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Transcription from the P_3 Promoter of the *Bacillus subtilis spx* Gene Is Induced in Response to Disulfide Stress▽

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The *spx* gene of *Bacillus subtilis* encodes a global regulator that controls transcription initiation in response to oxidative stress by interaction with RNA polymerase (RNAP). It is located in a dicistronic operon with the *yjbC* gene. The *spx* gene DNA complements an *spx* null mutation with respect to disulfide stress resistance, suggesting that *spx* is transcribed from a promoter located in the intergenic region of *yjbC* and *spx*. Transcription of the *yjbC-spx* operon has been reported to be driven by four promoters, three (P_1, P_2, and P_B) residing upstream of *yjbC* and one (P_M) located in the intergenic region between *yjbC* and *spx*. Primer extension analysis uncovered a second intergenic promoter, P_3, from which transcription is elevated in cells treated with the thiol-specific oxidant diamide. P_3 is utilized by the σ^A form of RNA polymerase in vitro without the involvement of a transcriptional activator. Transcriptional induction from P_3 did not require an Spx-RNAP interaction and was observed in a deletion mutant lacking DNA upstream of position −40 of the P_3 promoter start site. Deletion mutants with endpoints 3′ to the P_3 transcriptional start site (positions +5, +15, and +30) showed near-constitutive transcription at the induced level, indicating the presence of a negative control element downstream of the P_3 promoter sequence. Point mutations characterized by *bgaB* fusion expression and primer extension analyses uncovered evidence for a second *cis*-acting site in the P_3 promoter sequence itself. The data indicate that *spx* transcription is under negative transcriptional control that is reversed when disulfide stress is encountered.

The *spx* gene of *Bacillus subtilis* was discovered to be the site of mutations that suppressed null alleles of *clpX* and *clpP* with respect to competence development, sporulation gene expression, and growth in minimal medium (14, 27). It was later characterized as an RNA polymerase (RNAP)-binding protein that repressed activator-stimulated transcription and activated transcription initiation at the *trxA* (thioredoxin) and *trxB* (thioredoxin reductase) promoters in response to oxidative stress (16–18, 20). Recent reports indicated that Spx is an important regulatory factor in the stress response in *Staphylococcus aureus* (21) and during *Listeria monocytogenes* infection (3). Spx concentration and activity increase when cells encounter a toxic oxidant that brings about disulfide stress (16, 17). Increases in Spx protein concentration are associated with a variety of stress conditions (24) including heat, salt, disulfide, and peroxide stress.

The *spx* gene resides in the *yjbC-spx* dicistronic operon located between the *opp* operon and the *mecA* gene (Fig. 1A). Previous studies uncovered the complexity of the *yjbC-spx* operon's transcriptional organization (1). Four kinds of RNAP holoenzymes recognize promoter DNA sequences at the 5′ end of the operon and in the intergenic DNA between *yjbC* and *spx*. Upstream of the *yjbC* coding sequence are three putative transcription start sites uncovered by primer extension studies: P_1, recognized by the σ^A form of RNAP, and P_2 and P_B, utilized by the σ^W and σ^B RNAP holoenzymes, respectively. In the *yjbC-spx* intergenic region, the P_M promoter was identified (25), which is utilized by the σ^M form of RNAP. The σ^B RNAP holoenzyme is a crucial component of the general energy and environmental stress responses (6). The σ^W holoenzyme form is required for the transcription of genes that are induced by envelope stress, antimicrobial agents, and superoxide stress (2, 8, 9). Hence, part of the reason why the Spx concentration increases in response to stress might be the activities of the RNAP holoenzyme forms that utilize promoters of the *yjbC-spx* operon.

In this report, the discovery of a fifth promoter in the *yjbC-spx* operon intergenic region is described. It is activated when *B. subtilis* cells are treated with the thiol-specific oxidant diamide (10). The P_3 promoter is utilized by the σ^A form of RNAP and is associated with two negative control elements. The accompanying paper describes the two repressors that target the *spx* P_3 promoter region (10a).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* strains used in this study are derivatives of JH642 and are listed in Table 1. Cells were cultivated in a shaking water bath at 37°C in Difco sporulation medium (DSM) for β-galactosidase assays or TSS minimal medium (4) for diamide treatment experiments (17). Diamide was purchased from Sigma-Aldrich and used at a concentration of 1 mM to induce disulfide stress.

Construction of *yjbC* and *spx* transcriptional fusions. The *yjbC-spx* promoter region was amplified by PCR using primers oML02-17 and oML02-6 (Table 2). The product was digested with BamHI and BclI and then inserted into pUC19 digested with BamHI to generate pML11. The cloned *yjbC-spx* DNA was verified by DNA sequencing and was then digested with BamHI and SmaI to release the fragment containing 420 bp upstream of the *yjbC* start codon to 187 bp down-

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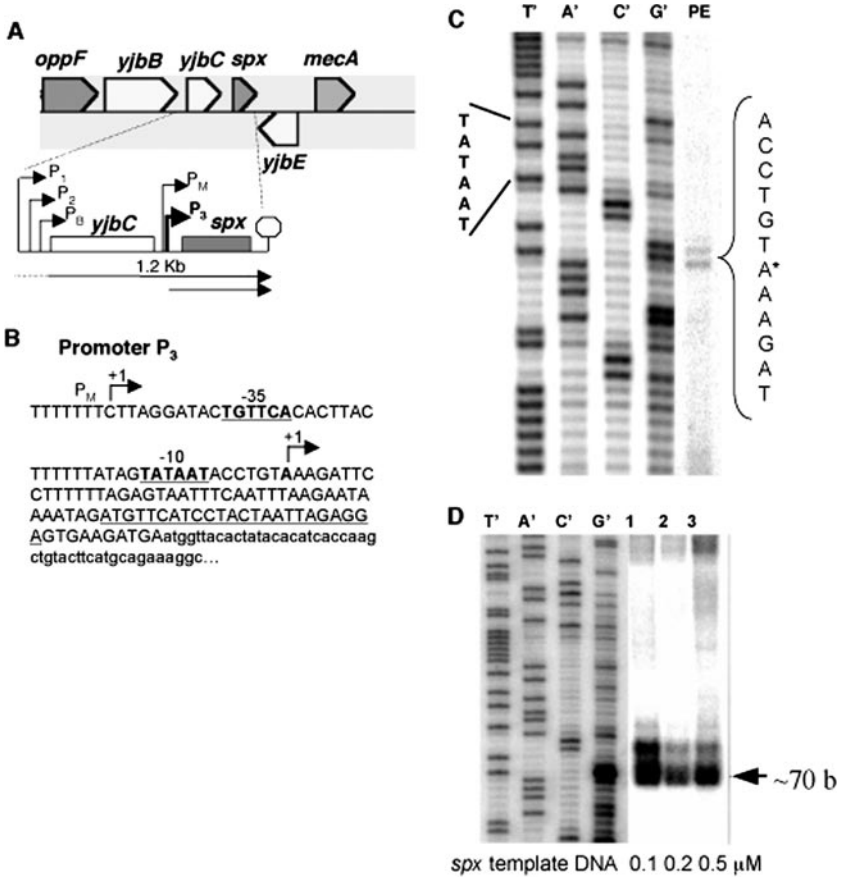

FIG. 1. (A) Organization of the *yjbC-spx* operon. Promoters (P_1, P_2, and P_B) of *yjbC* are indicated by bent arrows, as is the P_M promoter of the *spx* gene recognized by σ^M RNAP. The arrow beneath the operon diagram indicates 1.2-kb mRNA identified by Northern blot analysis (hybridization-labeled probe specific for *yjbC*) (1). The *spx* promoter P_3 is indicated by the bold, bent arrow. (B) Sequence of the *spx* P_3 promoter region. The −10 and −35 promoter sequences are indicated by boldface, underlined type. The P_3 transcription start site is marked by +1 and an arrow. The nucleotide sequence of the labeled primer used in the primer extension experiments is underlined. The nucleotide sequence of the *spx* coding sequence is shown in lowercase type. (C) Denaturing polyacrylamide gel profile of a primer extension (PE) reaction using RNA extracted from JH642 cells. The sequencing reaction and primer extension utilized the primer described in B. (D) Denaturing polyacrylamide gel profile of in vitro runoff transcription products of reaction mixtures containing purified RNAP (100 nM), *spx* promoter DNA (0.1, 0.2, and 0.5 μM as indicated), and radiolabeled nucleotide triphosphate mix. Reaction mixtures were incubated at 37°C for 20 min.

stream of the start codon. The resulting fragment was then subcloned into plasmid pDL to construct pML18, a plasmid bearing a *bgaB* fusion, encoding thermostable β-galactosidase (26), and the *yjbC-spx* intergenic region. The fragment released from pML18 by EcoRI digestion and containing 538 bp upstream of the P_3 start site to 187 bp downstream of the *spx* start codon was inserted into plasmid pDL to generate pML19, the *spx-bgaB* fusion.

Constructions of promoter deletion mutations. The *spx* 3′ promoter region was amplified by PCR using primer oML02-7 in combination with oML02-22 (+50 region) (wild type), oML02-26 (+5 region), oML02-27 (+15 region), oML02-28 (+30 region), or oML02-29 (+40 region) (Table 2). The products were digested with BamHI and KpnI and then inserted into pUC19 digested with the same enzymes to generate pML25, pML27, pML28, pML29, and pML33, respectively. The *spx* sequences were verified by DNA sequencing. The plasmids were digested with BamHI and EcoRI to release a fragment extending from 330 bp upstream from the P_3 start site to the 3′ deletion endpoint. The fragments were then inserted into plasmid pDL that was digested with the same enzymes to generate pML26, pML30, pML31, pML32, and pML34, respectively. The *spx* 5′ promoter deletion region was amplified by PCR using primer oML02-22 in combination with oML02-23 (−40 region), oML02-24 (−60 region), or oML02-25 (−100 region) (Table 2). The products were digested with BamHI and EcoRI and then inserted into pDL that was digested with the same enzymes to generate pML20, pML21, and pML22, respectively. The fragments extended from the 5′ deletion endpoint to 50 bp downstream of the P_3 transcription start site. The *spx* sequences were verified by DNA sequencing. The strains bearing wild-type *spx* and 5′ and 3′ *spx* deletion promoter-*bgaB* fusions were ORB5058 (wild type), ORB4980 (−40 region), ORB4981 (−60 region), ORB4982 (−100 region), ORB5077 (+5 region), ORB5078 (+15 region), ORB5079 (+30 region), and ORB5115 (+40 region). Cells bearing the promoter-*bgaB* fusions were grown in DSM until the optical density at 600 nm (OD_{600}) reached ~0.4 to 0.5. After further incubation for 30, 60, and 120 min, samples of cells were harvested and prepared for β-galactosidase assays.

Site-directed mutagenesis. T−26A, T−20G, T−19G, A−14T, A+3G, T+7C, and T+24C mutant alleles of *spx* were generated by PCR-based site-directed mutagenesis. First-round PCR was performed in two separate reactions with primer oMLbgaB in combination with oML02-38, oML02-40, oML02-42, oML02-44, oML02-46, oML02-48, or oML02-50 (Table 2) using pML26 as a template and primer oML02-37 in combination with oML02-39, oML02-41, oML02-43, oML02-45, oML02-47, oML02-49, or oML02-51 (Table 2) using pSN16 (19) as a template. The PCR products were hybridized and subsequently amplified by a second round of PCR using oML02-37 and oML02-22. The products from a second round of PCR were then digested with EcoRI and BamHI restriction enzymes (to release the PCR fragment from positions −330 to +50 relative to the P_3 transcription start site) and inserted into pDL digested with the same enzymes to generate pML42, pML43, pML44, pML46, pML45, pML47, and pML48, respectively. The *spx* sequences in the plasmids were verified by DNA sequencing. The *spx* promoter point mutant strains used for β-galactosidase assays and primer extension analyses were ORB6030 (T−26A),

TABLE 1. *Bacillus subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or description[a]	Reference or source
Strains		
JH642	*trpC2 pheA1*	J. A. Hoch
ORB3621	*trpC2 pheA1 rpoA*(*Y263C*)	15
ORB3834	*trpC2 pheA1 spx*::*neo*	14
ORB4888	*trpC2 pheA1 amyE*::*PyjbC-spx-bgaB cat*	This study
ORB4889	*trpC2 pheA1 amyE*::*Pspx-bgaB cat*	This study
ORB4980	*trpC2 pheA1 amyE*::*Pspx*?−40-*bgaB cat*	This study
ORB4981	*trpC2 pheA1 amyE*::*Pspx*Δ−60-*bgaB cat*	This study
ORB4982	*trpC2 pheA1 amyE*::*Pspx*Δ−100-*bgaB cat*	This study
ORB5058	*trpC2 pheA1 amyE*::*Pspx*Δ+50(wt)-*bgaB cat*	This study
ORB5077	*trpC2 pheA1 amyE*::*Pspx*Δ+5-*bgaB cat*	This study
ORB5078	*trpC2 pheA1 amyE*::*Pspx*Δ+15-*bgaB cat*	This study
ORB5079	*trpC2 pheA1 amyE*::*Pspx*Δ+30-*bgaB cat*	This study
ORB5107	*trpC2 pheA1 amyE*::*Pspx*Δ+50(wild type)-*bgaB cat spx*::*neo*	This study
ORB5115	*trpC2 pheA1 amyE*::*Pspx*Δ+40-*bgaB cat*	This study
ORB6030	*trpC2 pheA1 amyE*::*Pspx*(T−26A)-*bgaB cat*	This study
ORB6031	*trpC2 pheA1 amyE*::*Pspx*(T−20G)-*bgaB cat*	This study
ORB6032	*trpC2 pheA1 amyE*::*Pspx*(T−19G)-*bgaB cat*	This study
ORB6033	*trpC2 pheA1 amyE*::*Pspx*(A−14T)-*bgaB cat*	This study
ORB6034	*trpC2 pheA1 amyE*::*Pspx*(A+3G)-*bgaB cat*	This study
ORB6035	*trpC2 pheA1 amyE*::*Pspx*(T+7C)-*bgaB cat*	This study
ORB6036	*trpC2 pheA1 amyE*::*Pspx*(T+24C)-*bgaB cat*	This study
Plasmids		
pSN16	*yjbC-spx-lacZ* fusion plasmid	19
pML11	*yjbC-spx* transcriptional fusion in pUC19	This study
pML18	*yjbC-spx* transcriptional fusion in pDL	This study
pML19	*spx* transcriptional fusion in pDL	This study
pML20	*spx* promoter fragment (−40) in pDL	This study
pML21	*spx* promoter fragment (−60) in pDL	This study
pML22	*spx* promoter fragment (−100) in pDL	This study
pML23	*yjbC* promoter fragment in pDL	This study
pML25	*spx* promoter fragment (wild type +50) in pUC19	This study
pML26	*spx* promoter fragment (wild type +50) in pDL	This study
pML27	*spx* promoter fragment (+5) in pUC19	This study
pML28	*spx* promoter fragment (+15) in pUC19	This study
pML29	*spx* promoter fragment (+30) in pUC19	This study
pML30	*spx* promoter fragment (+5) in pDL	This study
pML31	*spx* promoter fragment (+15) in pDL	This study
pML32	*spx* promoter fragment (+30) in pDL	This study
pML33	*spx* promoter fragment (+40) in pUC19	This study
pML34	*spx* promoter fragment (+40) in pDL	This study
pML35	Random mutagenesis of *spx* promoter in pUC19	This study
pML36	Random mutagenesis of *spx* promoter in pDL	This study
pML42	*spx*(*T−26A*) in pDL	This study
pML43	*spx*(*T−20G*) in pDL	This study
pML44	*spx*(*T−19G*) in pDL	This study
pML45	*spx*(*A3G*) in pDL	This study
pML46	*spx*(*A−14T*) in pDL	This study
pML47	*spx*(*T7C*) in pDL	This study
pML48	*spx*(*T24C*) in pDL	This study

[a] wt, wild type.

ORB6031 (T−20G), ORB6032 (T−19G), ORB6033 (A−14T), ORB6034 (A+3G), ORB6035 (T+7C), and ORB6036 (T+24C).

Assays of β-galactosidase activity. Assays of BgaB activity were performed according to previously published methods (23). Activity was expressed as Miller units (12). The data from β-galactosidase assays are presented with standard deviations for three to four independent experiments.

Primer extension analysis. Cultures of wild-type and P_3 promoter mutant strains were grown at 37°C in TSS medium. At mid-log phase, the culture was split into two cultures, and diamide was added to one culture to a final concentration of 1 mM. Aliquots (20 ml) of cultures were withdrawn for RNA preparation at time zero (mid-log phase) and after 10 min with or without diamide (1 mM) treatment. The culture was mixed with an equal volume of ice-cold methanol; after centrifugation, the cell pellet was frozen at −80°C. The total RNA was prepared by using an RNeasy Mini kit (QIAGEN, Chatworth, CA). The primer corresponding to the *bgaB* sequence downstream of the inserted fragment (oMLbgaB) as well as primer oML02-15 (specifying the endogenous *spx* sequence) were used to examine the transcript of *spx* fusions and endogenous *spx*. Primer oML02-10 was used to examine the transcript of *yjbC*. The same amount of RNA used in the primer extension was applied to a formaldehyde-agarose gel. 16S rRNA was visualized by staining with ethidium bromide to confirm that comparable amounts of total RNA were used for each reaction mixture.

In vitro transcription assay. A linear DNA template for the *spx* promoter was generated by PCR with primers oML02-7 and oML02-15 using plasmid pML18 as the template (encoding a ~70-base transcript). The 0.1, 0.2, and 0.5 μM DNA templates were mixed with 100 nM RNAP in a solution containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumin, and 5 mM dithiothreitol at 37°C for 10 min. A nucleotide mixture (200 μM ATP, GTP, and CTP, 10 μM UTP, 10 μCi [α-^{32}P]UTP) was added to the reaction

TABLE 2. Oligonucleotides used in this study[a]

Oligonucleotide	Sequence
oMLbgaB	5′-CCCCCTAGCTAATTTTCGTTTAATTA-3′
oML02-6	5′-GATGATACGATCTATTAGTTTGCCAAAC-3′
oML02-7	5′-CCCAAGCTTGCATTTCAAGCATGCTCAG-3′
oML02-10	5′-TCAATTGGAAAGTACTCGCTAAGC-3′
oML02-15	5′-TCCTCTAATTAGTAGGATGAACAT-3′
oML02-22	5′-CGGGATCCCGAACATCTATTTTATTC-3′
oML02-23	5′-GGAATTCCGATACTGTTCACACTT-3′
oML02-24	5′-GGAATTCCTTGACACATTTTTTTC-3′
oML02-25	5′-GGAATTCCAGAATAAGAACATATC-3′
oML02-26	5′-CGGGATCCTCTTTACAGGTATTA-3′
oML02-27	5′-CGCGGATCCAAAAAAGGAATCTTT-3′
oML02-28	5′-CGCGGATCCAATTGAAATTACTC-3′
oML02-29	5′-CGCGGATCCTTTATTCTTAAATTG-3′
oML02-37	5′-GGAATTCCGGTACCGGCGCCGGTCATT-3′
oML02-38	5′-GTTCACACaTACTTTTTTATAG-3′
oML02-39	5′-AAAAGTAtGTGTGAACAGTATCC-3′
oML02-40	5′-CACTTACTTgTTTATAGTATAATACC-3′
oML02-41	5′-CTATAAAcAAGTAAGTGTGAAC-3′
oML02-42	5′-CACTTACTTTgTTATAGTATAATACC-3′
oML02-43	5′-CTATAAcAAAGTAAGTGTGAAC-3′
oML02-44	5′-ACTTTTTTATtGTATAATACCTG-3′
oML02-45	5′-CTATAAcAAAGTAAGTGTGAAC-3′
oML02-46	5′-ACCTGTAAgGATTCCTTTTTTAGAG-3′
oML02-47	5′-AGGAATCcTTACAGGTATTATACT-3′
oML02-48	5′-GTAAAGATcCCTTTTTTAGAGTAA-3′
oML02-49	5′-CTAAAAAAGGgATCTTTACAGGT-3′
oML02-50	5′-GAGTAATcTCAATTTAAGAATAA-3′
oML02-51	5′-TAAATTGAgATTACTCTAAAAAAGG-3′

[a] Bases in lowercase type are mutagenic.

mixture. The reaction mixtures (20 μl) were further incubated at 37°C for 20 min, and the transcripts were precipitated by ethanol. Electrophoresis was performed as described previously (11).

UV irradiation mutagenesis. ORB5107 (*Pspx-bgaB spx::neo*) was grown in liquid DSM until the OD_{600} reached 0.7 to 1.0. Cells were harvested and washed with 0.1 M $MgSO_4$. Cells were resuspended in 0.1 M $MgSO_4$ and then irradiated (UV Stratalinker 1800) for 4 min. Irradiated cells were kept on ice and in the dark. Serial dilutions of untreated cells and UV-treated cells were plated onto DSM plates for counting isolated colonies to determine the survival rate. The frequency of auxotrophic mutants among the UV-treated cells was determined by replica plating onto TSS agar containing 0.1% tryptophan and phenylalanine.

Error-prone PCR mutagenesis. The *spx* promoter region was amplified by PCR using primers oML02-7 and oML02-22. The error-prone PCR mixture contained error-prone PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 7 mM $MgCl_2$), 0.3 mM $MnCl_2$, 0.2 μM of each primers, 0.2 mM of dATP and dGTP, and 1 mM of dCTP and dTTP. The product was digested with KpnI and BamHI and then inserted into pUC19. The pooled plasmid was digested with EcoRI and BamHI, followed by subcloning into plasmid pDL. The recombinant pDL pool was used to transform JH642 to generate the strain bearing random nucleotide substitutions within the *spx* promoter region. The transformants were screened for blue colonies on the DSM plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The BgaB phenotype of the blue colony isolates was confirmed by β-galactosidase assays of samples collected from liquid DSM cultures. Confirmed $BgaB^+$ variants were analyzed by sequence analysis of the mutagenized *spx* promoter region.

RESULTS

Identification of the P_3 promoter within the *yjbC-spx* operon. Fig. 1 shows the genomic location and the organization of the *yjbC-spx* operon of *B. subtilis*. The *yjbC* operon resides between the *opp* (*spo0K*) operon and the *mecA* gene (Fig. 1A). Upstream of *yjbC* are three promoters, two of which are recognized by the σ^W (P_2) and σ^B (P_B) forms of RNAP (1, 2, 22). The *yjbC* coding sequence is followed by an intergenic region of 184 bp that contains the previously described P_M promoter of *spx* that is recognized by the σ^M form of RNAP. The *spx* gene coding sequence begins 123 bp downstream of the start point of the P_M promoter (Fig. 1B) (25). Because Spx activity and concentration are elevated upon oxidative stress, we felt that the transcription of the *spx* gene might be controlled in response to the presence of a toxic oxidant (diamide). Primer extension analysis of total JH642 RNA using primers specific for *spx* and *yjbC* was conducted to determine if transcription from the various promoters of the *yjbC-spx* operon was induced by diamide treatment. No elevation of *yjbC* transcript levels was observed upon disulfide stress (data not shown, but see Fig. 5D), but a transcript from a previously unidentified transcription start site was detected (Fig. 1C), and its concentration increased upon diamide treatment (see below). The start site of transcription from the new P_3 promoter resides 79 bp from the start of the *spx* coding sequence. The same start site was utilized by purified RNAP in vitro, as shown in the gel profile of the runoff transcription products in Fig. 1D. No transcription from the P_M promoter in vivo or in vitro was detected.

Diamide-dependent induction of transcription from P_3 does not require Spx. Transcription of *spx* from P_3 in cells of cultures that were subjected to disulfide stress was examined. RNA was isolated from wild-type or *spx* mutant cultures before and after 10 and 30 min of diamide treatment. Denaturing gel analysis of primer extension reactions showed that transcript levels increased after 10 min of diamide treatment of wild-type and *spx* mutant cells (Fig. 2A). The Spx-dependent induction of *trxA* (encoding thioredoxin) transcription by diamide (17) was included as a control (Fig. 2B). A similar pattern of transcriptional induction was observed in cells bearing the $rpoA^{cxs-1}$ mutation (Fig. 2B), which renders the RNAP α C-terminal

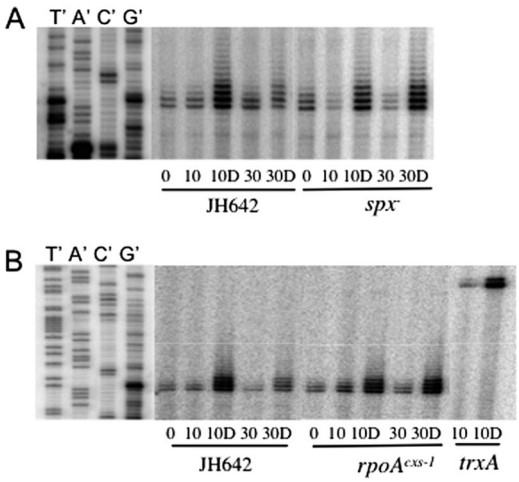

FIG. 2. Primer extension analysis shows the increase in *spx* transcript levels after diamide treatment. Total RNA was extracted from cells grown in TSS medium and harvested at mid-log phase (0) and then after cells were treated for 10 and 30 min with 1 mM diamide (10D and 30D, respectively) and without diamide (10 and 30, respectively). The labeled primer shown in Fig. 1B was used for primer extension reactions. The gel profile of the primer extension reaction using a *trxA*-specific primer (17) was a control for diamide-induced transcript accumulation. The dideoxy sequencing ladders are shown on the left. For dideoxynucleotide sequencing, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane (T′, A′, C′, and G′). (A) Total RNA was extracted from wild-type JH642 and ORB3834 (*spx* null) cells. (B) Total RNA extracted from wild-type JH642 and ORB3621 ($rpoA^{cxs-1}$) cells.

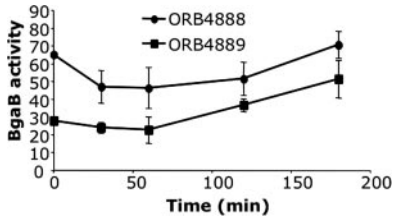

FIG. 3. Assay of *yjbC-spx*- and *spx*-directed β-galactosidase (BgaB) activity. Expression of *yjbC-spx-bgaB* and *spx-bgaB* was determined as BgaB activity in Miller units. Cells were grown in DSM. Time zero indicates the mid-log phase. ●, ORB4888 (*yjbC-spx-bgaB*); ■, ORB4889 (*spx-bgaB*).

domain unable to interact with Spx. Thus, it was shown that the induction of *spx* transcription from the P_3 promoter by diamide treatment did not require the interaction of Spx with RNAP, which is required for *trxA* and *trxB* transcription.

Examination of the primer extension signal at 30 min following diamide treatment shows that there is more *spx* transcript in the *spx* and *rpoA*$^{cxs-1}$ cells than in those of the wild-type strain. This suggests that while the Spx-RNAP interaction might not be required for *spx* transcription, this interaction is necessary for the negative control required to restore expression to the prestress state.

Transcription from the *yjbC-spx* intergenic region contributes substantially to the total expression of *spx*. The *yjbC-spx* operon encodes a 1.2-kb RNA, which specifies the YjbC and Spx proteins (1). Previously described results (14, 25) and data presented in Fig. 1 and 2 show that the intergenic DNA contains at least two promoters that could drive *spx* expression. Two β-galactosidase (*bgaB*) fusions were constructed, and their expression was examined to determine to what extent the *yjbC-spx* intergenic region contributes to the expression of *spx* under nonstress conditions. The *yjbC-spx-bgaB* fusion of strain ORB4888 contained a fragment extending from the stop codon of *spx* at its 3′ end to 420 bp upstream of the *yjbC* coding sequence. The *spx-bgaB* fusion of strain ORB4889 contains a fragment bearing the same 3′ end as in the *yjbC-spx-bgaB* fusion, but its 5′ end is 538 bp upstream of the P_3 start site, a point within the *yjbC* coding sequence. As shown in the time course experiment depicted in Fig. 3, the promoters of the *spx-bgaB* fusion account for between 45 and 70% of the total *bgaB* activity directed by the *yjbC-spx* operon. Because the cells were grown in DSM at 37°C, the stress conditions that would lead to the maximal induction of σ^W and σ^B activity were likely not encountered. Such conditions might result in a much larger contribution to *spx* expression by the *yjbC* promoter region. Nevertheless, the results shown in Fig. 3 indicate that the *spx* gene contains transcriptional signals that direct expression independently of the *yjbC* transcription initiation region. This is in keeping with the observation that a fragment bearing the *spx* gene and the upstream intergenic region is sufficient for the complementation of an *spx* null mutation (17).

Deletion analysis uncovers *cis*-acting negative control elements associated with the P_3 promoter. The results depicted in Fig. 1D show that the P_3 promoter can be utilized by purified σ^A RNAP in vitro without the presence of a positive control factor, suggesting that the diamide induction of P_3 transcription might be caused by a reversal of negative control. To identify *cis*-acting control sequences associated with the P_3 promoter, deletion and point mutational analyses were undertaken. Figure 4 summarizes the results of the deletion analysis of the *spx* promoter region. PCR-generated fragments in which sequences 5′ and 3′ of the P_3 promoter were omitted were introduced into the *bgaB* fusion vector. The levels of BgaB activity and the level of transcript determined by primer extension reactions were assessed. BgaB activity was used as a measure of uninduced expression, since diamide induction cannot be observed using the enzyme assay due to the inactivation of BgaB. Therefore, induction was observed by measuring the increase in the level of transcript from both the *spx-bgaB* fusion and the endogenous *spx* gene using primer extension. The 5′ deletions had modest effects on the basal expression of *spx*, but diamide induction was still observed.

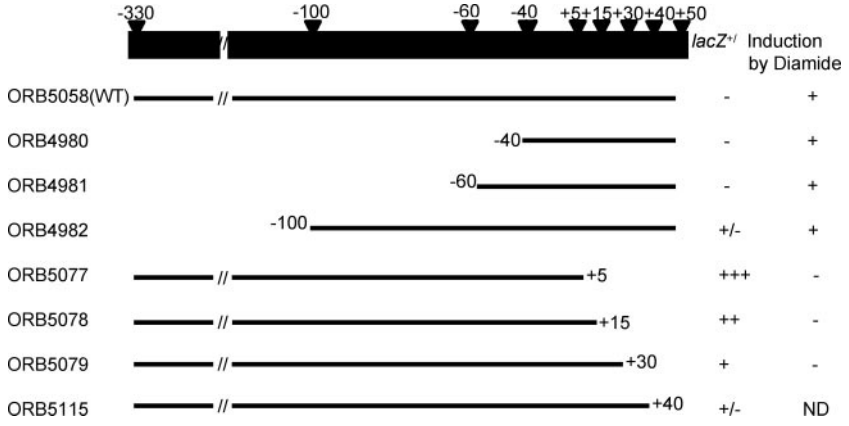

FIG. 4. Summary of deletion analysis of the *spx* P_3 promoter region. The top bar shows the intact *spx* promoter and the positions of deletion endpoints relative to the transcription start site. All constructs generated by PCR were fused with the promoterless *bgaB* gene as the reporter. The *bgaB* expression levels from the respective fusions after integration into the chromosome were examined by screening on DSM agar plates containing 40 μg/ml X-gal (blue [+], white [−], and pale blue [+/−]). Transcriptional induction was determined by the extraction of RNA from diamide-treated and untreated cells bearing *spx-bgaB* fusions followed by primer extension analysis. + indicates that transcriptional induction after diamide treatment was detected. − indicates that a loss of induction was detected after diamide treatment. ND, no data.

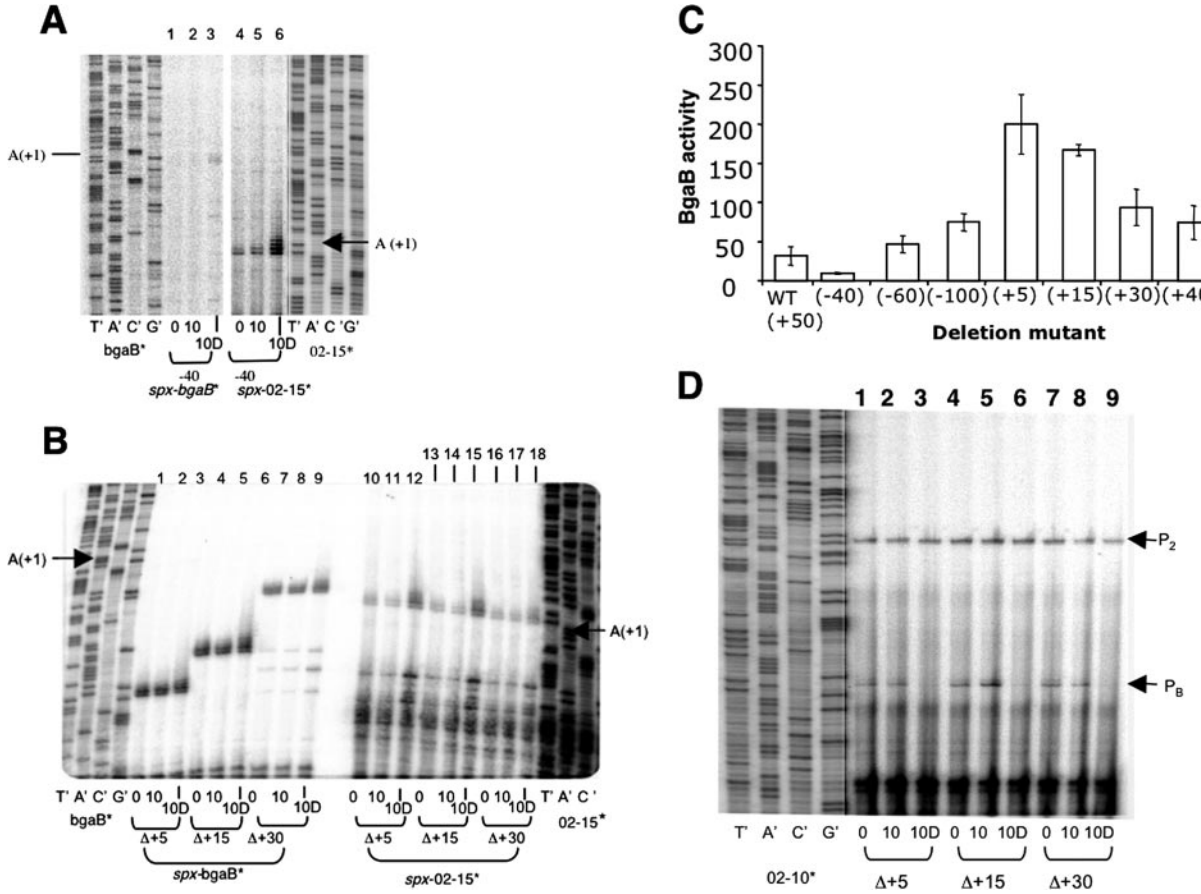

FIG. 5. Primer extension analysis of RNA extracted from −40 deletion ORB4980 cells (A) and 3′ deletion ORB5077 (Δ+5), ORB5078 (Δ+15), and ORB5079 (Δ+30) cells (B and C) in TSS cultures subjected to diamide treatment. Cells were treated with 1 mM diamide for 10 min (10D) and without diamide (0 and 10 min) after the OD_{600} reached 0.4 to 0.5. Labeled primers specific to the *bgaB* fusion (*bgaB*∗) and endogenous *spx* (02-15∗) were used to detect *spx* transcripts. Marker lanes of sequencing reactions show the nucleotide positions corresponding to the *spx-bgaB* (A and B) and endogenous *spx* (A and B) start points. (A) Primer extension analysis of *spx-bgaB* and endogenous *spx* transcripts in total RNA extracted from the −40 deletion strain (ORB4980). (B) Primer extension analysis of *spx-bgaB* and endogenous *spx* transcripts in total RNA extracted from 3′ deletion strains ORB5077 (Δ+5), ORB5078 (Δ+15), and ORB5079 (Δ+30). (C) Expression of *spx-bgaB* fusion derivatives bearing promoter deletions as determined by assays of *spx*-directed β-galactosidase activity. Cells were grown in DSM. The expression was determined as BgaB activity in Miller units 30 min after cultures reached the mid-log phase. WT, wild type. (D) Primer extension analysis of *yjbC* transcripts in total RNA extracted from 3′ deletion strains.

However, deletions of the sequences 3′ of the P_3 transcriptional start site resulted in higher basal-level expression.

A negative control element resides downstream of the P_3 promoter sequence. As summarized in Fig. 4, deletions of the 5′ end had little effect on the diamide-dependent induction of P_3. This is shown in Fig. 5A, which is a gel profile of the primer extension reaction of RNA extracted from diamide-treated cells. A −40 deletion, which removes most of the DNA upstream of the P_3 promoter, does not change the induction pattern of P_3 transcription (Fig. 5A, lanes 1 to 3), which is also observed when the endogenous *spx* P_3 transcript is examined (lanes 4 to 6). Deletion of the DNA 3′ of the P_3 start site, with endpoints at positions +30, +15, and +5, results in an increase in basal-level transcription and a loss of diamide induction, as shown in primer extension experiments and in assays of *spx-bgaB* activity (Fig. 5B, lanes 1 to 9, and C). The same RNA used in the primer extension analysis of *spx-bgaB* promoter mutants was used to examine the level of endogenous *spx* transcript, which increased within 10 min of diamide treatment (Fig. 5B, lanes 10 to 18). The data shown in Fig. 5B are strong evidence for a negative control element in the region 3′ to the P_3 promoter sequence.

Primer extension analysis also showed that the *yjbC* promoters do not contribute to the diamide induction of *spx* expression (Fig. 5D). The P_2 and P_B promoter transcripts can be detected in RNA from untreated cells, while diamide treatment has no effect on the level of P_2 transcript but results in a loss of the P_B transcript. At present, we do not know if this repression of P_B is operon specific or due to a reduction of σ^B activity upon disulfide stress.

Point mutations suggest the presence of a second negative control site within the P_3 promoter. Experiments were conducted to uncover mutations in *trans*-acting loci, with hopes of identifying the putative repressor controlling transcription from P_3. Cultures of *spx-bgaB* fusion-bearing cells were mutagenized by UV irradiation and examined for elevated BgaB activity on agar medium containing X-gal. Of the four variants isolated, all bore mutations in the intergenic region of *yjbC-spx*.

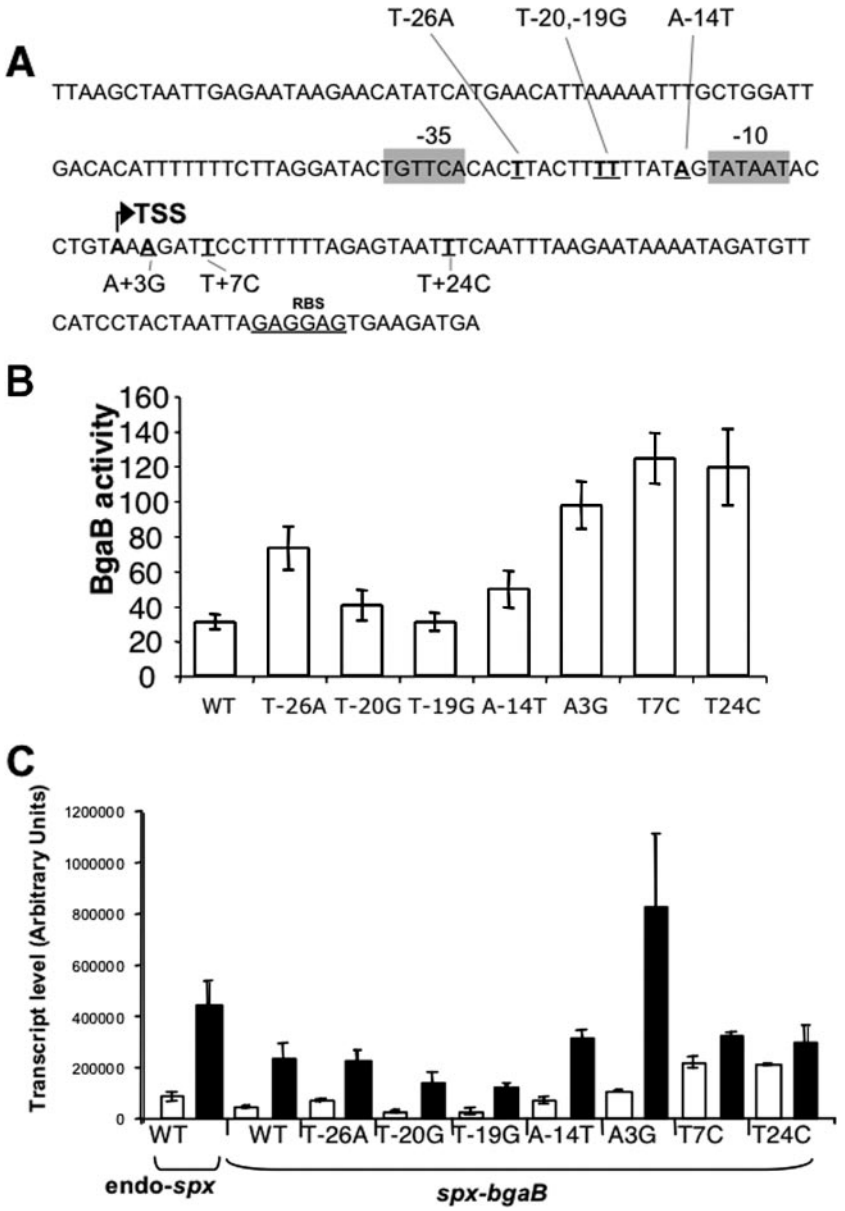

FIG. 6. Analysis of *spx* promoter P_3 point mutations. (A) *spx* promoter region. The *spx* sequence along with the locations of seven nucleotide substitutions, as indicated by underlining (T−26A, T−20G, T−19G, A−14T, A+3G, T+7C, and T+24C), are shown. The bent arrow indicates the transcription start site (TSS). RBS, ribosome binding site. −10 and −35 promoter sequences are indicated by shaded boxes. (B) The PCR fragments of each point mutation bearing the *spx* sequence from position −330 to +50 were inserted into plasmid pDL (the *bgaB* fusion vector). The constructed plasmids were integrated into the *amyE* locus of *B. subtilis*. Cells were grown in DSM. The expression was determined as BgaB activity in Miller units 30 min after cultures reached the mid-log phase. (C) Primer extension analysis of point mutations in the P_3 sequence of the *spx-bgaB* fusion after diamide treatment. The transcripts of the *spx* fusions and endogenous *spx* (endo-*spx*) of each point mutant without (white bars) and with (black bars) diamide treatment for 10 min are shown. RNA was extracted from cells harvested from cultures that reached an OD_{600} of 0.4 to 0.5. *spx-bgaB* transcripts were detected by primer extension analysis. Results, with standard deviations, for radiolabeled primer extension products from two independent RNA extracts and duplicate primer extension reactions for each extract are presented. endo-*spx* denotes the endogenous *spx* gene transcript level. WT, wild type.

In parallel experiments, in vitro mutagenesis by error-prone PCR was undertaken using a fragment of the P_3 promoter region as a template. The pool of mutagenized fragments was inserted into the pDL *bgaB* fusion vector. All of the mutants obtained by the two procedures bore multiple nucleotide substitutions. Therefore, site-directed oligonucleotide mutagenesis was carried out to create single substitutions, the locations of which were chosen from the mutant sequences obtained from the UV mutagenesis and error-prone PCR experiments. Seven single-site mutants obtained (Fig. 6A) were analyzed by measuring *spx*-directed BgaB activity (Fig. 6B) and by primer extension analysis (Fig. 6C). Of the seven mutants, five showed elevated basal *spx-bgaB* expression (T−26A, A−14T, A3G, T7C, and T24C) (Fig. 6B) and higher levels of transcripts as

shown in primer extension reactions with RNA from untreated cells (T−26A, A−14T, A3G, T7C, and T24C) (Fig. 6C). While the A3G substitution results in a generally higher level of transcript in both treated and untreated cells, the other mutations showing higher basal expression and transcript levels showed no increase in diamide-induced expression and, hence, have reduced induction ratios compared wild-type cells, indicating that these mutations reduce transcriptional repression under nonstress conditions. Our results provide evidence for a second *cis*-acting negative control region within the P_3 promoter region.

DISCUSSION

The transcription of *spx* is driven by five promoters of the *yjbC-spx* operon, although not all of the promoters are active under the culture growth conditions used in the study described herein. The promoters P_M and P_3 reside in the intergenic region of *yjbC-spx*, but only transcription from P_3 is observed in the primer extension analysis of RNA from cells grown in minimal TSS medium, before and after diamide treatment. The P_3 promoter contains a consensus −10 sequence that is utilized by the major σ^A form of RNA polymerase but has a −35 sequence that shows a 3-of-6-nucleotide match for the consensus −35 region. Levels of transcript synthesized from P_3 increase 10 min after diamide treatment. Transcription from P_3 is catalyzed by the σ^A holoenzyme in vitro without the requirement for an additional transcription factor, which suggested that transcription from P_3 is under negative control. The intergenic region contributes significantly to *spx* transcription, as shown in experiments using *yjbC-spx* and *spx-bgaB* fusions and by complementation of an *spx* null mutation with respect to diamide sensitivity using a single copy of the *spx* gene with the accompanying *yjbC-spx* intergenic region. The Spx concentration increases after diamide treatment, which was shown to be due in part to posttranscriptional control of *spx* expression (17), perhaps by the downregulation of ClpXP-dependent proteolysis of Spx. However, the observation that *spx* transcription can be induced by disulfide stress suggests that transcriptional control can also contribute to elevated Spx levels during oxidative stress.

Mutational analysis of the regulatory region of the *spx* gene uncovered two negatively *cis*-acting elements. Deletions of sequences located downstream of the transcriptional start site of P_3 resulted in higher basal-level transcription and a reduced diamide induction ratio. Based on these data, we propose that the mutations define an operator with which a negative transcriptional regulator interacts. Attempts at identifying a regulator involved a mutant search for *trans*-acting loci that might confer constitutive transcription from P_3 and encode a repressor. No extragenic mutations conferring such a phenotype were isolated, but several *cis*-acting mutations were uncovered by screening mutants after UV mutagenesis. Five mutations, two downstream of the P_3 transcriptional start site and three upstream and within the P_3 promoter sequence, were observed to cause elevated basal transcription. Of these, four mutations, T−26A, A−14T, T7C, and T24C, reduced the diamide induction ratio and increased basal-level transcription. The T7C and T24C mutations likely affect the downstream operator defined by deletion analysis. We propose that the T−26 and A−14 positions define a second operator that is the interaction site of a second negative regulator. Our inability to uncover *trans*-acting loci that affect the control of P_3 utilization suggested that there might be two mechanisms of negative control, and both must be reversed to ensure the proper induction of *spx* during disulfide stress; such a double mutant would likely be very rare.

Several transcriptome studies to identify the genes that are induced by oxidative stress or that are controlled by the peroxide response regulator PerR have been carried out (5, 7, 13). The study described previously by Hayashi et al. identified *spx* as being a member of the PerR regulon (5). The putative operator in the +5-to-+30 region contains at least one sequence bearing a resemblance to a PerR box. Indeed, as we show in the accompanying paper, the PerR protein binds to this region and, in doing so, represses *spx* transcription from P_3 in vitro (10a). Furthermore, a second repressor encoded by the *yodB* gene was observed to bind to the second putative operator defined by the T−26A and A−14T mutations within the P_3 promoter sequence. The description of this dual negative control of *spx* transcription appears in the accompanying paper (10a).

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