold increase in transcript levels after 1 h of starvation. It therefore appears that all of the B. subtilis S-box elements except that preceding the cysH operon are functional in vivo under the conditions tested, although there is extensive variability in the level of expression when methionine is abundant, the level of response to starvation for methionine, and the kinetics of induction.

We also monitored the effect of methionine starvation on SAM pools in cells grown under the conditions used for measurements of S-box gene expression, using the SAM-dependent in vitro transcription termination assay (25, 27). Total SAM pools were at a ∼300 μM concentration during growth in the presence of methionine, consistent with previous results (27, 41). After methionine was removed from the culture medium, SAM pools remained constant for 1 h and then dropped rapidly at 1.25 to 1.5 h. Expression of the yitJ-lacZ fusion began to increase immediately after the drop in SAM pools was observed, further supporting the model that S-box genes are induced in response to a decrease in SAM pools in vivo. These results are consistent with the other measurements of S-box gene expression in vivo, which showed an increase in transcript levels, followed by an increase in fusion expression, at time points that parallel the measured drop in SAM pools. It is important to note that the extracts include both free SAM and SAM that is in complex with other cellular components. It is likely that the free SAM pool, which is available for interaction with S-box transcripts, decreases prior to the decrease in total SAM, allowing induction of S-box gene transcription to begin prior to a detectable drop in total SAM pools.

The in vitro transcription termination assay was also used to compare the sensitivity to SAM of each of the S-box leaders to the affinity for SAM of the isolated RNA. The genes varied in both their termination efficiency in the absence of SAM and the concentration of SAM required to promote efficient termination. Termination in vitro should depend not only on the affinity for SAM but also on the efficiency of the terminator element, competition between the terminator and the antiterminator, and competition between the antiantiterminator and the antiterminator. Previous mutational analysis of S-box leaders showed that disruption of elements important for SAM binding or formation of helix 1 results in constitutive antitermination in vivo and in vitro (8, 25, 26, 44), suggesting that antitermination is the default state of the system. The predicted stabilities of the terminators and antiterminators are variable and in contrast to the model do not always show significantly greater stability of the antiterminator (data not shown). During transcription, the antiterminator is synthesized prior to the terminator, which may facilitate formation of the antiterminator element even if the terminator helix has greater intrinsic stability.

A 250-fold range in $K_d$ values was demonstrated in the binding assays. In general, binding affinity for SAM correlates with sensitivity to SAM in vitro, such that genes with lower $K_d$ values (e.g., metE, yitJ, yjcI, and ykrT) responded to low concentrations of SAM in the in vitro termination assay (Table 3). The ykrW gene is exceptional in that its affinity for SAM was identical to that of yitJ ($K_d$ value of 19 nM) but it required 20-fold more SAM for a half-maximal response in vitro. Similarly, ysjG and yoaD exhibited similar $K_d$ values but a 10-fold difference in the amount of SAM required for a half-maximal response in vitro. The genes with the greatest sensitivity to SAM in vitro also exhibited the highest induction ratio in vivo. This correlation is more consistent than the $K_d$ value, since ykrW exhibited a moderate induction that compares to its re-
requirement for a higher concentration of SAM in vitro despite the high affinity of the RNA for SAM. These results indicate that while affinity for SAM of the SAM binding domain is an important parameter for regulation, other parameters (e.g., the relative strengths of the competing structural elements) play a major role in the calibration of the system.

The stability of the individual SAM/S-box RNA complexes varied from 1.6 to 11 min, and in general the genes with the highest affinity, greatest sensitivity to SAM in vitro, and tightest regulation in vivo also exhibited high levels of complex stability. The rate of transcription in vivo has been estimated at ~40 nt s⁻¹ for *Escherichia coli* growing in glucose minimal medium (40). S-box leaders are 200 to 250 nt in length. Assuming that suggests that for the genes tested here, the S-box riboswitch is the antiterminator conformation prior to termination. This to allow dissociation of SAM and refolding of the RNA into synthesis of the terminator helix and be of significant duration the complete SAM binding element (helices 1 to 4) and before but these pausing events would have to occur after synthesis of of transcription may be modulated by transcriptional pausing, elongation complex has reached the termination site. The rate of transcription may be modulated by transcriptional pausing, but these pausing events would have to occur after synthesis of the complete SAM binding element (helices 1 to 4) and before synthesis of the terminator helix and be of significant duration to allow dissociation of SAM and refolding of the RNA into the antiterminator conformation prior to termination. This suggests that for the genes tested here, the S-box riboswitch is essentially irreversible.

The response of the *yusC* leader RNA to SAM differs from that of all other S-box leader RNAs. The $t_{1/2}$ value of the SAM/yusC leader RNA complex was comparable to that of the SAM/yitJ leader complex, but the affinity of the yusC leader RNA for SAM was 170-fold lower than that of the yitJ leader RNA. This indicates that the association rate of the SAM/yusC leader RNA interaction must be lower than that of the SAM/yitJ leader RNA interaction. The yusC leader RNA showed a high level of termination in the absence of SAM, suggesting that the antiterminator does not effectively compete with the terminator, resulting in low expression even under inducing conditions. These results are consistent with the observation that expression of yusC is low even under inducing conditions and is not completely repressed in the presence of methionine in vivo compared to other S-box leader RNAs which are more tightly regulated. As noted above, the role of the *yusCBA* genes in methionine transport is consistent with less-stringent regulation of expression by SAM.

Many riboswitches include long-range loop-loop interactions that are required for binding of the molecular effector. Certain riboswitch RNAs may also prefold into a tertiary structural arrangement similar to that induced by binding of the effector molecule (15, 20, 26, 31, 37), which could facilitate the response. The yitJ RNA was previously shown to have a stronger predisposition to form the pseudoknot interaction prior to SAM binding than the ykrW leader RNA (26). This difference could be responsible for the higher sensitivity of the yitJ leader to SAM in the in vitro termination assay but is not reflected by differences in $K_d$ values or expression in vivo. While destabilization of the pseudoknot diminishes SAM binding (26), it is not clear that stability of this interaction correlates with the response to SAM.

The crystal structure of the SAM-RNA complex revealed that SAM is buried within the binding pocket of the RNA and contacts several residues in the RNA, which results in highly specific recognition (21, 29). Residues shown to be involved in the formation of the SAM binding pocket and residues that directly contact functional groups in SAM are highly conserved in the S-box leader RNAs. All S-box leaders are predicted to have a very similar tertiary structure, yet there is considerable variability in the extent of response to SAM. A key open question is the identification of elements within the S-box RNAs that are responsible for the observed differences in SAM binding and the regulatory response. It is likely that affinity for SAM, stability of the SAM-RNA complex, and the regulatory response are determined by the combination of a large number of factors, and extensive analyses will be required to dissect the contributions of each of those factors.

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**REFERENCES**

43. Reference deleted.