JOURNAL OF BACTERIOLOGY, June 2005, p. 4042–4049 Vol. 187, No. 12
0021-9193/05/$08.00+0 doi:10.1128/JB.187.12.4042–4049.2005

Sulfate-Dependent Repression of Genes That Function in Organosulfur Metabolism in *Bacillus subtilis* Requires Spx

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Received 13 January 2005/Accepted 7 March 2005

Oxidative stress in *Bacillus subtilis* results in the accumulation of Spx protein, which exerts both positive and negative transcriptional control over a genome-wide scale through its interaction with the RNA polymerase α subunit. Previous microarray transcriptome studies uncovered a unique class of genes that are controlled by Spx-RNA polymerase interaction under normal growth conditions that do not promote Spx overproduction. These genes were repressed by Spx when sulfate was present as a sole sulfur source. The genes include those of the *ytmI*, *yxeI*, and *ssu* operons, which encode products resembling proteins that function in the uptake and desulfurization of organic sulfur compounds. Primer extension and analysis of operon-*lacZ* fusion expression revealed that the operons are repressed by sulfate and cysteine; however, Spx functioned only in sulfate-dependent repression. Both the *ytmI* operon and the divergently transcribed *ytlI*, encoding a LysR-type regulator that positively controls *ytmI* operon transcription, are repressed by Spx in sulfate-containing media. The CXXC motif of Spx, which is necessary for redox sensitive control of Spx activity in response to oxidative stress, is not required for sulfate-dependent repression. The *yxeL-lacZ* and *ssu-lacZ* fusions were also repressed in an Spx-dependent manner in media containing sulfate as the sole sulfur source. This work uncovers a new role for Spx in the control of sulfur metabolism in a gram-positive bacterium under nonstressful growth conditions.

Spx is a global transcriptional regulator of the oxidative stress response in *Bacillus subtilis* and is highly conserved among low-G+C-content gram-positive bacteria (34, 35, 43). It was initially identified as a protein encoded by a gene that was the site of suppressor mutations in *clpP* and *clpX* mutants (29). Spx concentration is proteolytically controlled by the ATP-dependent protease ClpXP (35, 36). The high concentrations of Spx in *clpX* and *clpP* mutants result in poor growth and sporulation and the loss of competence development as well as reduced anaerobic metabolism. Spx was discovered to exert global negative transcriptional control by blocking activator-RNA polymerase (RNAP) interaction (35). This it does by binding to the C-terminal domain of the RNAP α subunit at a site bearing Tyr263, which is a highly conserved amino acid position in the RNAP α of gram-positive bacteria. This interaction was determined to be responsible for the defects in growth and development observed in *clpX* and *clpP* mutants. Spx exhibited no DNA-binding activity in vitro.

High concentrations of Spx also resulted in the induction of genes whose products function in thiol homeostasis and the biosynthesis of cysteine, as shown by probing *B. subtilis* RNA with genomic microarrays (34). The *trxA* and *trxB* genes, encoding thioredoxin and thioredoxin reductase, respectively, are transcriptionally activated under conditions of oxidative stress by a mechanism that involves Spx-RNAP interaction. Reconstitution of transcription initiation in vitro demonstrated that Spx could stimulate transcription from the *trxA* and *-B* promoters by direct interaction with RNAP (33). Spx-dependent activation of *trxA* and *-B* was observed only under oxidative conditions and does not involve initial binding of Spx to DNA. The oxidative activation of Spx activity is attributed to the CXXC motif at the N terminus of Spx protein, which constitutes a disulfide/thiol switch. High concentrations of Spx and the accompanying increase in Spx-dependent positive and negative transcriptional control were observed in cells treated with the thiol-specific oxidant diamide (34). Spx bears no resemblance to any other transcription factor but instead shows secondary structure similarity to the ArsC protein (arsenate reductase) of *Escherichia coli* plasmid R773 (25, 43).

The aforementioned microarray experiments uncovered a unique class of Spx-regulated genes whose products function in alternative sulfur source utilization (see Results). These are repressed by low concentrations of Spx under nonstressful growth conditions and include the *ytmI*, *ssu*, and *yxeI* operons, which encode highly conserved proteins involved in the transport and utilization of alternative sulfur sources such as organic sulfonates and sulfate esters (1, 3, 6, 17–21, 40, 41). The expression of the operons in diverse bacterial species is regulated in response to sulfur source availability (3–5, 7, 18, 22, 39, 41). Transcriptional repression is observed in the presence of the preferred sulfur sources, sulfate or cysteine, while the operons are derepressed in the presence of methionine or taurine (1). This pattern of control has been reported in the cases of the *ytmI* and *ssu* operons of *B. subtilis* (7, 39).

In *E. coli*, the regulation of transcription in response to sulfur source is attributed to two transcriptional regulators, CysB and Cbl (15, 23, 26, 28, 37, 42). Cbl is a LysR homolog

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TABLE 1. *Bacillus subtilis* strains

Strain	Relevant genotype	Source and/or reference
BFS71	*trpC2 ytmI′-lacZ erm ΔytmI*	7
BSIP1214	*trpC2 ytlI*::*aphA3*	7
SB24	*trpC2 amyE*::*ssu*$_{21\text{-}289}$-*lacZ*	39
JH642	*trpC2 pheA1*	J. A. Hoch
ORB3621	*trpC2 pheA1 rpoA*$^{cxs\text{-}1}$	32
ORB3834	*trpC2 pheA1 spx*::*aphA3*	29
ORB3909	*trpC2 pheA1 spx*::*spc*	This study
ORB4269	*trpC2 pheA1 yxeL*::*pMMN519* (*yxeL-lacZ*)	M. M. Nakano
ORB4271	*trpC2 pheA1 amyE*::P$_{spank\text{-}hy}$-*spx*	34
ORB4785	*trpC2 pheA1 yxeL*::*pMMN519* (*yxeL-lacZ*) *rpoA*$^{cxs\text{-}1}$	This study
ORB4786	*trpC2 pheA1 yxeL*::*pMMN519* (*yxeL-lacZ*) *spx*::*aphA3*	This study
ORB4794	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI*	This study
ORB4795	*trpC2 pheA1 ytlI*::*aph*A3	This study
ORB4801	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI rpoA*$^{cxs\text{-}1}$	This study
ORB4802	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI spx*::*aphA3*	This study
ORB4803	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI spx*::*spc*	This study
ORB4804	*trpC2 pheA1 ytmI′-lacZ ytlI*::*aphA3*	This study
ORB4807	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI spx*::*aphA3 amyE*::P$_{spank\text{-}hy}$-*spx*	This study
ORB4814	*trpC2 pheA1 amyE*::P$_{spank\text{-}hy}$-*spx(C10A)*	This study
ORB4815	*trpC2 pheA1 amyE*::P$_{spank\text{-}hy}$-*spx(C13A)*	This study
ORB4863	*trpC2 pheA1 ytmI′-lacZ erm ΔytmI spx*::*neo amyE*::P$_{spank\text{-}hy}$-*spx(C10A)*	This study
ORB4864	*trpC2 pheA1 ytmI′*-lacZ erm ΔytmI spx::neo amyE::*P$_{spank\text{-}hy}$*-spx*(C13A)*	This study
ORB4866	*trpC2 pheA1 amyE*::*ytlI-lacZ cat*	This study
ORB4870	*trpC2 pheA1 amyE*::*ytlI-lacZ cat rpoA*$^{cxs\text{-}1}$	This study
ORB4871	*trpC2 pheA1 amyE*::*ytlI-lacZ cat spx*::*aphA3*	This study
ORB4873	*trpC2 pheA1 amyE*::*ytlI-lacZ cat ytlI*::*aphA3*	This study
ORB4891	*trpC2 pheA1 amyE*::*ssu*$_{21\text{-}289}$-*lacZ*	This study
ORB4896	*trpC2 pheA1 amyE*::*ssu*$_{21\text{-}289}$-*lacZ rpoA*$^{cxs\text{-}1}$	This study
ORB4898	*trpC2 pheA1 amyE*::*ssu*$_{21\text{-}289}$-*lacZ spx*::*neo*	This study
ORB4962	*trpC2 pheA1 amyE*::Pspac-*ytlI*	This study
ORB4977	*trpC2 pheA1 ytmI′-lacZ amyE*::Pspac-*ytlI*	This study
ORB4979	*trpC2 pheA1 ytmI′-lacZ amyE*::Pspac-*ytlI ytlI*::*aphA3*	This study
ORB4983	*trpC2 pheA1 ytmI′-lacZ amyE*::Pspac-*ytlI ytlI*::*aphA3 spx*::*spc*	This study

that activates *ssu* operon transcription in the absence of sulfate and is inhibited by the anti-inducer adenosine 5′-phosphosulfate (APS), which is an intermediate in the assimilation of sulfate. CysB down-regulates *ssu* transcription in the presence of cysteine and is sensitive to the inducer *N*-acetyl-L-serine. The *ssu* and *ytmI* operons of *B. subtilis* appear to be similarly regulated, but until recently no regulator conferring Cys- or sulfate-dependent control had been identified. The *ytmI* operon is regulated by the product of the divergently transcribed gene *ytlI*, which encodes a LysR homolog dedicated to *ytmI* operon transcriptional control (7). YtlI is necessary for activation of *ytmI* transcription in the absence of Cys and sulfate, but the inducer and/or coactivator for YtlI have not been uncovered.

In this report, evidence is presented that Spx negatively controls *ssu*, *yxeI*, and *ytmI* operon gene transcription in response to the presence of sulfate but not cysteine. A null mutation of the *spx* gene and a mutation of *rpoA* that blocks Spx-RNA polymerase interaction result in the derepression of *ssu*, *yxeI*, and *ytmI* operon expression when sulfate is the sole source of sulfur. The *ytlI* gene is also under Spx negative control when sulfate is the sole sulfur source. This function of Spx is carried out under nonstressful growth conditions that do not result in elevated Spx concentration, unlike the situation in cells undergoing oxidative stress, in which Spx concentration and activity are upregulated (33, 34).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. subtilis* strains used in this study are listed in Table 1 and are derivatives of strain JH642. *E. coli* cells were grown at 37°C in 2× yeast extract-tryptone (YT) liquid or on Luria-Bertani solid medium containing 1.2% agar (Difco). *B. subtilis* cells were grown at 37°C in 2× YT, Difco sporulation medium (13), or TSS minimal medium (10) with some modifications (37.4 mM NH_4Cl, 2 mM K_2HPO_4, 49.5 mM Tris, 5 mM $MgCl_2$, 0.5% glucose, 0.004% $FeCl_3$, 0.004% citric acid, 0.1% L-glutamate, 0.005% auxotrophic requirements) and supplemented with one of the following sulfur

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype or property	Source or reference
pDG1727	Contains Sp^r cassette	11
pDH32	Creates *lacZ* fusions	D. Henner
pDR66	Allows IPTG-dependent gene expression	14
pDR111	Allows IPTG-dependent gene expression	2
pFH3	pUC18 with *spx*	29
pKE17	pDR66 with *ytlI* under control of Pspac	This study
pSN95	pDR111 with *spx*(*C10A*) under control of P$_{spank\text{-}hy}$	This study
pSN96	pDR111 with *spx*(*C13A*) under control of P$_{spank\text{-}hy}$	This study
pSN104	pUC19 with *ytlI*	This study
pSN105	pDH32 with *ytlI*	This study
pYZ81	pFH3 with *spx*::*spc*	This study

TABLE 3. Primers used in this study

Primer	Sequence
oKE18	5′-ACGCGTCGACGCATACTCTGATCTTTGACTAATC-3′
oKE19	5′-ACATGCATGCCGTGAAACCATCTCCTGATGAATC-3′
oSN03-66	5′-CACATCACCAAGCGCGACTTCATGCAGAA-3′
oSN03-67	5′-TTCTGCATGAAGTCGCGCTTGGTGATGTG-3′
oSN03-68	5′-CAAGCTGTACTTCAGCGAGAAAGGCGAGAG-3′
oSN03-69	5′-CTCTCGCCTTTCTCGCTGAAGTACAGCTTG-3′
oSN03-70	5′-CACCAAGCGCGACTTCAGCGAGAAAGGCGAG-3′
oSN03-71	5′-CTCGCCTTTCTCGCTGAAGTCGCGCTTGGTG-3′
oSN03-73	5′-GGAATTCACCCTCTCCTTCTGTACATGTCAGC-3′
oSN03-74	5′-CCGGATCCGTTGTGTTTAAGCGCCTCGTATTGC-3′
Pspac-up	5′-GACTTTATCTACAAGGTGTG-3′
Pspac-down	5′-AAATGATGACCTCGTTTCCA-3′
oMN01-173	5′-CGAGGAAGCTTAGATGTTCATCCTACTA-3′
oMN01-174	5′-TACCAGCAGGTCGACAAATAAAAGAAGG-3′
o-sn04-90	5′-GAGTATTTCTGCAGCTTTATAGAAGCTTCC-3′
o-sn04-91	5′-AGCTCCGATGCATCTTCAACAGTTGCC-3′

sources at a concentration of 1 mM: L-cysteine, L-methionine, or $MgSO_4$. The solid medium was Difco sporulation medium supplemented with 1.2% agar. Sulfate-free solid medium was prepared by adding 1% agarose to TSS. X-Gal was added to plates at a concentration of 40 μg/ml. When necessary, the following antibiotics were added (at the concentrations shown in parentheses): ampicillin (25 μg/ml), neomycin (5 μg/ml), chloramphenicol (5 μg/ml), erythromycin plus lincomycin (1 and 25 μg/ml, respectively), and spectinomycin (75 μg/ml).

Plasmid and strain construction. Plasmids constructed in this study are listed in Table 2. Primers used in this study are listed in Table 3.

B. subtilis strain ORB3909 (*spx*::*spc*) was constructed as follows. A spectinomycin resistance cassette (Sp^r) was isolated from pDG1727 (11) by digestion with BamHI and XhoI. The fragment was filled in to create blunt ends and inserted into pFH3 digested with BclI, creating a product that was also filled in. The ligated product was pYZ81, which was introduced by transformation into JH642.

The strain ORB4269 contained *yxeL-lacZ* integrated at the *yxeI* operon locus of the chromosome. A 600-bp fragment that contains the 3′ end of *yxeK* and the 5′ end of *yxeL* was amplified by PCR using oligonucleotides oMN02-210 (5′-CTGCGGAATTCGGACGTGAT-3′) and oMN02-211 (5′-AACTGCAAGGCGGATCCGTGGCTT CA-3′) and chromosomal DNA prepared from JH642. The PCR product, after being digested with EcoRI and BamHI, was inserted into pTKlac (16). The resultant plasmid, pMMN519, was used to transform JH642 to generate ORB4269. The *yxeL* gene is the fourth open reading frame of the *yxeI* operon, and the introduction of the *yxeL-lacZ* plasmid by a single recombination event disrupts the operon.

B. subtilis strain ORB4814 [$P_{spank\text{-}hy}$-*spx*(*C10A*)] was constructed as follows. Two PCRs were performed to create a site-directed mutation. Primers oMN01-173 and oSN03-67 generated the first fragment, and oMN01-174 and oSN03-66 generated the second fragment. The fragments were ligated, and primers oMN01-173 and oMN01-174 were used to amplify the full-length mutagenized fragment. The resultant fragment was digested with HindIII and SalI and subsequently ligated to pDR111 (2) digested with the same enzymes, giving pSN95, which was introduced by transformation into strain JH642.

B. subtilis strain ORB4815[$P_{spank\text{-}hy}$-*spx*(*C13A*)] was constructed in a manner that was similar to that used for the construction of ORB4814, except that the first PCR was performed with mutagenic primers oSN03-69 and oSN03-68 replacing oSN03-67 and oSN03-66, respectively. The plasmid constructed was pSN96, which was introduced by transformation into JH642.

B. subtilis strain ORB4866 carrying a *ytlI-lacZ* fusion was constructed as follows. With oligonucleotides oSN03-73 and oSN03-74, a PCR fragment was obtained by using JH642 DNA as the template. The PCR product was digested with EcoRI and BamHI and ligated with pUC19, which was digested with the same enzymes, forming plasmid pSN104. Plasmid pSN104 was then digested with EcoRI and BamHI, and the resultant fragment was subcloned to plasmid pDH32, which had been digested with the same enzymes, forming pSN105. Plasmid pSN105 was then introduced by transformation into JH642, and chloramphenicol-resistant (Cm^r) transformants were tested for loss of amylase activity on LB-starch agar plates.

B. subtilis strain ORB4962 carries the *ytlI* coding sequence under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P*spac* promoter. To construct the strain, oligonucleotides oKE18 and oKE19 were used to perform PCR on JH642 chromosomal DNA previously digested with EcoRI and BamHI. The resultant 1,056-bp fragment corresponding to positions −81 to + 975 relative to the *ytlI* translational start site was digested with SalI and SphI. The digested fragment was then ligated to pDR66 (14) digested with the same enzymes, forming pKE17. The sequence of *ytlI* was verified by sequencing the plasmid with both Pspac-up and Pspac-down primers (Table 3). Plasmid pKE17 was transformed into JH642, resulting in recombination at *amyE*. Cm^r clones were picked and checked for loss of amylase activity.

β-Galactosidase assays. Cells from a frozen stock were used to inoculate to 1 or 2 ml of 2× YT plus antibiotics. The culture was allowed to grow for several hours, after which it was diluted 100-fold into 2 ml of TSS supplemented with 1 mM of different sulfur sources. After overnight growth, the culture was inoculated to 20 ml of TSS containing the same sulfur source to a starting optical density at 600 nm (OD_{600}) of 0.05. After growth with agitation to an OD_{600} of 0.7 to 0.8 (or 0.7 to 1.0 in the case of the experiment with cells bearing the *ytmI-lacZ* fusion [see Fig. 3A]), two 1-ml samples were taken. Cells were pelleted by centrifugation and stored at −80°C until assayed for β-galactosidase activity. When required, 1 mM IPTG was added at the initial OD_{600} of 0.05.

β-Galactosidase activity was determined as previously described (30) (27). The two samples from a single time point were averaged to give the Miller units. Experiments were repeated three times, and the data are presented as the mean of three independent experiments ± one standard deviation.

RESULTS

Genes that are derepressed in the *rpoA*$^{cxs\text{-}1}$ mutant background. The mutant *rpoA*$^{cxs\text{-}1}$ allele of the RNAP α subunit gene, *rpoA*, encodes a product bearing a Y263C substitution in the C-terminal domain of the RNAP α subunit that was shown to impair interaction with the Spx protein (35). Previous microarray studies (34) were conducted that were designed to identify genes whose expression was affected by the overproduction of a protease-resistant form of Spx, Spx^{DD}, but was not so affected in the *rpoA*$^{cxs\text{-}1}$ background. This strategy succeeded in the discovery of a number of genes controlled by the Spx-RNAP interaction.

The microarray experiment uncovered a class of Spx-controlled genes in *B. subtilis* that was not expressed in a *rpoA*$^+$ genetic background but was derepressed in an *rpoA*$^{cxs\text{-}1}$ mutant. While derepression of these genes was observed in the *rpoA*$^{cxs\text{-}1}$ background, repression occurred when the protease-resistant form of Spx, Spx^{DD}, was produced by IPTG-dependent induction of a $P_{spank\text{-}hy}$-*spx*^{DD} construct despite the weakened Spx interaction with the $RpoA^{cxs\text{-}1}$ RNAP subunit. The

TABLE 4. Genes derepressed in an *rpoA*$^{cxs\text{-}1}$ mutant background and negatively controlled by Spx[a]

Gene (operon)	Product(s)
cssS	kinase, protein secretion stress
ytmI, ytmO, ytmJ, ytmN, ytmL, ytmK, ytmM, ytnI, ytnJ, ytnM, ribR (*ytmI* operon)	sulfonate utilization
ytlI	LysR-like regulator of ytmI operon
yxeK, yxeO, yxeP, yxeN, yxeL, yxeQ, yxeR, yxeD (*yxe* operon)	sulfonate utilization
ssuD, ssuA, ssuC, ssuB, ygaN, *yhzA* (*ssu* operon)	sulfonate; DMS utilization
yjnA	permease
cysC, cysP, sat, ylnE, ylnD, ylnF (*cysP* operon)	sulfate transport, APS, sulfite reduction
yubD	MDR transporter
metE	cobalamin-independent methionine synthase

[a] From data from reference 34.

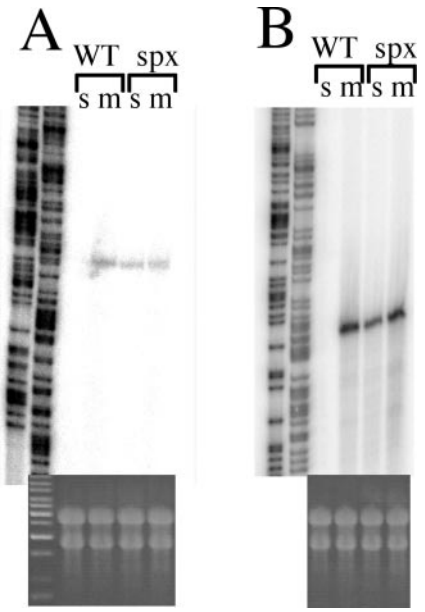

FIG. 1. Primer extension analysis of *ytmI* and *ytlI* RNA in wild-type and *spx* mutant cells. Cultures were grown in TSS medium containing either $MgSO_4$ or methionine (see Materials and Methods) to OD_{600}s of 0.7. RNA was harvested as described previously (35). Primer extension reactions were performed using oligonucleotides oSN04-90 (for *ytlI*) and oSN04-91 (for *ytmI*). Sanger dideoxynucleotide termination sequence reactions were performed using the same primers. Products were resolved by polyacrylamide-urea gel electrophoresis and visualized by phosphorimaging. WT, wild type (JH642); spx, *spx* mutant (ORB3834); s, sulfate; m, methionine. (A) Primer extension products of *ytlI* RNA. Sequencing reactions are shown on the left. (B) Primer extension products of *ytmI* RNA. At the bottoms of the primer extension product lanes are photographs of formaldehyde agarose gels of total RNA from each RNA sample.

unique class of Spx-controlled genes was not expressed in *rpoA*$^+$ cells incubated in minimal medium; hence, the production of Spx^{DD} had no observable effect on expression in the wild-type background.

Nearly all of the genes identified as members of this unique class encode products that function in alternative sulfur source utilization (Table 4). The *ytmI*, *ssu*, and *yxeI* operons encode ABC transport components and products showing homology to flavin mononucleotide or F420-dependent monooxygenases that function in the desulfurization of sulfur-containing organic compounds (18). Products of the *ssu* genes had also been implicated in dimethyl sulfide utilization in *Pseudomonas* (8).

Spx represses YtlI-dependent transcription of the *ytmI* operon in cultures containing sulfate as the sole sulfur source. One of the Spx-controlled operons identified was the *ytmI* (*ytmIJKLMNOytnIJribRytnLM*) operon (7). The operon is induced in the absence of cysteine and sulfate and in the presence of methionine (7). It encodes products resembling components of an ABC-type transport system, two FMN-dependent monooxygenases, and proteins that appear to function in flavin cofactor synthesis. A low-molecular-weight glutaredoxin-like protein, YtnI, may function as a monooxygenase-protecting antioxidant to eliminate reactive oxygen species, which are by-products of the FMN reductase-catalyzed reaction (19).

A recent report provides evidence that the divergently transcribed *ytlI* gene encodes a LysR-like product that functions in *ytmI* operon transcriptional activation (7). Primer extension analysis (Fig. 1) shows that the start sites of transcription for the *ytmI* operon and the *ytlI* gene are only 15 bp apart, with their −10 regions overlapping and with opposite orientations (Fig. 2). The primer extension shows that *ytmI* and *ytlI* are transcribed when methionine is present but are repressed by sulfate. Figure 1 also shows that SO_4^{2-}-dependent repression of *ytmI* and *ytlI* is not observed in cells of an *spx* mutant.

Spx-dependent repression of *ytmI* and *ytlI* in the presence of sulfate is not the result of Spx overproduction of the kind that characterizes cells of a *clpX* or *clpP* mutant or cells treated with the thiol-specific oxidant diamide. Western analysis with anti-Spx antiserum failed to detect Spx protein from extracts of cells grown in sulfate, cysteine, or methionine media (data not shown), indicating that changing the sulfur source does not significantly affect *spx* expression.

Spx is required for sulfate- but not cysteine-dependent repression. The expression of the *ytmI-lacZ* fusion of strains ORB4794, ORB4804 (*ytlI*::*aphA3*), ORB4801 (*rpoA*$^{cxs-1}$), and ORB4802 (*spx*::*aphA3*) was examined in cultures containing sulfate, cysteine, or methionine as sole sulfur sources. As shown in Fig. 3, *ytmI-lacZ* expression was repressed in cells of cultures grown in sulfate or cysteine (Fig. 3A, lanes 1 and 5). Strains bearing either the *rpoA*$^{cxs-1}$ or the *spx* null mutation had higher levels of *ytmI-lacZ* activity in sulfate-containing medium (Fig. 3A, lanes 2 and 3), but this activity was still repressed in cysteine medium (Fig. 3A, lanes 6 and 7). Cells of cultures grown in medium containing methionine exhibited derepressed *ytmI-lacZ* expression, and the introduction of the *spx*

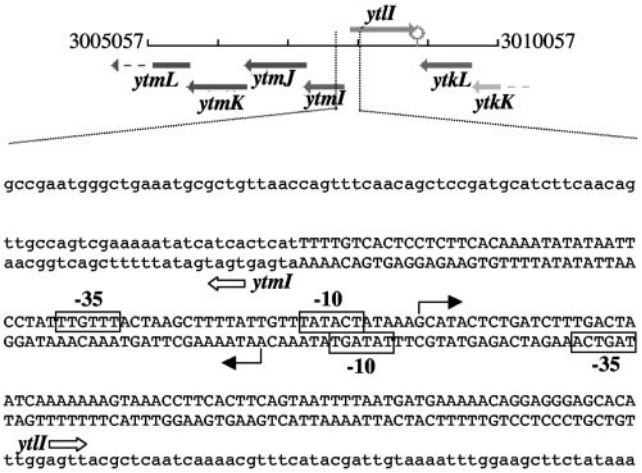

FIG. 2. Diagram and nucleotide sequence of the *ytmI-ytlI* promoter region. At the top is a diagram of the *ytmI-ytlI* locus from the SubtiList website (http://genolist.pasteur.fr/SubtiList/). The numbers at either end refer to the nucleotide positions in the chromosome. At the bottom is the nucleotide sequence of the *ytmI-ytlI* promoter region. Uppercase letters denote the noncoding, intergenic region between *ytmI* and *ytlI*. Lowercase letters specify the coding sequences of *ytmI* and *ytlI*. The ATG and TTG start codons are labeled with gene names and arrows for *ytmI* and *ytlI*. Solid arrows mark the transcriptional start sites for both genes as identified by primer extension analysis (Fig. 1). The −10 regions of both promoters are labeled, and the sequences are enclosed in rectangles.

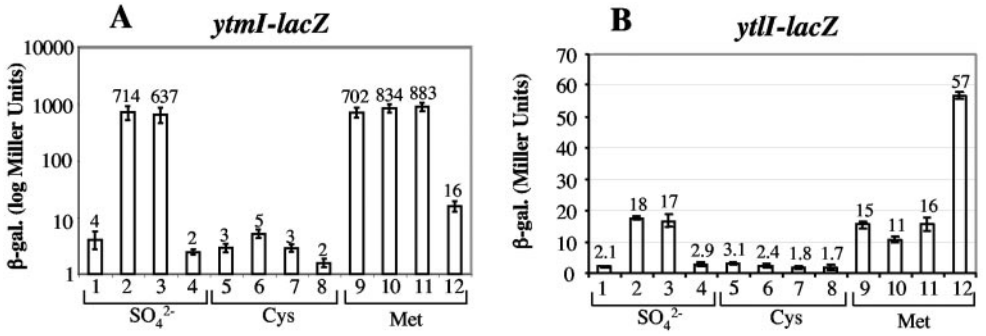

FIG. 3. Expression of *ytmI*- and *ytlI-lacZ* fusions in wild-type, *ytlI*, *spx*, and *rpoA*$^{cxs-1}$ strains. Cells of each fusion-bearing strain were grown in TSS media containing sulfate, cysteine, or methionine as the sole sulfur source. Samples were collected at late log phase, where maximum β-galactosidase activity was observed. The samples were assayed for β-galactosidase activity, which was expressed in Miller units (27). Experiments were performed in triplicate. (A) Bar graph of β-galactosidase activity in cells bearing the *ytmI-lacZ* fusion. Lanes 1, 5, and 9, strain ORB4794 (*ytmI-lacZ*); lanes. 2, 6, and 10, ORB4801 (*ytmI-lacZ rpoA*$^{cxs-1}$); lanes 3, 7, and 11, ORB4802 (*ytmI-lacZ spx*); lanes 4, 8, and 12, ORB4804 (*ytmI-lacZ ytlI*). (B) Bar graph of β-galactosidase activity in cells bearing the *ytlI-lacZ* fusion. Lanes 1, 5, and 9, ORB4866 (*ytlI-lacZ*); lanes 2, 6, and 10, ORB4870 (*ytlI-lacZ rpoA*$^{cxs-1}$); lanes 3, 7, and 11, ORB4871 (*ytlI-lacZ spx*); lanes 4, 8, and 12, ORB4873 (*ytl-lacZ ytlI*).

or the *rpoA*$^{cxs-1}$ mutation resulted in no further increase in expression (Fig. 3A, lanes 9 to 11). A mutation in the *ytlI* gene resulted in reduced expression in the presence of each of the three sulfur sources, although expression is relatively high in the methionine-grown cultures (Fig. 3A, lanes 4, 8, and 12).

As reported previously (7), YtlI exerts positive control over the *ytmI* operon. It is therefore possible that Spx indirectly affects *ytmI* expression by controlling the expression of the divergently transcribed *ytlI* gene. To examine this possibility, a *ytlI-lacZ* fusion was constructed and introduced into the *ytlI*, *spx*, and *rpoA*$^{cxs-1}$ mutants. The expression of the fusion was repressed in media containing either sulfate or cysteine (Fig. 3B, lanes 1 and 5) but was derepressed in medium containing methionine (Fig. 3B, lane 9). Thus, the expression patterns of *ytlI* with respect to the sulfur sources resembled those of the *ytmI-lacZ* fusion. Introduction of the *rpoA*$^{cxs-1}$ or *spx* mutations resulted in derepression in sulfate-containing medium (Fig. 3B, lanes 2 and 3) but not in medium containing cysteine (Fig. 3B, lanes 6 and 7), which again is similar to the pattern of *ytmI* operon regulation. As with *ytmI*, *ytlI-lacZ* was derepressed in medium containing methionine, and this expression was not elevated further by the introduction of the *rpoA*$^{cxs-1}$ and *spx* mutations (Fig. 3B, lanes 9 to 11). Unlike *ytmI* expression, however, a *ytlI* mutation resulted in overexpression in methionine-containing medium (Fig. 3B, lane 12), which is suggestive of YtlI-mediated negative autoregulation. This negative autoregulation is not involved with cysteine-dependent repression of *ytlI*, since repression is observed in wild-type and *ytlI* mutant cells. The results show that there are two modes of negative control, one involving Spx in sulfate medium and one that is dependent on exogenous cysteine.

Mutations in the CXXC motif of Spx do not eliminate sulfate-dependent negative control. The CXXC motif of Spx is required for redox sensitive control of Spx activity (33). The transcription of the *trxA* and *trxB* genes requires Spx in its oxidized form, in which it bears a disulfide linkage between the cysteine residues of the redox active site. The requirement of the CXXC motif in sulfate-dependent repression was tested by expression in an *spx* mutant background of the wild-type and mutant alleles of Spx, the latter bearing C-to-A codon substitutions at C10 and C13. The *spx* alleles were expressed as IPTG-inducible constructs from the *amyE* locus of the *B. subtilis* chromosome. In the presence of SO_4^{2-}, somewhat elevated *ytmI-lacZ* expression is observed in cells in the absence of IPTG (Fig. 4A, lanes 1 to 3). The induction of wild-type and mutant *spx* resulted in the complete repression of *ytmI* in the presence of sulfate (Fig. 4A, lanes 4 to 6). When the cells were grown in methionine, complete derepression was observed in both the presence and the absence of IPTG, whether or not the wild-type or mutant alleles were expressed. The low-level derepression in the absence of IPTG in sulfate medium suggests that the IPTG-inducible constructs are leaky, allowing some expression in the absence of inducer. This derepression was higher in the case of the mutant alleles, suggesting that these encode slightly defective Spx proteins. However, the mutants are capable of mediating SO_4^{2-}-dependent repression of *ytmI*.

Constitutive expression of *ytlI* does not render *ytmI* transcription independently of Spx-mediated repression in sulfate medium. Spx could regulate *ytmI* only indirectly, by controlling the expression of its positive regulator, YtlI. To determine if this was true, we expressed the *ytlI* gene from an IPTG-inducible P*spac* promoter so as to render the *ytlI* expression independent of Spx. The expression of *ytmI-lacZ* was then examined in both the presence and the absence of IPTG and sulfate. As shown in Fig. 4B, lanes 1 and 3, in *spx*$^+$ cells, the expression of *ytmI-lacZ* is repressed in sulfate medium in the presence or the absence of IPTG induction of *ytlI*. A much higher level of *ytmI-lacZ* expression is observed upon IPTG addition to cultures of the *spx* mutant (Fig. 4B, lanes 2 and 4). Repression of *ytmI* by cysteine is retained in the presence or the absence of IPTG in *spx* and *spx*$^+$ strains (Fig. 4B, lanes 5 to 8), showing again that cysteine-dependent repression does not involve Spx. In methionine medium (Fig. 4B, lanes 9 to 12), IPTG addition results in high-level *ytmI-lacZ* expression in *spx*$^+$ and *spx* mutant backgrounds. Thus, Spx was shown to exert negative control at the *ytlI* and the *ytmI* promoters when cells are grown in medium containing sulfate.

Spx exerts negative control of the *ssu* and *yxeI* operons in cells grown in sulfate medium. Like the *ytmI* operon, the *ssu* and *yxeI* operons encode products resembling ABC-type trans-

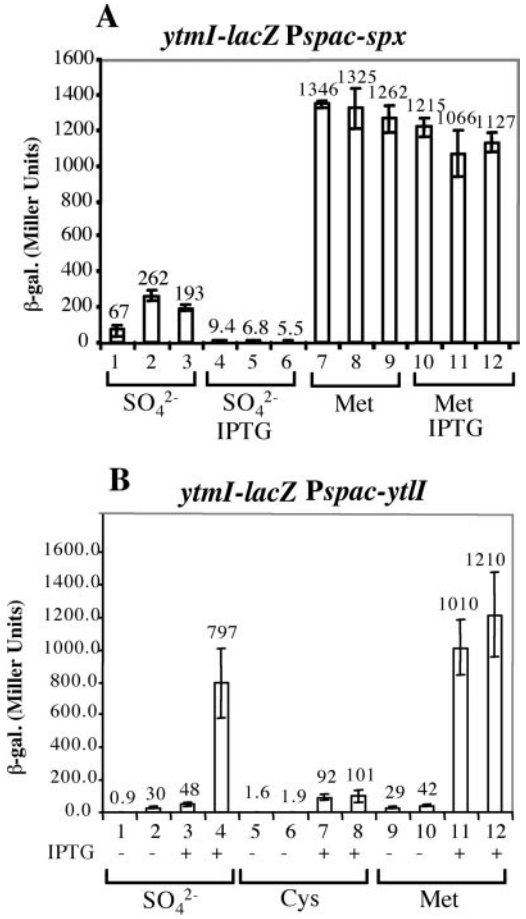

FIG. 4. (A) Complementation of *spx* with wild-type and CXXC mutant alleles of *spx*. Cells were grown in TSS media containing either sulfate or methionine as sole sulfur sources in the absence (lanes 1 to 3 and 7 to 9) and the presence (lanes 4 to 6 and 10 to 12) of IPTG. Samples were collected as described in the legend to Fig. 3. β-Galactosidase activity was expressed in Miller units. Experiments were performed in triplicate. Lanes 1, 4, 7 and 10, ORB4807 (*ytmI-lacZ spx amyE*::$P_{spank\text{-}hy}$-*spx*); lanes 2, 5, 8, and 11, ORB4863 [*ytmI-lacZ spx amyE*::$P_{spank\text{-}hy}$-*spx(C10A)*]; lanes 3, 6, 9, and 12, ORB4864 [*ytmI-lacZ spx amyE*::$P_{spank\text{-}hy}$-*spx(C13A)*]. (B) The *ytmI-lacZ* fusion is still controlled by Spx when *ytlI* is constitutively expressed. Strains containing *ytmI-lacZ* and $P_{spank\text{-}hy}$-*ytlI* with or without an *spx* mutation were grown in TSS containing either sulfate, cysteine, or methionine as the sole sulfur source. The absence (−) and the presence (+) of IPTG is indicated. Lanes 1, 3, 5, 7, 9, and 11, ORB4979 (*ytmI-lacZ amyE*::P_{spac}-*ytlI ytlI*::*aphA3*); lanes 2, 4, 6, 8, 10 and 12, ORB4983 (*ytmI-lacZ amyE*::P_{spac}-*ytlI ytlI*::*aphA3 spx*::*spc*).

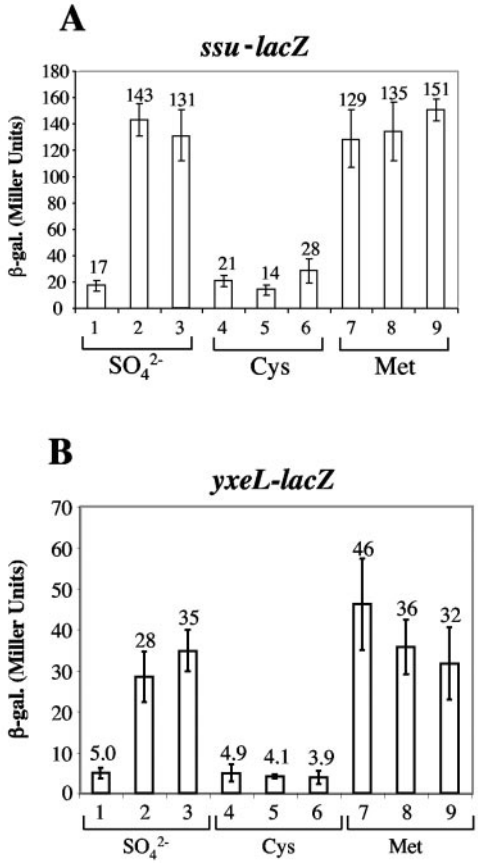

FIG. 5. (A) The expression of *ssu-lacZ* is negatively controlled by Spx in the presence of sulfate. The strains bearing *amyE*::$ssu_2$1_2$_{89}$-*lacZ* (39) in a wild-type, *spx*, or $rpoA^{cxs\text{-}1}$ background were grown in TSS containing either sulfate, cysteine, or methionine as the sole sulfur source. Samples were collected and assayed for β-galactosidase activity as described for Fig. 3. Lanes 1, 4, and 7, ORB4891 ($ssu_2$1_2$_{89}$-*lacZ*); lanes 2, 5, and 8, ORB4896 ($ssu_2$1_2$_{89}$-*lacZ* $rpoA^{cxs\text{-}1}$); lanes 3, 6, and 9, ORB4898 ($ssu_2$1_2$_{89}$-*lacZ spx*). (B) The expression of *yxeL-lacZ* is negatively controlled by Spx in the presence of sulfate. The strains bearing *yxeL-lacZ* in a wild-type, *spx*, or $rpoA^{cxs\text{-}1}$ background were grown in TSS containing either sulfate, cysteine, or methionine as the sole sulfur source. Samples were collected and assayed for β-galactosidase activity as for Fig. 3. Lanes 1, 4, and 7, ORB4269 (*yxeL-lacZ*); lanes 2, 5, and 8, ORB4785 (*yxeL-lacZ spx*); lanes 3, 6, and 9, ORB4786 (*yxeL-lacZ* $rpoA^{cxs\text{-}1}$).

port components and FMN-dependent monooxygenases, characteristics of operons whose products function in the metabolism of organosulfur compounds. Both fall into the class of operons that are derepressed in the $rpoA^{cxs\text{-}1}$ mutant but repressed by Spx overproduction. The expression of an *ssu-lacZ* fusion (obtained from J. R. van der Ploeg) and that of a *yxeL-lacZ* fusion (constructed by M. M. Nakano; unpublished) was examined in wild-type, *spx*, and $rpoA^{cxs\text{-}1}$ cells grown in sulfate, cysteine, or methionine media. Both fusions are repressed by sulfate and cysteine but are expressed to their highest levels in methionine medium (Fig. 5). Sulfate-dependent repression requires the *spx* gene and the wild-type allele of *rpoA* (Fig. 5A and B, lanes 1 to 3). As was the case with *ytmI* and *ytlI* expression, the *spx* and $rpoA^{cxs\text{-}1}$ mutations do not confer any increase in expression further than that observed in wild-type cells incubated in methionine medium.

DISCUSSION

The *ytmI*, *ssu*, and *yxeI* operons encode products resembling proteins that function in organic sulfonate uptake and desulfonation. The *ssu* operon of *B. subtilis* has been reported to be required for growth in medium containing short-chain alkanesulfonates, sulfoacetate, taurine, or isethionate as sole sulfur sources (40). Each of the operons encodes not only the components of organic sulfonate uptake but also the FMNH2- or coenzyme F420-requiring monooxygenases that catalyze the

oxygenolytic desulfonation of the substrate. The desulfonation of organic sulfonates yields sulfite, which is subsequently reduced to sulfide, a substrate for cysteine synthase. The transport components of the *ytmI* operon constitute one of three systems that can function in the uptake of cystine (4). All three operons are induced by sulfur limitation, which is a consequence of low exogenous concentrations of the preferred sulfur sources sulfate or cysteine. Sulfate is normally transported by the sulfate/thiosulfate transporter encoded by *cysP*. Sulfate sulfur assimilation proceeds with the formation of adenosine 5′-phosphosulfate (APS), which is generated by ATP sulfurylase using SO_4^{2-} and ATP followed by the phosphorylation of APS by APS kinase to yield PAPS (phosphoadenosine phosphosulfate). The product of the *cysH* gene, PAPS sulfotransferase (or phosphoadenylyl sulfate reductase), generates sulfite, which is reduced by sulfite reductase to sulfide for cysteine synthesis. In organic sulfonate utilization, sulfite is generated by a process that bypasses the sulfate reduction pathway (18).

Sulfate-dependent control of sulfonate utilization genes is accomplished through the LysR-like transcriptional activator Cbl in *E. coli* (15). APS, an intermediate in sulfate sulfur assimilation, serves as an antiactivator preventing the transcription of the *ssu* operon (5). The *ssu* operon is also under negative control by CysB, another LysR-type regulator that is sensitive to *O*-acetyl serine, a precursor of cysteine biosynthesis that accumulates under low cysteine concentrations (5). Until recently, there have been no such regulators identified in gram-positive bacteria. The evidence presented herein indicates that Spx is at least a component of the sulfate-dependent control system. This brings the number of regulatory factors controlling sulfur metabolism at the level of transcription initiation in gram-positive bacteria to five, with the others being CmbR of *Lactococcus lactis* (9), CysL (12), YtlI, and, putatively, YrzC of *B. subtilis* (38). CmbR is a LysR homolog that activates the *metC* (cystathionine β-lyase) *cysK* (cysteine synthase) operon in response to low cysteine and high *o*-acetylserine (OAS) concentrations. CmbR was reported to be a functional homolog of CysB. While there are many LysR-type regulators encoded in the *B. subtilis* genome, there are none that are close homologs of CmbR. OAS has been reported to exert a positive influence on expression of the *cysH* operon in *B. subtilis* (24). The specific regulator that mediates this control, the hypothetical CysR factor, has not been uncovered. CysL, another LysR-like regulator, is required for transcription of the *cysIJ* dicistronic operon, which encodes sulfite reductase. CysL-dependent transcription is activated in response to the presence of sulfate as the sole sulfur source and is repressed by thiosulfate (12). YtlI and YrzC have been implicated in the control of the *ytmI* operon. At present, it is not known if YtlI activity is controlled in response to cysteine or sulfate availability. The *yrzC* gene is the site of mutations that lead to the derepression of *ribR*, a gene that resides within the *ytmI* operon and encodes riboflavin kinase (38). It is presumed that YrzC is a negative regulator of *ytmI* operon expression, but here too it is not known how YrzC is controlled in response to sulfur source availability. Spx exerts a negative effect on both the *ytlI* gene and the *ytmI* operon and is responsible for sulfate-dependent repression. It is not clear if this is accomplished in conjunction with or independently of YrzC.

The finding that Spx can function in the control of genes involved in sulfur metabolism was not entirely unexpected, since microarray studies have shown that genes whose products function directly in cysteine biosynthesis are induced when Spx is overproduced as a protease-resistant variant (34). However, this and other Spx activities have been heretofore observed only when Spx is present in a high concentration (29, 31, 34, 35). Spx functions in sulfate-dependent control under normal laboratory culture growth and at concentrations far below the levels at which Spx was previously observed to activate the *trxA* gene and repress activator-stimulated transcription. Western blot analysis using anti-Spx antiserum did not result in the detection of Spx in cells of cultures grown in sulfate, cysteine, or methionine (data not shown), while Spx is easily detected after diamide treatment or in a *clpX* mutant. One could imagine that the *ytmI* operon is repressed by Spx interaction with the RNA polymerase α subunit, which prevents YtlI-RNA polymerase contact. But this kind of activation interference had been observed only when Spx concentration was high. Further complicating attempts to explain Spx function in controlling *ytmI* operon transcription is the finding that the divergently transcribed *ytlI* gene is also under sulfate-dependent Spx control.

At present, it is not known in what capacity Spx functions in the negative control of *ytmI* and *ytlI*. Even less is known about the role Spx plays in the sulfate-dependent transcriptional control of the *yxeI* and *ssu* operons, as there have been no regulators, aside from Spx, that have been uncovered. It is not clear how sulfur availability might influence the activity of Spx. The CXXC motif which constitutes the thiol/disulfide switch governing redox sensitive control does not play a significant role in sulfate-dependent control. The recently solved crystal structure of Spx has revealed two sulfate ions that are coordinated in the vicinity of the Cys10 and C13 positions (K. Newberry, S. Nakano, P. Zuber, and R. Brennan; unpublished data). It is possible that Spx directly senses the presence of sulfate and responds through a conformational change that affects Spx activity.

ACKNOWLEDGMENTS

We thank I. Martin-Verstraete for the *ytmI′-lacZ* fusion and the *ytlI* null mutant, J. R. van der Ploeg for the *ssu-lacZ* fusion construct, and Michiko M. Nakano for reagents and helpful discussions and for reading the manuscript.

The research presented herein was supported by grant GM45898 from the National Institutes of Health and a grant from the Oregon Medical Research Foundation.

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