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 media 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 (*trxB*) 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 *trxB* 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.

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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). α -kb, α -ketobutyrate; O-ahs, O-acetylhomoserine; pyr, pyruvate; AI-2, autoinducer-2 (48).

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*, *yxel*, and *ssu* operons in the presence of sulfate, while only CymR is required for negative control in cysteine medium (13, 14) (see below).

As previously observed in microarray transcriptome analysis, the Spx-RNAP interaction increased *yrrT* 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 *yrrT* 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 *yrrT* 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 *yrrT* operon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. subtilis* strains used in this work are listed in Table 1 and are all derivatives of strain JH642. *Escherichia coli* DH5 α was grown at 37°C in 2 $\times$ YT (yeast extract, tryptone) liquid or on LB agar medium. *B. subtilis* cells were grown at 37°C in 2 $\times$ YT, Difco sporulation medium (20), or TSS minimal medium (15) with some modification (37.4 mM NH $_4$ Cl, 2 mM K $_2$ HPO $_4$, 49.5 mM Tris pH 7.5, 5 mM MgCl $_2$, 0.5% glucose, 0.004% FeCl $_3$, 0.004% citric acid, 0.1% L-glutamate, 0.005% amino acids for auxotrophic requirements) and supplemented with one of the following sulfur sources at a concentration of 1 mM: L-cysteine, L-methionine, or MgSO $_4$. Iso-propyl- β -D-thiogalactopyranoside (IPTG) was added to culture media at a concentration of 1 mM where appropriate. When necessary, the following antibiotics were added (at the concentration shown in parentheses): ampicillin (25 μ g/ml), neomycin (5 μ g/ml), chloramphenicol (5 μ g/ml), erythromycin plus lincomycin (1 and 25 μ g/ml, respectively), and spectinomycin (75 μ g/ml).

Plasmids and construction of *Bacillus* strains. Plasmids constructed in this study are listed in Table S1 of the supplemental material. Primers used in this study are listed in Table S2 of the supplemental material.

B. subtilis strain ORB5724 (*cymR::spx*) was constructed as follows. The *B. subtilis* JH642 chromosomal DNA region covering the complete coding sequence of *cymR* (nucleotides -303 to +617 relative to the translational start site) was amplified by PCR using oligonucleotides oSY2 and oSY3 with the creation of EcoRI and AseI sites. The amplified DNA fragment was inserted into the EcoRI and NdeI sites of pUC18, producing pSY5. A spectinomycin resistance cassette (Sp r) was isolated from pDG1727 (7) by digestion with StuI and EcoRV, inserted into pSY5 digested with NdeI, and then filled in using T4 DNA polymerase and deoxynucleotide triphosphates. The ligated recombinant pSY7, which bears the *spx* cassette in the same orientation with the *cymR* transcription unit, was introduced into JH642. Growth of the resultant mutant was normal, indicating that there was no adverse effect on the expression of the downstream genes of the *cymR* operon, one of which is the essential *tmu* gene (1). Plasmid pSY11 is a derivative of pDG795 that contains the *cymR* gene and promoter region. The *cymR* fragment was synthesized by PCR using primers oSY4 and oSY5 (see Table S2 in the supplemental material) and then treated with BamHI and EcoRI. The fragment was then inserted into BamHI-EcoRI-cleaved pDG795 (43).

B. subtilis strain ORB6082 carries the *cymR* coding sequence under the control of the IPTG-inducible P_{spank-hy} promoter (6). The P_{spank-hy} construct was made as follows. Plasmid pSY13 was constructed by inserting a 450-bp PCR fragment (created using primers oSY07 and oSY05) containing the *cymR* coding sequence and ribosome-binding site into SphI/SalI-cleaved pDR66. Plasmid pSY18 was constructed by obtaining the SphI-SalI fragment bearing the *cymR* coding sequence and ribosome-binding site from pSY13 and inserting it into SphI/SalI-cleaved pDR111, thus placing the *cymR* gene under P_{spank-hy} control. Plasmid pSY20 was created by exchanging the EcoRI-BamHI fragment of pDR66 (containing the Pspac promoter and associated *lacI* gene) with the homologous

TABLE 1. *Bacillus subtilis* strains

Strain	Genotype	Source or reference
MH5636	<i>trpC2 pheA1 rpoC_{His10}</i>	5
ORB3834	<i>trpC2 pheA1 spx::aphA3</i>	32
ORB4028	<i>trpC2 pheA1 rpoC_{His10} spx::aphA3</i>	This study
ORB4794	<i>trpC2 pheA1 ytmI'-lacZ erm ΔytmI</i>	13
ORB4802	<i>trpC2 pheA1 ytmI'-lacZ ΔytmI spx::aphA3</i>	13
ORB4871	<i>trpC2 pheA1 amyE::ytmI'-lacZ spx::aphA3</i>	13
ORB5691	<i>trpC2 pheA1 amyE::ytmI'-lacZ</i>	This study
ORB5724	<i>trpC2 pheA1 cymR::spx</i>	This study
ORB5730	<i>trpC2 pheA1 ytmI'-lacZ erm ΔytmI cymR::spx</i>	This study
ORB5732	<i>trpC2 pheA1 ytmI'-lacZ erm ΔytmI ytmI::aphA3 cymR::spx</i>	This study
ORB5733	<i>trpC2 pheA1 amyE::ytmI'-lacZ cymR::spx</i>	This study
ORB5809	<i>trpC2 pheA1 amyE::ytmI'-lacZ cymR::spx thrC::cymR (pSY11)</i>	This study
ORB6080	<i>trpC2 pheA1 SPβc2 del2::Tn917::cymRTL-lacZ</i>	This study
ORB6081	<i>trpC2 pheA1 SPβc2 del2::Tn917::cymRTL-lacZ (pSY25) spx::aphA3</i>	This study
ORB6082	<i>trpC2 pheA1 amyE::ytmI'-lacZ thrC::P_{spank-hy}-cymR (pSY27) spx::aphA3</i>	This study
HNU25	<i>trpC2 pheA1 yrrT'-lacZ erm ΔyrrT</i>	This study
HNU26	<i>trpC2 pheA1 yrrT'-lacZ erm ΔyrrT cymR::spx</i>	This study
HNU27	<i>trpC2 pheA1 yrrT'-lacZ erm ΔyrrT spx::aphA3</i>	This study

EcoRI-BamHI fragment of pSY18, thus placing the $P_{\text{spank-hy-cymR}}$ construct with a linked chloramphenicol resistance (*cat*) marker. pSY24 was created by inserting a PCR fragment (obtained by using primers oSY12 and oSY05) containing the *cymR* gene and upstream DNA to the transcription start site into SphI/SalI-cleaved pSY20, thus exchanging the shorter *cymR* fragment with the complete *cymR* transcription unit. Plasmid pSY27 was created by obtaining the EcoRI-BamHI fragment of pSY24, containing the $P_{\text{spank-hy-cymR}}$ construct with associated *lacI* gene, and inserting it into EcoRI-BamHI-cleaved pDG795, thus creating a $P_{\text{spank-hy-cymR}}$ plasmid that could integrate into the *thrC* locus. The sequence of *cymR* was confirmed by sequencing the plasmid with both Pspac-up and Pspac-down primers. Strain ORB4871 was transformed with plasmid pSY27 with selection for erythromycin resistance (Erm^r) and screening for Thr^- , which created strain ORB6082. ORB5809 was constructed by transforming ORB5733 with plasmid pSY11 with selection for Erm^r and screening for Thr^- .

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 *cymR* was amplified by PCR from the chromosome of strain JH642 using primers oSY13 and oSY14. The amplified 350-bp fragment corresponding to position -303 to +49 relative to the *cymR* translational start site was digested with SalI and ligated with plasmid pTKlac (22) digested with the same enzyme, which placed the *cymR* coding sequence in frame with the *lacZ* coding sequence. The resulting plasmid, pSY25, was then introduced into *B. subtilis* ZB307A by 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 JH642 and ORB3834 (*spx::aphA3*), respectively, with the lysate prepared from the ZB278 transductants.

The *yrrT-lacZ* fusion strain HNU25 was constructed as follows. The 330-bp DNA containing 5' promoter and coding sequence of *yrrT* 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 used for transformation of *E. coli* DH5 α . After the sequence of the cloned DNA was confirmed by DNA sequencing, the resulting plasmid, pSY42, was introduced into the chromosome of *B. subtilis* JH642 by a single crossover event for the integrational disruption of *yrrT*. The disruption was confirmed by resistance to erythromycin and PCR analysis of the chromosomal DNA of the erythromycin-resistant recombinant. *Bacillus subtilis* strain ORB4028 (*rpoC*^{His10} *spx::aphA3*) was constructed by transforming strain MH5636 (38) with chromosomal DNA derived from ORB3834 (*spx::aphA3*).

β -Galactosidase assays. Cells from a frozen stock or an isolated colony were used to inoculate 2 ml of 2 $\times$ 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 at 600 nm (OD_{600}) of 0.05. 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 $\pm$ 1 standard deviation.

Primer extension analysis. ORB4794 (*spx* [wild type]) and ORB4802 (*spx::aphA3*) were grown at 37°C in TSS medium containing either MgSO_4 , cysteine, or methionine to an OD_{600} of 0.7. The purification of the total RNA and the primer extension reactions were performed as described previously (34). Primer oSY9 was used to detect the *cymR* transcript. As a control, the *rpsD* transcript was detected by primer extension using oligonucleotide primer oSN03-87.

Overproduction and purification of CymR protein. For production of recombinant CymR protein, the IMPACT system (New England BioLabs), which utilizes the inducible self-cleaving intein tag, was used. The *cymR* gene was amplified by PCR using JH642 chromosomal DNA and oSY10 and oSY11 primers. The amplified DNA fragment, after being digested with NcoI and SmaI, was inserted into pTYB4 (New England BioLabs) digested with the same enzymes to generate pSY22. The nucleotide sequence of cloned *cymR* was verified by DNA sequencing. *E. coli* ER2566 (New England BioLabs) carrying pSY22 was grown in 2 $\times$ YT at 37°C. When the culture reached an OD_{600} of 0.8, 0.5 mM IPTG was added to induce *cymR* expression. Incubation was continued overnight at 17°C. The procedure for further purification was described previously (34).

RNAP purification. RNAP was purified according to the method previously described (27, 38) with minor modifications. After elution from a Ni-nitrilotriacetic acid (QIAGEN) column, the proteins were then passed through a heparin-agarose (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_2 , and 50% glycerol and stored at -20°C.

In vitro transcription assay. In vitro transcription experiments were carried out as described elsewhere (33). Linear DNA templates for *rpsD* and *trxB* promoters were generated as previously described (33, 35), while *cymR* and *yrrT* fragments were derived using primers oSY04 and oDYR05-025 (encoding an 86-base transcript) and oyrrT1 and oyrrT2 (encoding a 79-base transcript), respectively.

Microarray transcriptome analysis. RNA was purified from JH642 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).

DNase I footprinting. A DNA fragment (180 bp) containing the *yrrT* promoter DNA was synthesized by PCR amplification using primers oyrrT-1 and oyrrT-2. Plasmid pJSY3-3 (see Table S1 in the supplemental material) was used for the PCR template. The coding strand primer (oyrrT-1) was treated with T4 polynucleotide kinase and [γ - ^{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_2 , 0.5 mM β -mercaptoethanol. Proteins were incubated with 100,000 cpm-labeled probe at 37°C for 20 min prior to DNase I treatment. The reactions were then precipitated by ethanol and subjected to 8% polyacrylamide-8 M urea gel electrophoresis.

RESULTS

CymR is required for both sulfate- and cysteine-dependent repression of the *ytlI* operon. *Spx* is known to control sulfur metabolism in *B. subtilis* under steady-state, log-phase growth conditions (13). As reported previously (13), one of the *Spx*-controlled operons identified was the *ytmI* operon (*ytmI-JKLMNO ynlIribRytnLM*) (8, 14), which encodes highly conserved 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 *ytlI* gene, exerts positive control over the *ytmI* operon (8), and *Spx* indirectly regulates *ytmI* expression by controlling the expression of the *ytlI* gene in medium containing sulfate. Recently, it was also reported that CymR is a negative regulator of the *ytmI* operon and *ytlI* gene expression (8, 14, 42). To investigate the role of CymR as a negative regulator for *ytlI* expression, we expressed the *ytlI'-lacZ* fusion (13) in wild-type (ORB5691), *spx* (ORB4871), and *cymR* mutant (ORB5733) strains. As shown in Fig. 2A, a much higher level of *ytlI'-lacZ* expression was observed in the *cymR* mutant and in the *spx* mutant grown in medium containing sulfate as a sole source of sulfur. In contrast to the *spx* mutant, which showed increased *ytlI* expression only in sulfate medium, the *cymR* mutant exhibited derepression of *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 *ytmI* operon is induced in the presence of methionine, and induction requires the LysR-like regulator YtlI (8, 14). The expression of *ytmI'-lacZ* and *ytlI-lacZ* was examined in strains ORB4794, ORB5730 (*cymR::spx*), and ORB5732 (*cymR::spx ytlI::aphA3*) grown with sulfate or cysteine as a sole sulfur source (Fig. 2B). CymR has a negative effect on *ytmI*

FIG. 2. Control of *ymtI* and *ytII* by Spx and CymR. A. Expression of *ytII'*-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 *ytII* (ORB5732) eliminates the derepression of *ytmI* in the *cymR* mutant. C. Complementation of *cymR* with a *thrC*::*cymR* construct. *ytII*-directed β -galactosidase activity of the *cymR* mutant strain bearing a *ytII*-lacZ fusion (strain ORB5733) and the fusion-bearing partial diploid strain, ORB5809, with a *thrC*::*cymR*⁺ construct are shown.

expression, as shown by the observed derepression of *ytmI* in the *cymR* mutant background. An additional mutation in the *ytII* 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 expression of the *ytII* gene encoding its activator.

The *cymR* mutation is an insertion of a spectinomycin resistance cassette into the coding sequence. Although the resistance 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-thiouridylate)-methyltransferase, an essential protein of *B. subtilis* (1), the product of which is required for thiouridine formation in tRNA. Hence, a complementation experiment was undertaken to determine whether repression of *ytII*-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 confirms that CymR is a negative regulator for the *ytII* gene. In the *cymR* mutant, repression of *ytII* 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 P_{spank-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 *ytII* and the *ytmI* operon.

Expression of *cymR* is reduced in the *spx* mutant. As shown above, *ytII*-lacZ expression is derepressed in both *spx* and *cymR* mutants in cultures grown with sulfate as sole sulfur source. We sought to determine if *cymR* expression is affected by Spx, indicating that Spx exerts its control of *ytII* through the expression 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 translation start point of *cymR* and downstream of the coding sequence 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* (ribosomal 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 *ytII* and *ytmI* expression in the *spx* mutant.

Western analysis was performed on two sets of protein extracts 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 medium (Fig. 5A). However, in cysteine medium, only a slight

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).

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 *ytII* in an *spx* mutant. Expression of the *cymR* gene under P_{spx} promoter control in the *spx* mutant

FIG. 4. Control of *cymR* expression by Spx. Expression of a translational *cymR-lacZ* fusion over time in ORB6080 (*cymR-lacZ*; diamonds) and ORB6081 (*spx::aphA3 cymR-lacZ*; squares) grown in TSS medium containing either MgSO₄ (SO₄) (A) or cysteine (Cys) (B) is shown. The x axis represents the time (in hours) of cell growth.

(ORB6082) restored repression of *ytII* (Fig. 5C) in the presence of IPTG, which indicates that the requirement of Spx in negative control of *ytII* 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 *ytII* 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 *ytII* 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.

Microarray hybridization analysis uncovers the CymR regulon. Previous studies showed that Spx exerts negative control of the *ytmI*, *yxel*, and *ssu* operons in medium containing sulfate as sole sulfur source. As with *ytmI*, the role of *spx* in *yxel* and *ssu* expression is likely the enhancement of *cymR* expression, which leads to repression of the two operons by the CymR protein. We sought to determine if this was the case by performing a genomic microarray hybridization experiment to uncover the genes that are negatively controlled by CymR. A

FIG. 5. Spx controls *cymR* indirectly. A. Western analysis of CymR protein levels in wild-type (WT) and *spx* mutant cells. Strains ORB5691 (*ytII-lacZ*) and ORB4871 (*ytII-lacZ spx::aphA3*) were grown in TSS medium containing MgSO₄ or cysteine as the sulfur source. Cleared lysates from mid-log-phase cells were obtained after lysis by French press and centrifugation. Lysate from ORB5724 (*cymR::aphA*) was used as a negative control. B. In vitro runoff transcription reactions of the *cymR* promoter fragment (5 nM) in the reaction mixtures containing purified RNAP in the presence and absence of Spx. Concentrations of Spx in the reaction mixtures are indicated. A positive control is the *trxB* promoter fragment (10 nM), which shows activation by Spx. The *rpsD* template DNA (20 nM) containing the ribosomal protein S4 gene promoter shows no effect by Spx. C. The CymR repressor, supplied with the Pspac promoter in the *spx* mutant, restores the repression of *ytII*. Cells of WT and *spx* mutant strains (indicated) bearing the *ytII-lacZ* fusion were transformed with plasmid pSY20, in which the *cymR* gene was placed under the control of P_{spac-hy}. Cultures were grown to mid-log phase in sulfate medium in presence or absence of IPTG. Samples were collected and assayed for β-galactosidase activity.

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

Gene	Function	Induction ^a
<i>ytmL</i>	<i>ytmI</i> operon, sulfonate utilization	6.72
<i>ytmI</i>	<i>ytmI</i> operon, sulfonate utilization	6.51
<i>ytmJ</i>	<i>ytmI</i> operon, sulfonate utilization	6.42
<i>ytmO</i>	<i>ytmI</i> operon, sulfonate utilization	6.42
<i>ssuB</i>	<i>ssu</i> operon, sulfonate utilization	6.35
<i>ytmN</i>	<i>ytmI</i> operon, sulfonate utilization	6.23
<i>ssuC</i>	<i>ssu</i> operon, sulfonate utilization	6.16
<i>ssuA</i>	<i>ssu</i> operon, sulfonate utilization	6.13
<i>ytmK</i>	<i>ytmI</i> operon, sulfonate utilization	6.07
<i>ytmI</i>	<i>ytmI</i> operon, sulfonate utilization	5.93
<i>ytmM</i>	<i>ytmI</i> operon, sulfonate utilization	5.75
<i>ygaN</i>	<i>ssu</i> operon, sulfonate utilization	5.39
<i>ytmI</i>	<i>ytmI</i> operon, sulfonate utilization	5.09
<i>yezD</i>	Unknown	4.97
<i>ssuD</i>	<i>ssu</i> operon, sulfonate utilization	4.55
<i>ytmM</i>	<i>ytmI</i> operon, sulfonate utilization	4.44
<i>ribR</i>	<i>ytmI</i> operon, sulfonate utilization	4.19
<i>yrhB</i>	Cysteine synthesis, cystathionine gamma synthase	3.67
<i>yrhA</i>	Cysteine synthesis	3.51
<i>yrpT</i>	S-Adenosylmethionine-dependent methyltransferase	3.45
<i>cah</i>	Cephalosporin C deacetylase	3.37
<i>ytII</i>	LTTR, ^b activator of <i>ytmI</i> operon	2.81
<i>katA</i>	Catalase	2.68
<i>ytIB</i>	Unknown	2.65
<i>yjnA</i>	Predicted permease	2.53
<i>yhcL</i>	<i>tcyP</i> , cystine transport	2.53
<i>yhzaA</i>	Ribosomal protein S14, <i>ytmI</i> operon	2.49
<i>yxep</i>	<i>yxel</i> operon, organosulfur utilization	2.4
<i>yxex</i>	<i>yxel</i> operon, organosulfur utilization	2.39
<i>yxen</i>	<i>yxel</i> operon, organosulfur utilization	2.37
<i>mtn</i>	Methylthioadenosine nucleosidase	2.31
<i>yxex</i>	<i>yxel</i> operon, organosulfur utilization	2.28
<i>yogM</i>	SPB phage gene	2.27
<i>yegN</i>	Similar to 1-pyrroline-5-carboxylate dehydrogenase	2.25
<i>yrhC</i>	<i>yrpT-mtn</i> operon, unknown	2.16
<i>yxex</i>	<i>yxel</i> operon, organosulfur utilization	2.15
<i>yxem</i>	<i>yxel</i> operon, organosulfur utilization	2.11
<i>yqxi</i>	Unknown, secreted	2.02
<i>tgl</i>	Transglutaminase	2.01
<i>tasA</i>	Translocation-dependent antimicrobial spore protein	2
<i>cysK</i>	Cysteine synthase	1.95
<i>yxel</i>	<i>yxel</i> operon, organosulfur utilization	1.94
<i>yxed</i>	<i>yxel</i> operon, organosulfur utilization	1.9
<i>ytoI</i>	CBS ^c domain protein, putative thioesterase	1.85
<i>yegM</i>	Similar to proline oxidase	1.83
<i>sipW</i>	Signal peptidase I	1.77
<i>ydbM</i>	Similar to butyryl-coenzyme A dehydrogenase	1.75
<i>yxek</i>	<i>yxel</i> operon, organosulfur utilization	1.72
<i>yuaB</i>	Unknown	1.7
<i>cssS</i>	Secretion stress sensor kinase	1.68
<i>cotJB</i>	Spore coat	1.67
<i>yqxi</i>	Unknown	1.63
<i>ywfD</i>	Similar to glucose 1-dehydrogenase	1.63
<i>ywcI</i>	Unknown	1.62
<i>pel</i>	Pectate lyase	1.53
<i>lrpB</i>	Leucine-responsive protein, represses <i>glyA</i> and <i>kinB</i>	1.53
<i>luxS</i>	SAM metabolism, autoinducer-2 production protein	1.53

^a Log₂ ratio of the *yrzC* mutant transcript level and wild-type transcript level.^b LTTR, LysR-type transcriptional regulator.^c CBS, cystathione beta synthase.

ing of our *cymR* mutant, we used an insertion of the *spc* resistance cassette, which bears a constitutively utilized promoter to drive expression of the downstream genes of the operon, while disrupting the *cymR* gene.

RNA was purified from triplicate, parallel TSS-sulfate cultures of wild-type JH642 cells and cells of the *cymR* mutant, ORB5724. The RNA was used as template for Cy3- and Cy5-labeled cDNA synthesis. The cDNA was then used for hybridization analysis of a *B. subtilis* genomic microarray using previously described procedures (2). Table 2 lists the genes whose expression is elevated in the *cymR* mutant. All of the genes of

the *ytmI*, *yxel*, and *ssu* operons show derepression in the *cymR* mutant background. It is likely, therefore, that the reduced expression of *yxel* and *ssu* in the *spx* mutant (13) is due to the reduced expression of *cymR* in the *spx* background. Additionally, the *yrpT* operon genes show derepression in the *cymR* mutant. Genes of the *yrpT* 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* transcriptome 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 *yrpT* operon by CymR and Spx. In contrast to the *ytmI*, *yxel*, and *ssu* operons, which are under the negative control of both Spx and CymR, the *yrpT* 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, *yrpT* is under CymR negative control (14), while Spx appears to exert positive control. We validated these results using a *yrpT* operon-*lacZ* fusion introduced into the *yrpT* locus. As shown previously (14), *yrpT-lacZ* is derepressed in the *cymR* cells (Fig. 6B) and, like *ytII* and the *ytmI* operon, repression in sulfate medium requires Spx (Fig. 6C). In methionine medium, *yrpT-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 *yrpT* promoter in vitro. To determine if Spx can directly stimulate transcription from the *yrpT* promoter, a fragment containing the intergenic region between the divergently organized *yrpT* and *yrzA* coding sequences (Fig. 7A) was obtained by PCR. This region contains the *yrpT* 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 *yrpT* 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 RNAP interaction with the -35 element. Using the downstream PCR primer that hybridizes to the first eight codons of *yrpT*, 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

FIG. 6. Spx and CymR control of the *yrrT* operon. A. Organization of the *yrrT* operon (see Fig. 1 for details of gene functions). B. Expression of *yrrT'*-*lacZ* in wild-type (WT) and *cymR* mutant cells. Cells were grown to mid-log phase in TSS medium containing either MgSO_4 (SO_4), cysteine (Cys), or methionine (Met) as the sole sulfur source. Samples were collected for assay of β -galactosidase activity. C. Expression of *yrrT'*-*lacZ* in wild-type and *spx* mutant cells. Cultures were grown and samples were processed as for panel B.

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 *cysII* (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 *yrpT* promoter is activated by Spx in vitro. A. Organization of the *yrpT* 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 *yrpT* (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 *yrpT* transcription by CymR. In vitro runoff transcription reaction mixtures contained RNAP (25 nM) and either *trxB* promoter (20 nM) or *yrpT* 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 *yrpT* 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 *yrpT* promoter with CymR. The *yrpT* promoter prepared by PCR using *oyrT*-1 and *oyrT*-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 μ M. 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.

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.

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 *trxA* and *trxB* 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 con-

trol (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 catabolism (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.

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