The Global Regulator Spx Functions in the Control of Organosulfur Metabolism in Bacillus subtilis†

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Spx is a global transcriptional regulator of the oxidative stress response in Bacillus subtilis. Its target is RNA polymerase, where it contacts the α subunit C-terminal domain. Recently, evidence was presented that Spx participates in sulfate-dependent control of organosulfur utilization operons, including the ytmI, yxeI, ssu, and yrrT operons. The yrrT operon includes the genes that function in cysteine synthesis from S-adenosylmethionine through intermediates S-adenosylhomocysteine, ribosylhomocysteine, homocysteine, and cystathionine. These operons are also negatively controlled by CymR, the repressor of cysteine biosynthesis operons. All of the operons are repressed in medium containing cysteine or sulfate but are derepressed in medium containing the alternative sulfur source, methionine. Spx was found to negatively control the expression of these operons in sulfate medium, in part, by stimulating the expression of the cymR gene. In addition, microarray analysis, monitoring of yrrT-lacZ fusion expression, and in vitro transcription studies indicate that Spx directly activates yrrT operon expression during growth in medium containing methionine as sole sulfur source. These experiments have uncovered additional roles for Spx in the control of gene expression during unperturbed, steady-state growth.

The global regulator Spx of Bacillus subtilis functions in the oxidative stress response by activating transcription of genes that function in thiol homeostasis (33, 34, 49). In this capacity, it is required for the transcriptional activation of the thioredoxin (trxA) and thioredoxin reductase (trxR) genes in response to disulfide stress. It also represses genes that function in a variety of metabolic and developmental pathways (34, 35). The activity and synthesis of Spx are stimulated when cells undergo accelerated disulfide generation resulting from encounters with toxic oxidants. The accumulated Spx interacts with RNA polymerase (RNAP) in part by contacting residues of the alpha subunit C-terminal domain (CTD), while showing no sequence-specific DNA-binding activity (36). An important feature of Spx is the N-terminal CxxC redox disulfide motif that controls Spx activity. Transcriptional activation from the trxA and trxR promoters requires that the two cysteines be in the oxidized, disulfide state. The presence of a reductant that converts the cysteines to the thiol state results in loss of transcription-stimulating activity (33). It is currently not known how the interaction of oxidized Spx protein with RNA polymerase activates transcription at promoters under Spx positive control.

In addition to its role in oxidative stress, recent studies indicate that Spx functions as a regulator of gene expression in cells undergoing steady-state growth. Spx was found to negatively affect the transcription of genes that function in the utilization of organosulfur compounds as alternative sources of sulfur (13). Such operons are repressed in minimal glucose media containing either of the two preferred sulfur sources, sulfate and cysteine. These operons are derepressed when cysteine or sulfate are replaced with methionine or an organosulfonate as sole sulfur source (8, 44, 46). The ytmI, yxeI, and ssu operons are derepressed in sulfate medium when cells bear an spx null mutation (13).

Recent studies have clarified the control of sulfur metabolism in B. subtilis, and these findings likely apply to sulfur control systems in other low-GC content gram-positive bacteria. First, cysteine, the product of sulfur assimilation, can be produced from the aforementioned organosulfur compounds, which undergo oxygenolytic cleavage to yield sulfite (10, 24) (Fig. 1), an intermediate in cysteine sulfur assimilation. Second, sulfite for cysteine production can also be obtained through uptake and reduction of inorganic sulfate. Third, cysteine can be made from methionine via the S-adenosyl homocysteine (SAH) shunt and reverse transsulfuration of homocysteine (18, 40) (Fig. 1). This is believed to involve the activities of the YrrT protein, an S-adenosylmethionine methyl (SAM) transferase; the Mtn protein, an S-adenosyl homocysteine nucleosidase; LuxS, the ribosyl homocysteinase; YrhA, a putative cystathionine β-synthase; and YrhB, a cystathionine γ-lyase. All of these proteins, except LuxS, are encoded by the yrrT operon (14, 40). Like the ytmI, yxeI, and ssu operons, the yrrT operon is repressed by sulfate and cysteine but is derepressed in methionine medium. Another route to cysteine synthesis from methionine involving the enzyme methionine-γ-lyase might exist in B. subtilis (18). This would generate methanethiol, which is a substrate for oxygenolytic cleavage, possibly catalyzed by the enzymes encoded by the ssu, ytmI, or yxeI operon, and would yield sulfite. However, no candidate gene encoding the methionine-γ-lyase has been identified.

† Supplemental material for this article may be found at http://jb.asm.org/.

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The repressor CymR (formerly YrzC) has been shown to function in the negative control of operons whose products participate in the biosynthesis of cysteine from a variety of sources (14) (Fig. 1). Both CymR and Spx exert negative control of the ytmI, yxl, and stu operons in the presence of sulfate, while only CymR is required for negative control in cysteine (13, 14) (see below).

As previously observed in microarray transcriptome analysis, the Spx-RNAP interaction increased yrt operon and cysK transcript levels (34) in cultures grown with sulfate as sole sulfur source. It also repressed genes that function in methionine biosynthesis (34) (Fig. 1). Thus, when Spx is overproduced, sulfur metabolism appears to undergo a shift that favors cysteine biosynthesis over methionine biosynthesis. An increase in yrt operon expression is characteristic of cells undergoing oxidative stress, as this was also observed in cells having depleted levels of the antioxidant thioredoxin (41) and cells treated with the thiol-specific oxidant diamide (26).

In this report, we show that Spx positively controls the cymR operon, which accounts, in part, for the role of Spx in sulfate-dependent negative control of the ytmI and yrtT operons. However, Spx has another role in organosulfur metabolism under steady-state growth. This report provides evidence that Spx controls the production of cysteine from methionine by regulating the transcription of the yrtT operon.

FIG. 1. Diagram of the organosulfur metabolic pathways of B. subtilis (18, 40). Cysteine can be produced from sulfate or from methionine/SAM. Production of sulfite is from sulfate and organosulfonates, and the genes believed to function in the process are shown. The SAH shunt-transsulfuration pathways (indicated) function in converting methionine to cysteine via SAM and SAH. The genes whose products function at each step are indicated, as are the effects of Spx activity; an arrowhead pointing up denotes Spx-dependent stimulation of expression, and an arrowhead pointing down indicates Spx-dependent repression (34). o-kh, o-ketobutyrate; O-ahs, O-acetylhomoserine; pry, pyruvate; AI-2, autoinducer-2 (48).
EcoRI-BamH1 fragment of pSxY18, thus placing the P\textsubscript{senkycmR} construct with a linked chloramphenicol resistance (cat) marker. pSxY24 was created by inserting a PCR fragment (obtained by using primers oSxY12 and oSxY05) containing the cmyr gene and upstream DNA to the transcription start site into SphI/Sall-cleaved pSxY20, thus exchanging the shorter cmyr fragment with the complete cmyr transcription unit. Plasmid pSxY27 was created by obtaining the EcoRI-BamH1 fragment of pSxY24, containing the P\textsubscript{senkycmR} construct with associated lacI gene, and inserting it into EcoRI-BamH1-cleaved pDG795, thus creating a P\textsubscript{senkycmR}\textsubscript{lacI} plasmid that could integrate into the hrc locus. The sequence of cmyr was confirmed by sequencing the plasmid with both Pspac-up and Pspac-down primers. Strain ORB4871 was transformed with plasmid pSxY27 with selection for erythromycin resistance (Erm\textsuperscript{r}) and screening for Thr\textsuperscript{r}, which created strain ORB6682. ORB6682 was constructed by transforming ORB7733 with plasmid pSxY11 with selection for Erm\textsuperscript{r} and screening for Thr\textsuperscript{r}.

A Bacillus subtilis strain carrying a cymR-lacZ translational fusion was constructed as follows. The DNA region containing the 5' regulatory and the first 16 codons of cmyr was amplified by PCR from the chromosome of strain JH462 using primers oSxY13 and oSxY14. The amplified 350-bp fragment corresponding to position –303 to +149 relative to the cmyr translational start site was digested with Sall and ligated with plasmid pTlac2 (digested with the same enzyme, which placed the cmyr coding sequence in frame with the lacZ coding sequence. The resulting plasmid, pSxY25, was then introduced into B. subtilis ZB307A with homologous recombination at the SPB prophage locus (50) and the phage lysate prepared from the transformants was used to transduce ZB278 (50). Strains ORB6080 and ORB6081, carrying the cymR-lacZ translational fusion, were constructed by transducing JH462 and ORB3834 (p::aph\textsubscript{AC}3), respectively, with the lysate prepared from ZB278 transduction.

The cymR-lacZ fusion in strain ENU25 (p::aph\textsubscript{AC}3) was constructed as follows. The 330-bp DNA containing 5' promoter and coding sequence of yprT was amplified by PCR using chromosomal DNA of strain 168 and the primer pair forward oHSY17 and reverse oHSY18. The PCR product was doubly digested with HindIII and BamHI and then ligated to the HindIII- and BamHI-cleaved pMUTIN2 plasmid. The ligated DNA was transformed for use of E. coli DH5\textalpha. After the sequence of the cloned DNA was confirmed by DNA sequencing, the resulting plasmid pSxY24, was transformed into the chromosome of B. subtilis HH482 with a single crossover event for the integrational disruption of cymR. The disruption was confirmed by resistance to erythromycin and PCR analysis of the chromosomal DNA of the erythromycin-resistant recombinant. Bacillus subtilis strain ORB4028 (p::aph\textsubscript{AC}3) was constructed by transforming strain HH5636 (38) with chromosomal DNA derived from ORB8384 (p::aph\textsubscript{AC}3).

\textbf{β-galactosidase assays.} Cells from a frozen stock or an isolated colony were used to inoculate 2 ml of 2\texttimes YT plus antibiotics. The culture was allowed to grow for several hours, after which it was diluted 100-fold into 1 ml of TSS medium supplemented with a 1 mM concentration of different sulfur sources. When required, 1 mM IPTG was added at the initial optical density of 0.600. Growth of cultures and assay of β-galactosidase activity were carried out as previously described (13). The two samples from a single time point were averaged to provide the β-galactosidase activity in Miller units (31). Experiments were repeated three times, and the data are presented as the mean of three independent experiments ±1 standard deviation.

\textbf{Primer extension analysis.} ORB4794 (spx [wild type]) and ORB4802 (spx\::aph\textsubscript{AC}3) were grown at 37°C in TSS medium containing either MgSO\textsubscript{4}, cysteine, or methionine to an OD\textsubscript{600} of 0.7. The purification of the total RNA was performed according to the method previously described (2, 27, 38) with minor modifications. After elution from a Ni-nitrilotriacetic acid (QIAGEN) column, the proteins were then passed through a heparinagarose (Sigma) column with a 100 to 800 mM NaCl gradient. The enzyme was further purified with Bio-Rad High-Q column chromatography with a 100 to 500 mM NaCl gradient prior to concentration. Finally, RNAP was dialyzed against 10 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM MgCl\textsubscript{2}, and 50% glycerol and stored at −20°C.

\textbf{In vitro transcription assay.} In vitro transcription experiments were carried out as described elsewhere (33). Linear DNA templates for proSx27 and trxA promoters were generated as previously described (33, 35), while cmyr and yrtL fragments were derived using primers oSY04 and oSY05-025 (encoding the 86-base transcript) and oYRT1 and oYRT2 (encoding a 79-base transcript), respectively.

\textbf{Microarray transcriptome analysis.} RNA was purified from HH462 and from ORB5724 cells using the previously reported procedure (34). RNA was used as template for the synthesis of dye-labeled cDNA, which was applied in a hybridization reaction with microarray slides of B. subtilis genomic DNA (2). Microarray slides were constructed from Corning UltraGAPS slides and an oligo set from Sigma-Genosys that represented 4,106 B. subtilis genes according to the Subtilist R16.1 release (http://genolist.pasteur.fr/Subtilist/). Analysis of hybridization data was carried out as previously described (2).

\textbf{DNase I footprinting.} A DNA fragment (180 bp) containing the yrtL promoter was synthesized by PCR amplification using primers oYRT1 and oYRT2. Plasmid pSxY3-3 (see Table S1 in the supplemental material) was used for the PCR template. The coding strand primer (oYRT1) was treated with 14 poly nucleotide kinase and [γ-\textsuperscript{32}P]ATP before PCR. The PCR product was separated on a nondenaturing polyacrylamide gel and purified with Elutip-d columns (Schleicher & Schuell). Dideoxy sequencing ladders were obtained using the Thermo Sequenase cycle sequencing kit (USB) and the same primer used for the footprinting reactions. DNase I footprinting reactions were performed in 10 mM Tris-HCl pH 8.0, 30 mM KCl, 10 mM MgCl\textsubscript{2}, 0.5 mM dNTPs, and 0.5 mM dithiothreitol. The reaction mix was supplemented with 100,000 cpm-labeled probe at 37°C for 20 min prior to DNase I treatment. The reactions were then precipitated with ethanol and subjected to 8% polyacrylamide–8 M urea gel electrophoresis.

\section*{RESULTS}

\textbf{CymR is required for both sulfate- and cysteine-dependent repression of the ytlI operon.} Spx is known to control sulfur metabolism in \textit{B. subtilis} under steady-state, log-phase growth conditions (13). As reported previously (13), one of the Spx-controlled operons identified was the \textit{ytmI} operon (\textit{ytmI-KLMO ytnI-RBIRytnLM}) (8, 14), which encodes highly conserved sulfur proteins functioning in the transport and utilization of alternative organosulfur sources such as sulfonates (3, 10, 23, 25, 45). A LysR homolog, encoded by a divergently oriented \textit{ytlI} gene, exerts positive control over the \textit{ytmI} operon (8), and Spx indirectly regulates \textit{ytmI} expression by controlling the expression of the \textit{ytlI} gene in medium containing sulfur. Recently, it was also reported that CymR is a negative regulator of the \textit{ytmI} operon and \textit{ytlI} gene expression (8, 14, 42). To investigate the role of CymR as a negative regulator for \textit{ytmI} expression, we expressed the \textit{ytlI-lacZ} fusion (13) in wild-type (ORB5691), spx (ORB4871), and \textit{cymR} mutant (ORB5733) strains. As shown in Fig. 2A, a much higher level of \textit{ytlI-lacZ} expression was observed in the \textit{cymR} mutant and in the spx mutant grown in medium containing sulfate as a sole source of sulfur. In contrast to the \textit{spx} mutant, which showed increased \textit{ytlI} expression only in medium containing sulfate, the \textit{cymR} mutant exhibited derepression of \textit{ytlI} in the presence of either cysteine or sulfate (Fig. 2A), suggesting that there is a mechanism governing CymR production or activity that is responsive to the presence of cysteine or sulfate.

The \textit{ytmI} operon is induced in the presence of methionine, and induction requires the LysR-like regulator \textit{YtlI} (8, 14). The expression of \textit{ytmI-lacZ} and \textit{ytlI-lacZ} was examined in strains ORB4794, ORB5730 (\textit{cymR::spc}), and ORB5732 (\textit{cymR::spc ytlI-aph\textsubscript{AC}3}) grown with sulfate or cysteine as a sole sulfur source (Fig. 2B). CymR has a negative effect on \textit{ytmI}.
expression, as shown by the observed derepression of ytmI in
the cymR mutant background. An additional mutation in the
ytlI gene eliminates the derepression of ytmI-lacZ expression in
the cymR mutant strain, consistent with the conclusion that
CymR exerts negative control over ytmI by repressing the ex-
pression of the ytlI gene encoding its activator.

The cymR mutation is an insertion of a spectinomycin resis-
tance cassette into the coding sequence. Although the resis-
tance gene is oriented such that it can drive transcription of the
downstream genes of the operon (Fig. 3A), polarity effects are
still possible. The cymR gene is the first gene of an operon (Fig.
3A) that includes the yrvO gene, encoding a putative NifS
homolog, and the trmU gene, encoding tRNA (5-methylamino-
methyl-2-thioriouridylate)-methyltransferase, an essential pro-
tein of B. subtilis (1), the product of which is required for thiou-
dine formation in tRNA. Hence, a complementation experi-
ment was undertaken to determine whether repression of ytmI-
lacZ would be restored if an ectopically expressed copy of
cymR were introduced into the unlinked thrC locus of the B.
subtilis genome. The complementation result in Fig. 2C con-
firms that CymR is a negative regulator for the ytlI gene. In the
cymR mutant, repression of ytlI is observed when a copy of
cymR (expressed from its own promoter) is supplied in trans
(ORB5809). The expression of cymR (under control of the
Pspank-hy promoter) also results in the repression of ytmI-lacZ
in the cymR mutant (data not shown), confirming that CymR is
responsible for negatively regulating the expression of ytlI and
the ytmI operon.

Expression of cymR is reduced in the spx mutant. As shown
above, ytmI-lacZ expression is derepressed in both spx and cymR
mutants in cultures grown with sulfur as sole sulfur source.
We sought to determine if cymR expression is affected by Spx,
indicating that Spx exerts its control of ytlI through the expres-
sion of the repressor gene, cymR. Primer extension analysis of
total RNA purified from a wild-type strain and an spx mutant
grown in TSS minimal medium containing sulfate, cysteine, or
methionine shows that the start site of transcription for the
cymR gene is at G located 57 bases upstream from the trans-
lation start point of cymR and downstream of the coding se-
quence belonging to the divergently oriented yrvN gene (Fig.
3A and B). The primer extension result indicates that cymR is
constitutively transcribed in the medium supplemented with
sulfate, cysteine, or methionine as a sole source of sulfur. The
spx mutation appeared to have no effect on the level of primer
extension product. The primer extension product of rpsD (ri-
bosomal protein S4 [16]), transcript levels of which are not
expected to change under the growth conditions tested, was
included as a control.

In order to examine whether expression of cymR is reduced
in spx mutant cells in vivo, a translational fusion of cymR-lacZ
was constructed and cymR-directed β-galactosidase activity in
wild-type (ORB6080) and spx mutant (ORB6081) cells was
assayed. Figure 4A and B show that in sulfate- or cysteine-
containing medium cymR-lacZ is reduced to 30% of the wild-
type level in the spx mutant. The result suggested that Spx was
required for optimal expression of cymR and that the reduced
production of repressor might account for the increase in ytlI
and ytmI expression in the spx mutant.

Western analysis was performed on two sets of protein ex-
tracts from sulfate- and cysteine-containing cultures of wild-
type and spx mutant strains. The results showed that the level
of CymR protein was twofold less in the spx mutant than in
wild-type cells when cultures were grown in TSS-sulfate me-
dium (Fig. 5A). However, in cysteine medium, only a slight

FIG. 2. Control of ytmI and ytlI by Spx and CymR. A. Expression of
ytmI-lacZ in the wild type (ORB5691) and spx (ORB4871) and cymR
(ORB5733) mutant strains. Cells of each fusion strain were grown in
TSS medium containing MgSO4 (SO4) or cysteine (Cys) as sole sulfur
source. Samples were collected at log phase and assayed for
β-galactosidase activity, which was expressed in Miller units. B. Expression of
ytmI-lacZ in wild-type (ORB4794), cymR (ORB5730), and cymR ytlI
(ORB5732) strains. Mutation of ytlI (ORB5732) eliminates the derep-
pression of ytmI in the cymR mutant. C. Complementation of cymR
with a thrC::cymR construct, ytlI-directed β-galactosidase activity of the
cymR mutant strain bearing a ytmI-lacZ fusion (strain ORB5733) and
the fusion-bearing partial diploid strain, ORB5809, with a thrC::cymR−
construct are shown.
A decrease in CymR protein level was observed when an spx mutation was introduced. To determine if the Spx enhances cymR transcription by interacting with RNAP, in vitro transcription was carried out using RNAP holoenzyme from an spx null mutant, with and without added Spx protein. There was no observable effect of Spx on in vitro transcription initiation from the cymR promoter DNA or the negative control promoter of the rpsD gene (Fig. 5B), while Spx was observed to activate transcription from the trxB promoter, as previously reported (33).

If Spx were necessary for optimal cymR expression, then releasing cymR expression from Spx control should result in repression of ytlI in an spx mutant. Expression of the cymR gene under Pspank-hy promoter control in the spx mutant

FIG. 3. A. The diagram top right shows the organization of the cymR operon containing the yrvO and trmU genes, based on primer extension analysis of cymR RNA in wild-type and spx mutant cells. Cultures were grown in TSS minimal medium containing either MgSO₄ (S), cysteine (C), or methionine (M) to an OD₆₀₀ of 0.7, and total RNA was isolated. Primer extension reactions and the sequencing reaction were performed using oligonucleotide oSY09. Primer extension products were resolved by polyacrylamide gel electrophoresis and visualized by phosphorimaging. Four lanes on the left are the gel profiles of sequencing reactions A, G, C, and T. Primer extension was also done with an rpsD-specific primer for a control of constitutive expression. B. Diagram of the nucleotide sequence of the cymR promoter region, showing the −10 sequence (TG promoter) and −35 along with the regions of dyad symmetry upstream of the promoter (dotted arrows). Coding sequences of the cymR and divergently oriented yrvN genes are shown with associated ribosome-binding sites (rbs) and a putative transcriptional start site for cymR (bent arrow).
(ORB6082) restored repression of ytlI (Fig. 5C) in the presence of IPTG, which indicates that the requirement of Spx in negative control of ytlI is due, at least in part, to its role in stimulating cymR expression.

The amount of CymR produced in the spx mutant in sulfate medium might not be sufficient to repress ytlI expression, while the amount produced by the mutant in cysteine medium is enough to repress the expression. The reduced amount of CymR in the spx mutant results in derepression of ytlI and enhanced expression of the ytmI operon, as shown in Fig. 2A. In cysteine medium, the low expression of cymR-lacZ in the spx mutant evidently is not an indication of the concentration of active CymR, since protein levels are higher in cysteine-grown spx mutant cells than in mutant cells grown in sulfate.

Transcriptional profile of the CymR regulon has recently been published by the Martin-Verstraete group (14); in this profile genes functioning in cysteine biosynthesis and those involved in the general stress response were derepressed in the cymR in-frame deletion mutant. To undertake transcriptional profil-
TABLE 2. Genes induced by cymR mutation

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<th>Function</th>
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<td>tuxA</td>
<td>Translocation-dependent antimicrobial spore protein</td>
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<tr>
<td>cyxM</td>
<td>Cysteine synthetase</td>
<td>1.95</td>
</tr>
<tr>
<td>yxeL</td>
<td>yxe operon, organosulfur utilization</td>
<td>1.94</td>
</tr>
<tr>
<td>yxeD</td>
<td>yxe operon, organosulfur utilization</td>
<td>1.9</td>
</tr>
<tr>
<td>ytoI</td>
<td>yto operon, organosulfur utilization</td>
<td>1.85</td>
</tr>
<tr>
<td>ygaM</td>
<td>yga operon, thioesterase</td>
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<tr>
<td>sqw</td>
<td>Signal peptide I</td>
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<tr>
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<td>yxe operon, organosulfur utilization</td>
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<tr>
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<tr>
<td>cssS</td>
<td>Secretion stress sensor kinase</td>
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<tr>
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<td>Spore coat</td>
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<tr>
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<td>yfeD</td>
<td>Similar to glucose 1-dehydrogenase</td>
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<tr>
<td>yxcl</td>
<td>Unknown</td>
<td>1.62</td>
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<tr>
<td>pel</td>
<td>Pectate lyase</td>
<td>1.53</td>
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<tr>
<td>hpr</td>
<td>Leucine-responsive protein, represses glyA and kinB</td>
<td>1.53</td>
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<tr>
<td>lacs</td>
<td>lacs operon, sodium utilization</td>
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The ytmL, yxeI, and ssu operons show derepression in the cymR mutant background. It is likely, therefore, that the reduced expression of yxeI and ssu in the spc mutant (13) is due to the reduced expression of cymR in the spc background. Additionally, the yrrT operon genes show derepression in the cymR mutant. Genes of the yrrT operon encode enzymes of the S-adenosylhomocysteine shunt and reverse transsulfuration pathway that yield cysteine from S-adenosylmethionine (Fig. 1 and 6A). Unlike the previously published cymR transcription profile (14), we observed little significant induction of general stress operons, although the katA gene, a member of the hydrogen peroxide-induced PerR regulon (7) and encoding the vegetative catalase, is significantly induced in the cymR mutant. It is possible that differences in expression profiles between microarray hybridization analyses reported herein and published previously could be the result of different growth media (TSS glucose-glutamate in our work and phosphate-buffered medium containing glucose and glutamine in the work of Even et al. [14]) used in cultures of wild-type and cymR mutant strains. The use of different cymR alleles could have also affected the results of the microarray analyses.

Control of the yrrT operon by CymR and Spx. In contrast to the ytmL, yxeI, and ssu operons, which are under the negative control of both Spx and CymR, the yrrT operon is positively controlled by Spx and is one of the most highly induced transcription units observed in microarray analysis of Spx-overexpressing cells (34). Thus, yrrT is under CymR negative control (14), while Spx appears to exert positive control. We validated these results using a yrrT operon-lacZ fusion introduced into the yrrT locus. As shown previously (14), yrrT-lacZ is derepressed in the cymR cells (Fig. 6B) and, like ytlI and the ytm operon, repression in sulfur medium requires Spx (Fig. 6C). In methionine medium, yrrT-lacZ is derepressed in wild-type and cymR mutant cells; however, derepression requires Spx. This is the first indication that Spx can activate transcription during log phase in the presence of an alternative sulfur source (methionine).

Spx activates transcription from the yrrT promoter in vitro. To determine if Spx can directly stimulate transcription from the yrrT promoter, a fragment containing the intergenic region between the divergently organized yrrT and yrrA coding sequences (Fig. 7A) was obtained by PCR. This region contains the yrrT operon promoter, the sequence for which can be discerned by inspection of the intergenic DNA sequence (Fig. 7A). The TG-type promoter —10 region (9) is located between 66 and 77 bp upstream of the start of the yrrT coding sequence. Promoters with extended —10 TG elements are more prevalent in B. subtilis than in E. coli, and such promoters have a reduced dependence on the RNAS interaction with the —35 element. Using the downstream PCR primer that hybridizes to the first eight codons of yrrT, template DNA was synthesized by PCR for in vitro transcription reactions. A transcript of 82 bases was generated in the in vitro transcription reaction containing the PCR product, as shown in Fig. 7B (the trxB transcript is 88 bases). This transcript corresponds to the predicted start point and promoter sequence indicated (Fig. 7A). Transcription from this promoter is stimulated by Spx, as evident from the elevated amount of transcript synthesized in reactions containing RNAP and Spx protein (Fig. 7B). As shown in previously reported work (33), the addition of the reductant
dithiothreitol (DTT) eliminates trxB transcription due to the conversion of the CxxC redox disulfide center of Spx to the thiol state. In contrast, the reaction containing the yrrT promoter DNA, RNAP, and Spx shows stimulation of yrrT transcription by Spx even in the presence of DTT, although the amount of transcript accumulation is less than in the absence of reductant. Consistent with the previous microarray transcriptome analysis (34), yrrT is a member of the Spx regulon and is under the direct positive control of Spx, but the oxidized form of Spx is not essential for the activation, as suggested by the β-galactosidase assay carried out with yrrT-lacZ fusion-bearing cells under steady-state, log-phase growth conditions (or under nonoxidative conditions in vitro).

We used purified CymR protein to determine if it could repress transcription of yrrT in transcription reactions containing purified RNAP and Spx. As shown in Fig. 7C, added CymR caused repression of yrrT in the presence and absence of Spx but had no effect on Spx-dependent transcription from the trxB promoter. This result is in keeping with the previously reported finding that CymR protein binds to DNA bearing the yrrT regulatory region (14). The direct binding of CymR to an operator element residing downstream of the transcription start site was reported (14). This was confirmed by DNase I footprinting using a fragment of yrrT promoter DNA and purified CymR protein. As shown in Fig. 7D, CymR protected a region between +11 and +28, which corresponds closely with the CymR-binding sequence suggested in the previously reported electrophoretic mobility shift assay analysis (14).

**DISCUSSION**

Organosulfur metabolism in *Bacillus* is controlled by a number of regulatory systems that act at the level of transcription initiation and during transcription elongation. An example of the latter is the S-box control mechanism that governs expression of genes that function in methionine synthesis in response to levels of SAM (17). SAM binds directly to the nascent RNA at a folded sequence known as the S-box, within the untranslated leader region encoded by *met* genes (30). High SAM levels stabilize a terminator that reduces transcription of the downstream coding regions. As for regulation of transcription initiation, LysR-type regulators show operon-specific activity. For example, CysL and YtlI activate transcription of *cysIJ* (sulfite reductase) and *ytmI* (sulfonate utilization and cysteine transport), respectively (8, 11, 19).

The work described herein has uncovered multiple roles for Spx in sulfur metabolism. First, Spx functions in sulfate-dependent control of organosulfur utilization operons through stimulation of cymR expression. Secondly, microarray transcriptome analysis reveals that transcript levels of genes required for cysteine synthesis, the yrrT operon and cysK, increase when Spx interacts with RNAP (34). The stimulation of homocysteine production and transsulfuration is characteristic of cells responding to oxidative stress (4, 12, 21, 28, 29, 37, 39, 47). In mammalian systems, cysteine production is accelerated during bacterial infection because of an increased need for glutathione. Oxidative stress stimulates the activity of cystathionine β-synthase, which catalyzes the first step of homocysteine transsulfuration. Thus, by stimulating the SAH shunt and homocysteine transsulfuration, Spx appears to perform a regulatory function that operates in species representing several phylogenetic levels.

The CymR repressor exerts wide control over a number of sulfur metabolism operons and is responsible for their repression when the preferred sulfur sources, sulfate and cysteine, are present (14). Evidence was reported that CymR binds
FIG. 7. Transcription from the yrrT promoter is activated by Spx in vitro. A. Organization of the yrrT operon. Dotted arrows indicate regions of dyad symmetry. The $-10$ TG promoter is indicated, as is the $-35$ sequence. The dashed arrow indicates the complementary sequence of the downstream primer used to construct the transcription promoter fragment by PCR. The bent arrow indicates the approximate location of the transcription start site as deduced from the size of the in vitro transcript. B. Gel profiles of transcription reactions containing promoter fragments trxB or yrrT (10 nM) and RNAP (50 nM) in the presence and absence of Spx and DTT. Concentrations of Spx and DTT are indicated. C. Repression of yrrT transcription by CymR. In vitro runoff transcription reaction mixtures contained RNAP (25 nM) and either trxB promoter (20 nM) or yrrT promoter (10 nM) DNA. Spx and CymR were added to the reaction mixtures at the indicated concentrations. The lower panel shows a plot of band intensities of the in vitro runoff transcription of yrrT shown in the upper panel. Band intensities were quantified using ImageQuant version 5.2, and error bars were derived from two repeats of the experiment. D. DNase I footprinting of the yrrT promoter with CymR. The yrrT promoter prepared by PCR using oyrrT-1 and oyrrT-2 is shown. Reaction mixtures contained CymR and promoter DNA with a radiolabeled coding strand. The concentrations of CymR used were 0.5, 1, 2, and 4 μM. The positions relative to the transcription start site are shown on the left. The $-10$ and $+1$ are shown next to the dideoxy sequencing ladders. The protected region is indicated by the solid line on the right side.
directly to the promoter DNA of genes under its control (14). Optimal levels of CymR protein require the activity of Spx as shown herein by Western blot analysis and by examining the expression of a cymR-lacZ fusion. The contribution of Spx to control of cymR is likely indirect, as Spx has no effect on transcription from the cymR promoter in vitro. That the promoter of cymR is utilized by purified RNAP in vitro suggests that cymR does not require a positive transcriptional control factor for expression. Spx could function indirectly by affecting the expression of a gene encoding the direct transcription factor. Alternatively, through its control of other genes that function in organosulfur metabolism and cysteine biosynthesis, Spx could affect the concentration of a metabolite, perhaps an intermediate of sulfur assimilation or organosulfur utilization, which serves as an effector of cymR expression control.

The third function of Spx in sulfur metabolism is the derepression of certain organosulfur metabolism genes in methionine medium. The yrrT gene is derepressed in a cymR mutant, but this derepression requires Spx (Fig. 6). This is observed under nonstress conditions when methionine is the sole sulfur source. Consistent with this observation is the finding that Spx can stimulate yrrT transcription in vitro in the presence or absence of reductant, unlike the control it exerts in the transcription from the trxB and trxA promoters (33, 34). Spx can interact with RNA polymerase in vitro in the presence of reductant, as observed in reactions in which Spx repressed ComA-dependent transcription from the srf operon promoter (35; Y. Zhang and P. Zuber, unpublished data). From the evidence detailed herein, we can conclude that Spx can activate gene transcription under steady-state growth conditions.

A diagram (Fig. 8) is shown that summarizes the proposed Spx-dependent control that governs the expression of genes functioning in sulfur metabolism. Spx promotes negative control (by stimulating CymR production) of operons that function in cysteine synthesis from alternative sulfur sources but stimulates yrrT operon expression in the absence of cysteine, thus promoting cysteine synthesis through S-adenosyl methionine catalysis (Fig. 1). Microarray analysis (34) also suggests that Spx represses expression of genes required for methionine biosynthesis, a role of Spx that is currently under investigation (M. Derr, Y. Zhang, and P. Zuber, unpublished data). Our findings uncover an expanded role in global transcriptional control mediated by the Spx-RNAP interaction.

FIG. 8. Diagram summarizing the proposed role of Spx in the control of organosulfur metabolism. Spx is required for the optimal production of the CymR repressor, which exerts negative control of the organosulfur utilization operons ytmI, yxeI, and ssu, and the methionine utilization pathway that generates cysteine via reactions catalyzed by the products of the yrrT operon. Spx exerts direct positive control of yrrT transcription under conditions that result in elevated Spx protein concentration (oxidative stress) and when cells must produce cysteine from alternative forms of sulfur, such as methionine.


