Mutational Analysis of the *Bacillus subtilis* RNA Polymerase α C-Terminal Domain Supports the Interference Model of Spx-Dependent Repression†

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The Spx protein of *Bacillus subtilis* exerts both positive and negative transcriptional control in response to oxidative stress by interacting with the C-terminal domain of the RNA polymerase (RNAP) alpha subunit (αCTD). Thus, transcription of the *srf* operon at the onset of competence development, which requires the ComA response regulator of the ComPA signal transduction system, is repressed by Spx-αCTD interaction. Previous genetic and structural analyses have determined that an Spx-binding surface resides in and around the α1 region of αCTD. Alanine-scanning mutagenesis of *B. subtilis* αCTD uncovered residue positions required for Spx function and ComA-dependent *srf* transcriptional activation. Analysis of *srf-lacZ* fusion expression, DNase I footprinting, and solid-phase promoter retention experiments indicate that Spx interferes with ComA-αCTD interaction and that residues Y263, C265, and K267 of the α1 region lie within overlapping ComA- and Spx-binding sites for αCTD interaction. Evidence is also presented that oxidized Spx, while enhancing interference of activator-RNAP interaction, is not essential for negative control.

The *spx* gene of *Bacillus subtilis* was identified as the site of mutations that overcome the requirement for the protease ClpXP in the expression of genes that are transcriptionally activated by response regulator proteins (16, 25). The *srf* operon of *B. subtilis*, which contains genes encoding products that function in the control of competence development and in nonribosomal peptide synthesis (4, 11, 17, 33), is activated by the ComPA two-component signal transduction system (5, 6). ComA is a response regulator that becomes phosphorylated by interaction with the histidine kinase ComP when the latter autophosphorylates in response to the peptide pheromone ComX (9). This quorum-sensing system converts ComA to active ComA phosphate (ComA~P), which interacts as two dimers with the two ComA box elements residing upstream of the *srf* operon promoter (21, 30). ComA-dependent transcriptional activation is one of the regulatory events in *B. subtilis* that are negatively affected by Spx (25).

Competence development, as well as several other transition state processes of *B. subtilis*, is severely impaired in strains bearing mutations in *clpX* or *clpP* (12, 15, 16). Likewise, *srf* operon expression is diminished in *clpX* and *clpP* mutant cells (16, 19). Some of the suppressor mutations resulting in restored *srf* expression in a *clpX* background mapped to the *rpoA* gene, which encodes the RNAP α subunit. Codon substitutions in the region encoding the C-terminal domain of α (αCTD)

were uncovered through this suppressor analysis (19). One codon change conferring the *clpX* suppressor phenotype was Y263C, in the α1 helix of the αCTD. The other *clpX* suppressor locus is the *spx* gene (16), the product of which interacts with the RNAP to affect transcription initiation (25, 27). Subsequent structural analysis confirmed that Spx interacts with the αCTD of RNAP and that the binding surface includes residue Y263 of the α subunit (27). This interaction is necessary for Spx-dependent repression of *srf* operon transcription (25). Unlike other negative transcriptional regulators, however, Spx does not exhibit sequence-specific DNA binding activity (23).

Spx, while exerting negative control on activator-stimulated transcription, positively controls transcription of the thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) genes as well as several genes that function in the oxidative stress response and in cysteine synthesis (23, 24). Positive control is observed after thiol-specific oxidative stress and requires disulfide formation at the highly conserved N-terminal CXXC motif. The detailed mechanism of Spx-dependent transcriptional activation is not known at this time.

The repression of the *srf* operon by Spx during oxidative stress is at least partly the result of a higher Spx protein concentration and interaction with the αCTD of RNAP (24, 25). It is not known if oxidized Spx is required for repression. The mechanism of repression has been proposed to involve the interference of ComA interaction with RNAP by Spx (25, 35). As with other prokaryotic transcriptional activators, ComA-dependent activation involves interaction of activated ComA with RNAP αCTD (this report). Alanine-scanning mutagenesis of RNAP αCTD and the identification of *rpoA* mutant alleles that affect both Spx- and ComA-RNAP interaction are reported herein. The evidence presented supports the interference model of Spx-dependent repression.

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TABLE 1. *Bacillus subtilis* strains

Strain	Genotype	Reference
LAB545	*trpC2 pheA* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	21, this study
MH5636	*trpC2 pheA1* His$_{10}$ *rpoC*	29
OKB167	*trpC2 pheA1 comPA*::Erm *srfB*::Tn*917*	20
ORB3621	*trpC2 pheA rpoA(Y263C)*	This study
ORB4123	*trpC2 pheA* His$_{10}$ *rpoC rpoA^{cxs-1}*	23
ORB4342	*trpC2 pheA amyE*::pSN56	24
ORB4343	*trpC2 pheA rpoAY263C amyE*::pSN56	This study
ORB5501	*trpC2 pheA rpoA(C265A)* His$_{10}$ *rpoC*	This study
ORB5259	*trpC2 pheA rpoA(C265A)*	This study
ORB5262	*trpC2 pheA rpoA(K267A)*	This study
ORB5327	*trpC2 pheA rpoA(K267A)* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5422	*trpC2 pheA rpoA(C265A)* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5553	*trpC2 pheA rpoA(Y263C)* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5661	*trpC2 pheA1 comPA*:Erm SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6116	*trpC2 pheA rpoA(K267A)* His$_{10}$2 *rpoC*	This study
ORB6127	*trpC2 pheA rpoA(C265A) amyE*::pSN56 (pDR111-*spxLDD*)	This study
ORB6128	*trpC2 pheA rpoA(K267A) amyE*::pSN56 (pDR111-*spxLDD*)	This study
ORB6129	*trpC2 pheA amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6130	*trpC2 pheA rpoA(Y263C) amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6131	*trpC2 pheA rpoA(C265A) amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6132	*trpC2 pheA rpoA(K267A) amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6137	*trpC2 pheA amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6138	*trpC2 pheA rpoA(Y263C) amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6139	*trpC2 pheA rpoA(C265A) amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6140	*trpC2 pheA rpoA(K267A) amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6303	*trpC2 pheA1 amyE*::pDR111	This study
ORB6304	*trpC2 pheA1 amyE*::pZY14(*spxLDD(C10A)*)	This study
ORB6305	*trpC2 pheA1 amyE*::pDR111 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6307	*trpC2 pheA1 amyE*::pZY14(*spxLDD(C10A)*) SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. *Bacillus subtilis* strains used in the study are listed in Table 1. *B. subtilis* strains constructed with alanine-scanning αCTD alleles are listed in Table S1 in the supplemental material. Oligonucleotides used in the study, including those used for alanine-scanning mutagenesis of *rpoA*, are listed in Table S2 in the supplemental material. To express mutant *spx(C10A)* from the isopropyl-β-thiogalactopyranoside (IPTG)-inducible P*hyperspank* (P*spank-hy*) promoter (1), plasmid pZY14 was constructed. Plasmid pSN56 (24) was digested with BclI and SalI to obtain the 201-bp fragment containing the 3′ half of the *spxLDD* allele, which encodes the ClpXP-resistant form of Spx. The plasmid pSN95 (23) was digested with HindIII and BclI to obtain a 281-bp fragment containing the N-terminal portion of the *spx(C10A)* allele. Ligation of the two fragments with pUC18, which was digested with SalI and HindIII, was followed by transformation of *Escherichia coli* DH5α competent cells with the ligation mix. This resulted in construction of plasmid pZY11, which encodes the mutant SpxLDD(C10A). Plasmid pZY11 was digested with SalI and HindIII to obtain the 482-bp SpxLDD(C10A)-encoding fragment for ligation with pDR111, which was digested with SalI and HindIII, to yield plasmid pZY14 [P*spank-hy-spxLDD(C10A)*]. Plasmid pZY14 was used to transform *B. subtilis* strain LAB545 (*srfA-lacZ*, contains pMMN92 [21] integrated into SPβ*c2del2*::Tn*917*::pSK10Δ6 prophage [36]) to obtain ORB6307. SpxLDD-expressing strains having mutations in *rpoA* were obtained by transforming ORB5259 [*rpoA(C265A)*] and ORB5262 [*rpoA(K267A)*] with chromosomal DNA from ORB4342 [*amyE*::pSN56 (P*spank-hy-spxLDD*)] to yield ORB6127 and ORB6128. The *srf-lacZ* fusion was introduced into the resulting strains and into ORB4342 (*amyE*::pSN56 *rpoA$^+$*) by transduction with the SPβ phage lysate (36) carrying *srf-lacZ* (pMMN92) (21) with selection for chloramphenicol resistance (5 μg/ml) to yield ORB6127, ORB6128, and ORB6129. Construction of a *srf-35* promoter mutant plasmid (−34,−35TG to CC) was carried out by site-directed mutagenesis (23). Upstream and downstream fragments were synthesized via PCR by using the mutagenic oligonucleotides oYZ02-6 and oYZ02-3a along with the upstream primer oYZ02-3(−347) and downstream primer oYZ02-4 (+65). The two resulting PCR fragments were used as templates for PCR with primers oYZ02-3 and oYZ02-4. The PCR fragment digested with BamHI and HindIII was inserted

into pUC18 to obtain plasmid pZY6. The plasmid pMMN101, containing the mutant *srf* promoter fragment bearing a ComA box 2 mutation, was previously described (21).

Alanine-scanning mutagenesis of the *rpoA* CTD region. The *rpoA-rplQ* region of the *B. subtilis* chromosome was amplified by PCR using the primers oMN99-91 and oMN001-106 (19). The fragment was inserted into HindIII- and XbaI-cleaved pAG58-ble-1 (34) to yield pSN108. Alanine-scanning mutagenesis was conducted using mutagenic primers and the PCR amplification method of site-directed mutagenesis. Two primers (forward and reverse) specifying a single mutation were used to perform inverse PCR on whole pSN108 plasmid DNA. The mutation also introduced an additional restriction site in the mutated DNA insert. The PCR product was extracted with phenol-chloroform–isoamyl alcohol and precipitated with ethanol using yeast RNA as the carrier. The DNA was cleaved with DpnI to eliminate template DNA, and the restriction reaction was used to directly transform competent cells of *E. coli* strain DH5α. Introduction of the mutation into the *rpoA-rplQ* fragment was confirmed by nucleotide sequencing (Oregon National Primate Research Center, Core Facility, Beaverton). The primers used for alanine-scanning mutagenesis are listed in Table S2 in the supplemental material. The pSN108 derivative bearing the alanine codon substitution was used to transform competent cells of strain JH642. The plasmid integrated into the *rpoA* locus of the chromosome by a Campbell recombination mechanism. The selection for elimination of the plasmid vector DNA by loop-out recombination, thus leaving the alanine codon substitution in the *rpoA* gene, was accomplished according to a previous published procedure (19). The presence of the mutation was confirmed by PCR of the *rpoA* CTD region followed by cleavage with the restriction enzyme that recognizes the mutated sequence.

Diamide sensitivity. Wild-type *B. subtilis* strain JH642, ORB3621 (*rpoA^{cxs-1}*), ORB5259 [*rpoA(C265A)*], and ORB5262 [*rpoA(K267A)*] were grown in Difco sporulation medium (DSM) at 37°C with shaking until mid-log phase (optical density at 600 nm = 0.5). Viable-cell numbers were measured by plating 5 μl of cells from a dilution series onto DSM agar medium with or without 0.1 mM diamide. Cells were also spotted onto DSM plates without drug in the same way as above.
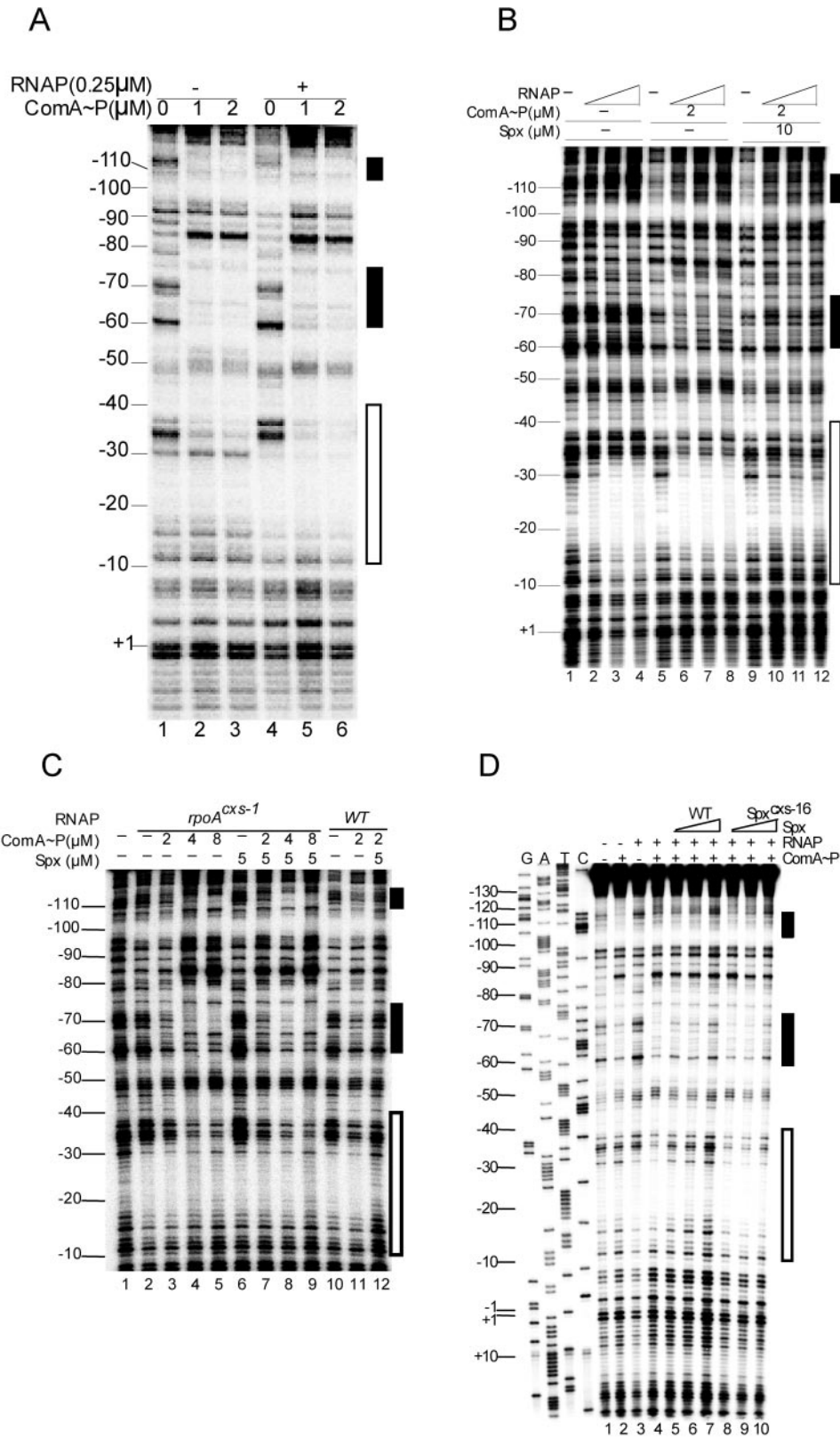
FIG. 1. Effect of Spx on binding of ComA~P and RNAP to the *srf* promoter region. (A) RNAP and ComA~P (25) were added to DNase I footprinting reaction mixtures containing the *srf* promoter fragment synthesized by PCR and end labeled on the noncoding strand. Concentrations of RNAP and ComA are indicated. Reaction conditions are described in Materials and Methods. The two black rectangles indicate the locations of the ComA binding elements, box 1 (upper) and box 2 (lower). The white rectangle marks the site of RNAP-promoter interaction. (B) RNAP and ComA~P were combined with end-labeled *srf* promoter DNA as described for panel A, but a gradient of RNAP concentration was tested in

Protein purification. RNAP containing a His$_{10}$-tagged RpoC (β') subunit was purified from *B. subtilis* MH5636 (wild type [WT]), ORB4123 (*rpoA^{cxs-1}*), ORB5501 [*rpoA(C265A)*], or ORB6116 [*rpoA(K267A)*] by using a procedure described previously (29). Intein-tagged ComA was purified using a procedure described previously (25). The self-cleavable affinity tag system IMPACT (New England Biolabs) was used to purify ComA from *E. coli* strain BL21(DE3)(pLysS). The ComA proteins obtained have a Pro-Gly extension at the C termini and were further purified by elution with a 100-to-600 mM KCl gradient from a High Q column (Bio-Rad). Intein-tagged Spx was purified by using a procedure described previously (26). His$_6$-tagged wild-type, Cxs-16, C10A, and C13A Spx proteins were purified using a previously published procedure (23).

In vitro transcription reactions. Linear DNA fragments for templates of promoters P*rpsD* (from −115 to about +71), P*srfA* (−347 to about +104) and P*trxB* (−220 to about +88) for in vitro transcription were generated by PCR. The oligonucleotides used in PCR to generate promoter fragments are listed in Table S4 in the supplemental material. The transcription reaction mixtures (20 μl) contained 40 mM Tris HCl (pH 7.9), 10 mM NaCl, 6 mM MgCl$_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT) (unless otherwise indicated), 10 units RNasin (Promega), 50 nM P*srf* or 20 nM P*trxB* or 50 nM P*rpsD* template, 0.05 μM RNAP, and 1.6 μM ComA phosphorylated by treatment with acetyl phosphate as previously described (25). The mixtures were incubated at 37°C for 10 min with or without Spx before the addition of 40 μM ATP, CTP, and GTP, 10 μM UTP, and 5 μCi [α-^{32}P]UTP. After incubation (times are indicated in figure legends and text), the reactions were stopped by addition of 10 μl stop buffer (1 M ammonium acetate, 0.1 mg/ml yeast RNA, 0.03 M EDTA) and then precipitated with 75 μl ethanol at −80°C. Electrophoresis was performed on 6% urea gel as described previously (13).

Assay of β-galactosidase activity. β-Galactosidase activity was determined as previously described (18) and is presented as Miller units (14).

DNase I footprinting experiment. A radioactively end-labeled fragment of the *srf* promoter (from −138 to +65) was made by PCR amplification using primers o-MN02-195 and o-YZ02-4 and JH642 chromosomal DNA as a template. To end-label the template or coding strand, one member of each primer set was treated with T4 polynucleotide kinase and [γ-^{32}P]ATP. The PCR products were separated on a nondenaturing polyacrylamide gel and purified with Elutip-d columns (Schleicher and Schuell). Dideoxy sequencing ladders were obtained using a Thermo Sequenase cycle sequencing kit (USB) with the primers used for the footprinting reactions. DNase I footprinting experiments were performed in 20 μl reaction buffer containing 10 mM Tris HCl (pH 7.9), 30 mM KCl, 10 mM MgCl$_2$, and 0.5 mM β-mercaptoethanol. Proteins were incubated with labeled probe (50,000 cpm) at 37°C for 20 min. The reaction mixtures were treated with 3 μl of 0.02-mg/ml DNase I (diluted in 5 mM MgCl$_2$, 5 mM CaCl$_2$) at room temperature for 15 s (without proteins) or 30 s (with proteins). The reactions were then stopped with 10 μl stop buffer (6.25 mM EDTA [pH 8.0], 0.125% sodium dodecyl sulfate (SDS), 0.375 M sodium acetate, 62.5 μg/ml yeast RNA). After phenol-chloroform–isoamyl alcohol extraction and ethanol precipitation, pellets were dissolved in loading dye and subjected to 6% polyacrylamide–8 M urea gel electrophoresis as previously described (23).

SPPR. Solid-phase promoter retention (SPPR) experiments used streptavidin-attached agarose beads that bind to biotinylated DNA fragments along with any interacting proteins. Biotinylated DNA fragments were synthesized by PCR with biotinylated 5′ upstream oligonucleotides and underivatized downstream oligonucleotides (see Table S3 in the supplemental material). Streptavidin agarose beads were equilibrated with binding buffer (10 mM Tris-HCl [pH 8.0], 100 mM KCl, 10 mM MgCl$_2$ and 0.5 mM β-mercaptoethanol). The beads were preincubated with the biotinylated DNA fragment for 30 min in binding buffer containing 1% Casamino Acids and 0.1 mg/ml bovine serum albumin (BSA) to shield the nonspecific binding sites on the agarose beads. After the unbound DNA fragment was washed out, protein mixtures were added to the beads in binding buffer containing 0.05 mg/ml yeast RNA, 0.08 mg/ml pUC18 plasmid, and 0.1 mg/ml BSA and then incubated for 1 h at room temperature with gentle shaking. After the unbound protein was washed out by suspension in and centrifugation from binding buffer, the beads were heated at 95°C in SDS loading dye to release the

proteins from the agarose beads. SDS-polyacrylamide gel electrophoresis was performed to examine the proteins that were immobilized on the biotinylated DNA-streptavidin agarose complex. The 12% SDS-polyacrylamide gel was stained by colloidal Coomassie G (3), and the images were taken with a UV Transilluminator with a visible-spectrum conversion filter.

RESULTS

Spx-RNAP interaction reduces ComA-assisted binding of RNAP to the *srf* promoter. Previous studies provided evidence for a model of Spx-dependent transcriptional repression that involves the direct interference of interaction between the promoter-bound transcriptional activator and RNAP (reviewed in reference 35). This was based on in vitro transcription studies using purified *srf* promoter DNA and ComA protein phosphorylated by treatment with acetyl phosphate. ComA~P had been shown to bind upstream of the *srf* promoter in two ComA boxes (30). However, electrophoretic mobility shift analysis (EMSA) suggested that ComA binding to *srf* promoter DNA requires its interaction with RNAP (25). The binding of ComA~P to *srf* promoter DNA was reexamined, and the effect of Spx-RNAP interaction was investigated. DNase I footprinting showed that ComA~P is able to bind to ComA boxes 1 and 2 upstream of the *srf* −35 sequence (Fig. 1A) as previously shown (30). Higher concentrations resulted in protection near the −35 region (Fig. 1A, lane 3). RNAP alone protected sequences between −70 and −90 and between −10 and −30, but protection was extended upstream to the −35 region when ComA~P was included in the reaction (Fig. 1A, lanes 4 to 6). The ComA-assisted binding of RNAP to the −35 region is observed again in Fig. 1B. While higher concentrations of RNAP seemed to interact with DNA in the region between −10 to −30 in the absence of ComA (Fig. 1B, lanes 2 to 4), protection in the −30 to −40 region was observed when ComA~P was present (Fig. 1B, lanes 6 to 8). Figure 1B also shows that the addition of Spx protein significantly reduced RNAP binding and weakened ComA~P binding (lanes 10 to 12).

The *rpoA^{cxs-1}* and *spx^{cxs-16}* mutations block Spx-dependent inhibition of ComA-assisted RNAP binding to the *srf* promoter. A concentration of Spx (5 μM) that prevents binding to WT RNAP to the *srf* promoter (Fig. 1C, lanes 11 and 12) did not have a significant negative effect on binding of RNAP bearing the mutant RpoA^{cxs-1} subunit (Fig. 1C, lanes 7 to 9). The binding of WT RNAP to the *srf* promoter in the presence of ComA~P, while inhibited by WT Spx (Fig. 1D, lanes 5 to 7), was not affected by the inclusion of the mutant inactive Spx^{cxs-16}, which was previously shown to confer reduced interaction between Spx and αCTD (Fig. 1D, lanes 8 to 10). Recently reported structural analysis has shown that the amino acid positions altered by the *rpoA^{cxs-1}* and *spx^{cxs-16}* mutations define part of the αCTD-Spx interaction interface (27).

The fact that ComA~P is able to interact with the *srf* pro-

the footprinting reaction. RNAP concentrations (from left to right and marked by the white ramped triangle) are 0.25, 0.5, and 1 μM. Spx is included in the rightmost reactions at the indicated concentration. ComA box 2 is indicated by the black rectangle (box 1 is obscured at the top of the gel image). (C) Footprinting reactions containing either WT or *rpoA^{cxs-1}* RNAP with and without ComA~P and Spx. White and black rectangles indicate RNAP and ComA binding sites as in panel A. Protein reaction components were applied in the concentrations indicated. (D) WT RNAP and ComA~P were added to footprinting reaction mixtures in the absence or presence of Spx or the mutant Spx^{cxs-16} protein. RNAP, 0.1 μM; ComA, 2 μM; Spx, 5, 10, and 20 μM; Spx^{cxs-16}, 5, 10, and 20 μM.
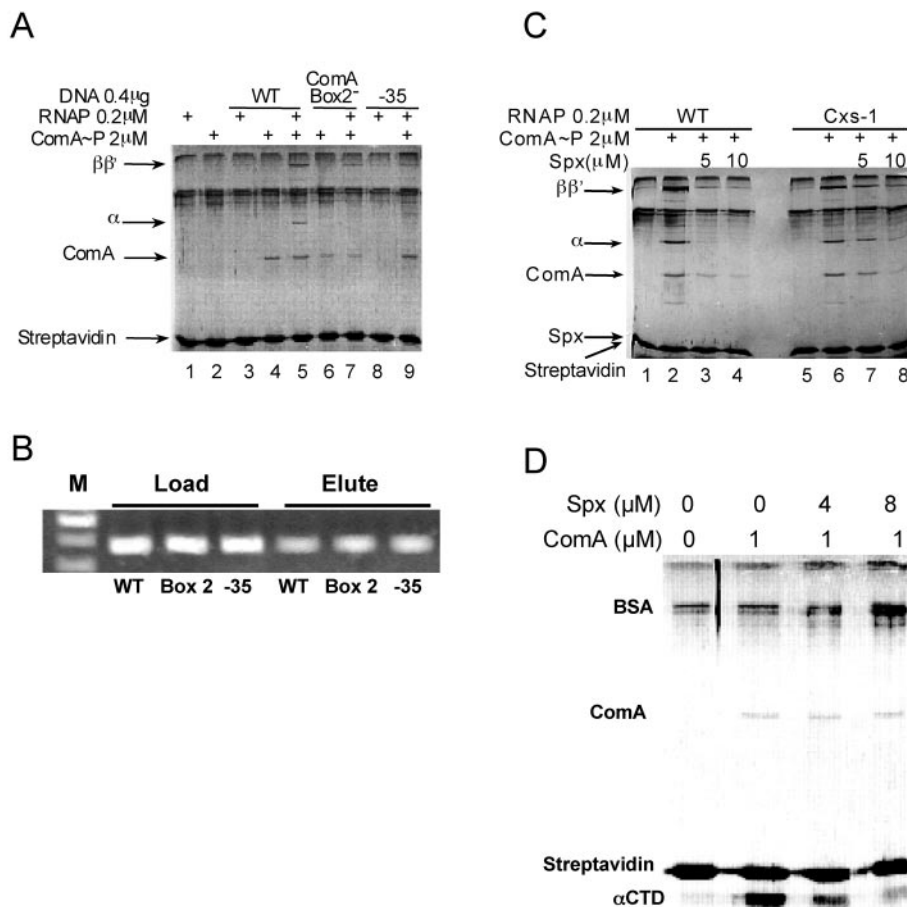
FIG. 2. Binding of ComA~P and RNAP to the *srf* promoter as observed using SPPR analysis. (A) Biotinylated *srf* or mutant *srf* promoter DNA bound to streptavidin beads was combined with RNAP and/or ComA. Bound protein was analyzed by SDS-polyacrylamide gel electrophoresis as outlined in Materials and Methods. WT, box 2 mutant, and −35 mutant *srf* promoter DNA was used in the indicated reactions. Protein concentrations: RNAP, 0.2 μM; ComA, 2 μM; Spx, 10 μM. (B) Ethidium bromide-stained DNA on a 1% agarose gel. Biotinylated DNA fragments of WT, box 2 mutant, and −35 mutant DNA were applied to the streptavidin beads and extracted with phenol-chloroform from streptavidin agarose beads. (C) Effect of Spx on RNAP and ComA binding to *srf* promoter DNA as determined by SPPR analysis. RNAP of WT and *rpoA*[cxs-1] (Cxs-1) strains was used in the reactions. ComA~P phosphorylated by acetyl phosphate and Spx were added in the concentrations indicated. (D) SPPR reactions with purified αCTD and ComA, untreated or treated with Spx. Amounts of proteins in reactions are indicated. The SPPR method is described in Materials and Methods.

moter region and, in doing so, assists RNAP interaction contradicts previously reported EMSA results, which showed poor ComA interaction in the absence of RNAP. Hence, we employed a third method to examine the function of ComA and Spx in RNAP-promoter interaction. The method, the SPPR method, involves immobilization of biotinylated promoter DNA to a streptavidin-agarose bead support. Proteins are added to the bead-bound DNA, and the proteins retained after washing are examined by SDS-polyacrylamide electrophoresis and a colloidal Coomassie blue staining protocol (3). A blocking solution containing a mixture of BSA and Casamino Acids (amino acid solution) was used to prevent nonspecific binding of protein to the streptavidin-agarose beads (Fig. 2A). RNAP β, β′ and α subunits are visible on the gels, but σ is obscured by the BSA band. RNAP binds poorly to the bead-bound *srf* promoter DNA (Fig. 2A, lane 3), and the addition of ComA~P to the mixture enhances RNAP retention (lanes 4 and 5). A ComA box 2 mutant version of *srf* promoter reduces

the amount of ComA binding and reduces RNAP retention (Fig. 2A, lanes 6 and 7). The same outcome was observed with a −35 mutant form of the *srf* promoter (Fig. 2A, lanes 8 and 9), which has been shown to eliminate ComA-stimulated *srf* transcription (data not shown). This is consistent with the result that the protection observed in the −35 region of *srf* promoter DNA in footprinting reactions containing RNAP and ComA~P (Fig. 1) is due to RNAP binding. Figure 2B shows that equal amounts of DNA were applied to the streptavidin-agarose beads and could be recovered by phenol-chloroform extraction from the beads. Thus, the SPPR method provides an authentic picture of ComA/RNAP interaction at the *srf* promoter.

The addition of Spx to the SPPR reaction mixture containing ComA~P and RNAP reduces binding of RNAP to the bead-bound *srf* promoter DNA (Fig. 2C). When mutant *rpoA*[cxs-1] RNAP was used in the reaction in place of WT RNAP, a reduction in Spx-dependent RNAP release was observed (Fig.
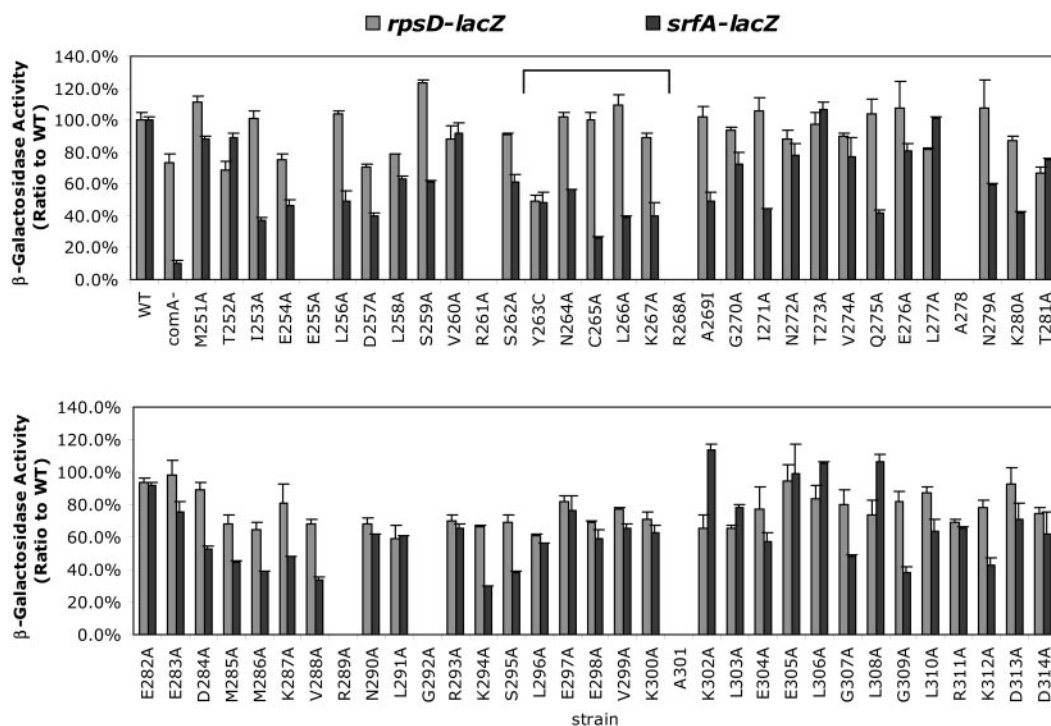
FIG. 3. Measurement of *lacZ* fusion activity in *B. subtilis* strains bearing alanine codon substitutions in the αCTD-coding region of the *rpoA* gene. β-Galactosidase activity was measured in culture samples collected at the beginning of stationary phase for *srf-lacZ*-bearing cells and mid-log phase for *rpsD-lacZ* cells. Activity is expressed as a percentage of the activity measured in *rpoA⁺* cells. The bracket shows the region around α1 of αCTD.

2C, lanes 6 to 8), a result consistent with the DNase footprinting data of Fig. 1 and previously published data (24). In both footprinting and SPPR experiments, Spx substantially reduced RNAP binding to the *srf* promoter but also reduced ComA-DNA interaction. We conclude from these experiments that ComA is capable of interacting with *srf* promoter DNA, as was previously shown, but that this interaction is strengthened when RNAP is present, as was observed in the footprinting experiments of Fig. 1.

The hypothesis that ComA interacts with RNAP by binding to αCTD was supported by the observation that ComA can recruit purified αCTD protein to the bead-bound *srf* promoter DNA. The SPPR experiment of Fig. 2D shows that αCTD cannot interact with *srf* promoter DNA unless ComA~P is present. Spx addition causes the release of αCTD. This indicates that promoter-bound ComA~P can interact with αCTD and this interaction is sensitive to Spx.

Alanine-scanning mutagenesis of RNAP αCTD uncovers residues required for ComA-dependent activation of *srf* transcription. We sought to employ *lacZ* fusion expression, in vitro transcription, footprinting, and SPPR analyses to study the effects of αCTD mutations on ComA and Spx function and to gain a better understanding of how Spx represses transcription. Our objective was to determine if ComA and Spx have overlapping binding surfaces on αCTD, indicating that Spx sterically hinders ComA-RNAP interaction.

The αCTD-coding region of the *B. subtilis rpoA* gene was subjected to alanine-scanning mutagenesis, and the resulting mutant alleles were introduced into the *rpoA* locus by a previ-

ously reported procedure (19). The expression of a *lacZ* fusion controlled by the *srf* promoter was examined in the αCTD mutants (Fig. 3). The activity of *srf-lacZ* was tested at the onset of stationary phase, while the mid-log expression of *rpsD-lacZ* was also monitored as a ComA- and Spx-independent control fusion. The *rpsD* gene encodes ribosomal protein S4 (10), and its expression is maximal during the middle of exponential phase.

The C265, L266, K267A, K287A, and G307A residues are required for optimal ComA-dependent *srf-lacZ* expression (Fig. 3). The Y263C mutation, previously shown to confer reduced interaction between Spx and αCTD, has a negative effect on both *srf-lacZ* and *rpsD-lacZ* fusions.

Since the region around α1 of αCTD (Fig. 3, bracket) contains the binding interface between RNAP αCTD and Spx, as shown by crystal structure analysis (27), the effects of mutations altering residues C265 and K267 on ComA- and Spx-dependent control of the *srf* promoter were further examined.

The *rpoA*(C265A) mutation affects ComA-activated *srf* transcription and RNAP binding to the *srf* promoter. The effect of the C265A mutation in *rpoA* on *srf* promoter utilization was examined using in vitro transcription and SPPR analysis. RNAP was purified from the *rpoA*(C265A) mutant cells and combined with ComA~P for time course transcription experiments. The reaction mixtures containing mutant RNAP and ComA~P showed a reduced rate of transcript accumulation (Fig. 4A and B), which was in keeping with the reduced expression of *srf-lacZ* in *rpoA*(C265A) mutant cells (Fig. 3). The mutant polymerase showed a level of *rpsD* transcript accumu-
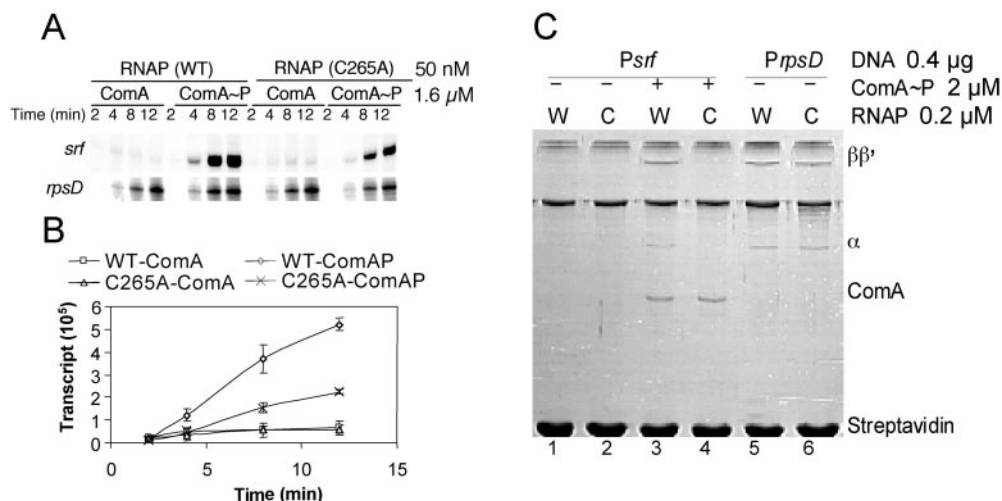
FIG. 4. Effect of *rpoA*(*C265A*) mutation on *srf* transcription and on ComA and RNAP binding to *srf* promoter DNA. (A) Time course in vitro runoff transcription experiment using *srf* promoter DNA as the template and untreated ComA (ComA) or ComA treated with acetyl phosphate (ComA~P), plus RNAP or *rpoA*(*C265A*) RNAP. (B) Plot of band intensities derived from three repeats of the experiment shown in panel A against time of incubation. (C) Binding of WT and mutant *rpoA*(*C265A*) RNAP with ComA~P to the *srf* promoter as determined by SPPR analysis. Reactions containing wild-type RNAP (W) and mutant *rpoA*(*C265A*) RNAP (C) are indicated.

lation similar to that of wild-type RNAP. SPPR analysis shows that ComA-assisted binding of *rpoA*(*C265A*) RNAP to the *srf* promoter is defective (Fig. 4C, lanes 3 and 4), while no defect in binding of mutant RNAP to the *rpsD* promoter is observed (Fig. 4C, lanes 5 and 6). The mutation has no detectable effect on Spx repression (see below). The C265A substitution has no effect on ResD-dependent transcriptional activation in vivo and in vitro (H. Geng and M. M. Nakano, unpublished data), indicating that the mutation has a specific effect on ComA-RNAP interaction and does not confer a general defect on RNAP activity.

The *rpoA*(*K267A*) mutation affects ComA- and Spx-activated transcription and the Spx-dependent negative control. Based on the recently published crystal structure of the αCTD-Spx complex, the K267 residue of αCTD contacts the conserved

R47 of Spx (27) and might be important for stable RNAP-Spx interaction. A *B. subtilis* strain bearing the *rpoA* allele with a K267A codon substitution is hypersensitive to diamide-induced thiol-specific oxidative stress (Fig. 5A), which is the phenotype associated with defective RNAP-Spx interaction. The C265A mutation has no significant effect on diamide resistance, and the Y263C mutation, as shown previously (24), confers hypersensitivity to diamide due to reduced Spx-RNAP interaction and consequent defective oxidative stress response. In vitro transcription analysis of the mutant *rpoA*(*K267A*) RNAP in the presence of Spx showed that Spx-stimulated transcription from the *trxB* promoter is reduced compared to the reaction containing WT RNAP (Fig. 5B). The mutation also appears to have a modest effect on *rpsD-lacZ* expres-
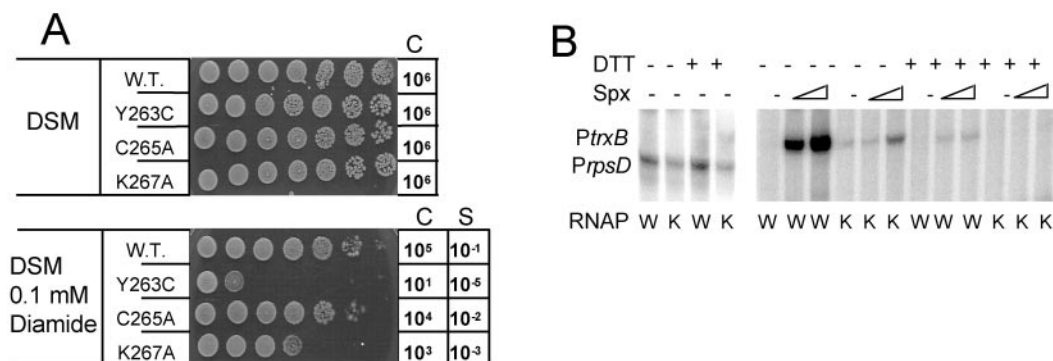

FIG. 5. In vivo and in vitro phenotypes of *rpoA* mutants. (A) Sensitivity of *rpoA*$^{cxs-1}$, *rpoA*(*C265A*), and *rpoA*(*K267A*) mutants to the thiol-specific oxidant diamide. Cultures of WT cells and those of each mutant grown in DSM to mid-log phase were serially diluted to 10^{-6}, and 5 μl of each dilution was spotted onto DSM agar and DSM agar containing diamide. C, control indicating the final dilution spotted that showed growth; S, sensitivity, shown as the approximate fraction of total cells surviving exposure to diamide [(C + diamide)/C − diamide)]. (B) Transcription from the *trxB* promoter catalyzed by WT RNAP (W) and mutant *rpoA*(*K267A*) RNAP (K) in the absence (−) and presence (+) of Spx (0.4 μM and 0.8 μM). Where used, DTT was added to a final concentration of 5 mM. The control transcription reaction mixture contained *rpsD* gene promoter DNA.
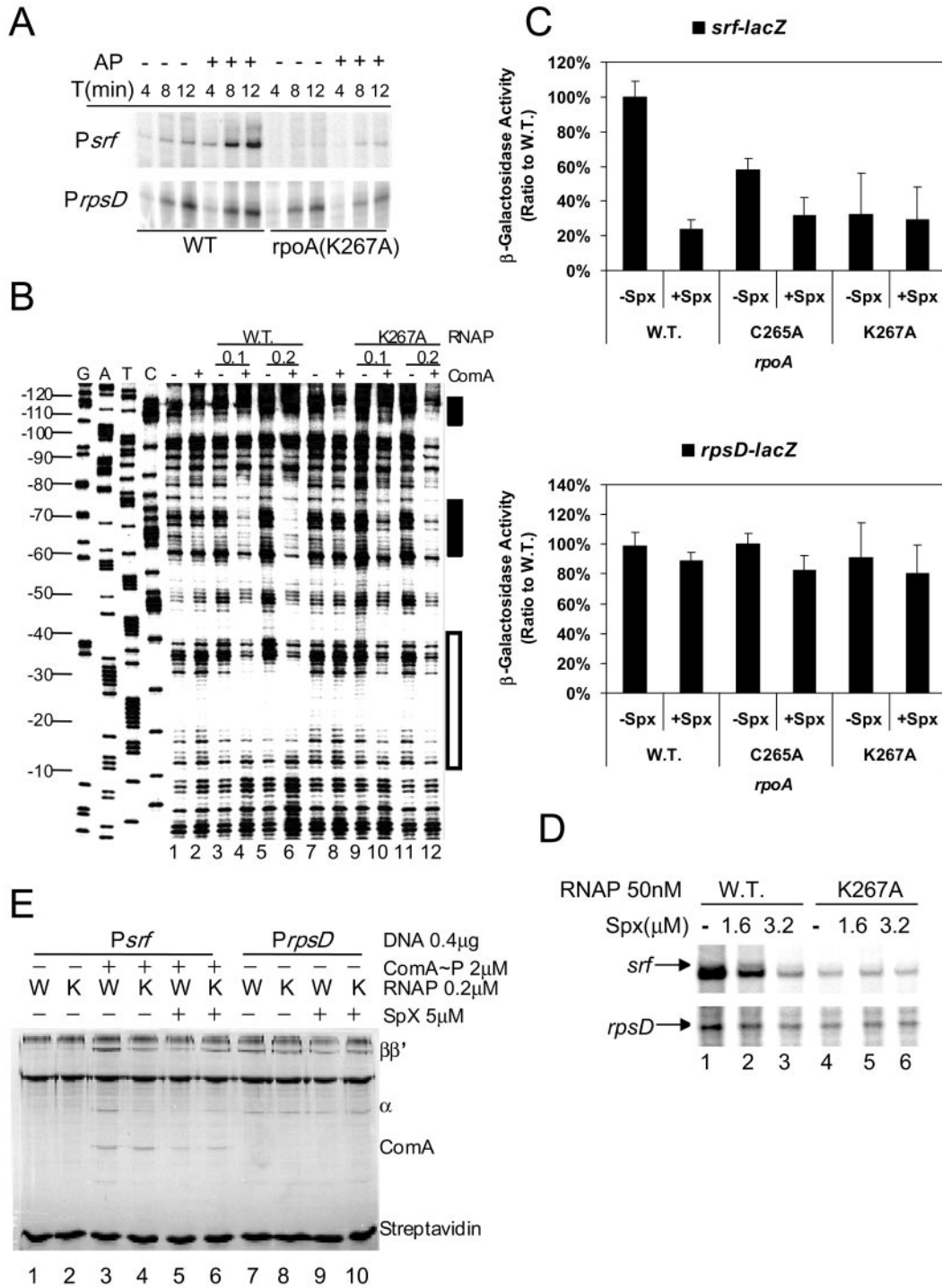
FIG. 6. Effect of *rpoA*(*K267A*) mutation on ComA-dependent *srf* transcription and Spx-dependent repression. (A) Time course in vitro transcription experiment showing the accumulation of *srf* transcript in reaction mixtures containing untreated (−) or acetyl phosphate (1.6 mM)-treated (+) ComA, and either wild-type RNAP (WT) or *rpoA*(*K267A*) RNAP (50 nM). (B) Denaturing gel analysis of DNase I footprint reactions containing P*srf* DNA with (+) and without (−) ComA~P in the presence of wild-type RNAP (WT) or RpoA(K267A) RNAP. RNAP was used at 0.1 μM and 0.2 μM as indicated. (C) Effect of SpxLDD production on levels of β-galactosidase activity in *srf-lacZ* (ORB6129, ORB6131, and ORB6132) and *rpsD-lacZ* (ORB6137, ORB6139 and ORB6140) cells bearing the wild-type *rpoA* allele or the *rpoA*(*C265A*) or *rpoA*(*K267A*) mutant allele. *srf-lacZ* fusion-bearing cells were collected from cultures at the end of exponential growth, while *rpsD-lacZ* cells were collected from mid-log-phase cultures. (D) Effect of Spx (1.6 μM) on transcription of *srf* in reaction mixtures containing ComA (1.6 μM), acetyl phosphate (1.6 mM), and either wild-type (WT) or *rpoA*(*K267A*) RNAP (50 nM). Control transcripts from reactions containing *rpsD* promoter DNA are shown at the bottom. (E) Effect of *rpoA* mutations on ComA-dependent RNAP binding to the *srf* and *rpsD* promoter and on Spx-dependent RNAP release from promoter DNA. SPPR reaction mixtures contained ComA (+) and either wild-type (W) or *rpoA*(*K267A*) (K) RNAP in the presence (+) or absence (−) of Spx.
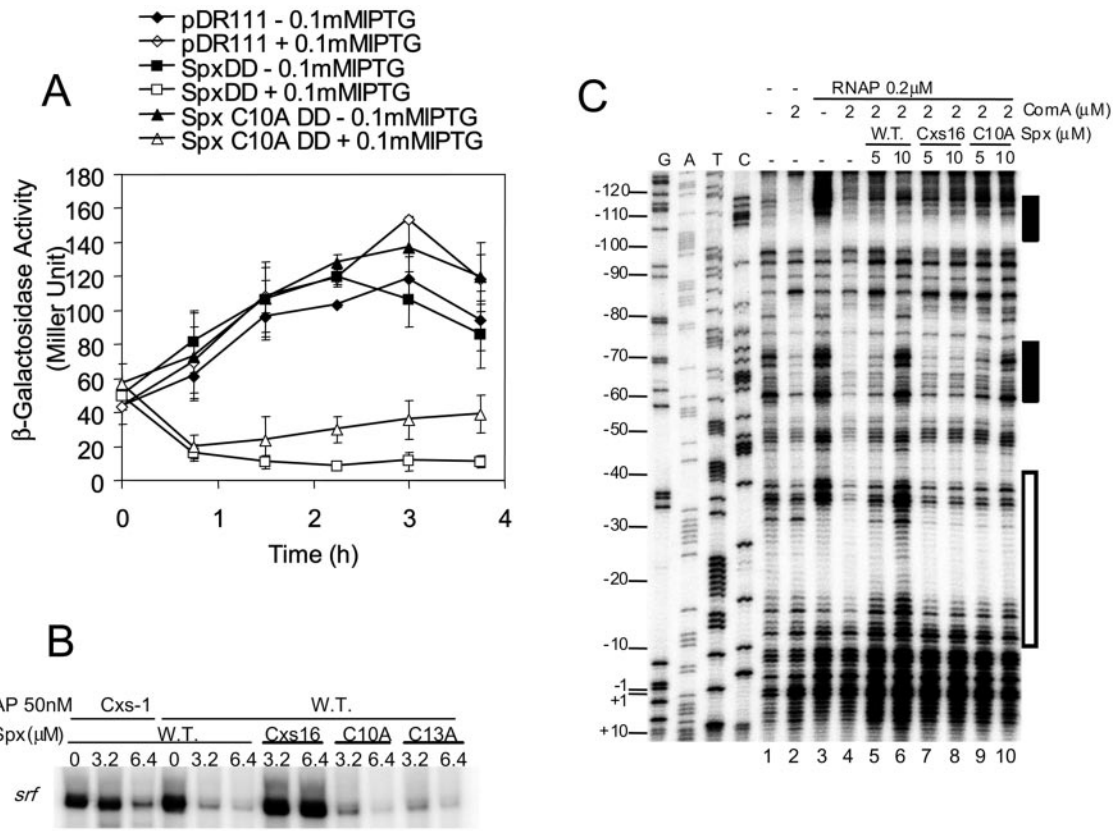
FIG. 7. Effect of amino acid substitutions in the CXXC motif of Spx on Spx-dependent repression of *srf* transcription. (A) Measurement of β-galactosidase activity in a time course experiment of cultures of *srf-lacZ* bearing cells expressing either SpxLDD or SpxLDD(C10A). *spxLDD* and mutant *spxLDD(C10A)* alleles were expressed from an IPTG-inducible construct derived from pDR111. Vector control cultures are indicated (pDR111), as are *spxLDD* and mutant *spxLDD(C10A)* cultures. Data are from two experiments. (B) In vitro transcription data from reactions with WT or *rpoA*^cxs-1^ RNAP, ComA~P (1.6 μM), and increasing concentrations of either Spx, Spx^cxs-16^, Spx(C10A), or Spx(C13A). (C) Image of denaturing polyacrylamide gel of DNase I footprinting reactions containing end-labeled *srf* promoter DNA, RNAP, ComA~P, and either Spx, Spx^cxs-16^, or Spx(C10A), at the concentrations indicated.

sion (Fig. 3) and transcription from the *rpsD* promoter in vitro (Fig. 5B).

The effect of the *rpoA(K267A)* mutation on ComA-activated *srf* transcription and its repression by Spx was examined. In vitro transcription analysis of time course reaction mixtures containing mutant RpoA(K267A) RNAP showed that ComA~P stimulates transcription catalyzed by the mutant RNAP, but the rate of transcript accumulation was significantly lower than that of reactions containing WT RNAP (Fig. 6A). Defective binding of mutant *rpoA(K267A)* holoenzyme in the presence of ComA~P was observed in gels of footprinting reactions. While protection in the −35 region was observed in footprint reactions containing P*srf*, wild-type RNAP, and ComA~P (Fig. 6B, lanes 3 to 6), reduced protection in the −35 region is apparent in the reactions in which the mutant RpoA(K267A) RNAP was added in place of wild-type RNAP (lanes 9 to 12).

The reduced in vitro transcriptional activity and ComA-assisted promoter binding of the mutant RpoA(K267A) RNAP is in keeping with the reduced in vivo activity observed in *srf-lacZ* strains bearing the *rpoA(K267A)* mutation (Fig. 6C). This low activity was not affected by Spx, as expression of the *spxLDD* allele (which encodes the protease-resistant form of Spx), while causing repression in *rpoA*^+^ *srf-lacZ* cells, did not

result in repression of *srf-lacZ* in *rpoA(K267A)* cells. No significant effect of SpxLDD expression or *rpoA(K267A)* mutation on the expression of *rpsD-lacZ* (Fig. 6C and D) was observed. The *rpoA(C265A)* mutation did not prevent Spx-dependent repression, in that reduced expression of *srf-lacZ* was observed when SpxLDD is produced (Fig. 6C and D), and ComA-dependent *srf* transcription in vitro was repressed when Spx protein was added to the transcription reaction mixture containing the mutant RpoA(C265A) form of RNAP (data not shown).

SPPR analysis shows that ComA-assisted RNAP binding to P*srf* is impaired by the mutant RpoA(K267A) subunit (Fig. 6E, compare lanes 3 and 4). However, interaction of RNAP with the *rpsD* promoter was not affected by the *rpoA(K267A)* mutation (Fig. 6E, lanes 7 to 10). Spx disrupted the ComA-RNAP complex at the *srf* promoter, as shown in SPPR reactions (Fig. 6E, lanes 3 and 5). However, Spx had no significant effect on ComA-assisted promoter binding of RpoA(K267A) RNAP (Fig. 6E, lanes 4 and 6). The K267 amino acid position in the αCTD is important for ComA-dependent transcriptional activation, for Spx-dependent repression, and for Spx-dependent transcriptional activation.
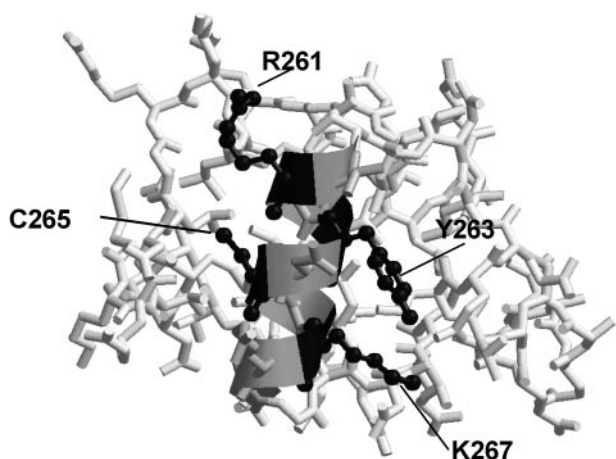
FIG. 8. Structure of *B. subtilis* RNA polymerase αCTD (27). Stick structures denote peptide backbone and amino acid side chains. The ribbon indicates the α1 helix. The side chains of residues R261, Y263, C265, and K267 are presented as black ball-and-stick structures.

The CXXC motif of Spx is not essential for repression of *srf* transcription. Transcriptional activation by *B. subtilis* Spx at the *trxA* and *trxB* promoters requires the oxidized form of Spx having an intrachain disulfide at the N-terminal CXXC motif (23). It was not known if the CXXC motif was also required for Spx-dependent repression. A protease-resistant form of Spx (24) bearing a C10A substitution in the CXXC motif was produced from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression system in a *B. subtilis* strain bearing a *srf-lacZ* fusion. Separate cultures expressing wild-type and C10A mutant forms of SpxLDD were analyzed by Western blotting, and equal amounts of SpxLDD protein were observed in each strain (data not shown). When the SpxLDD protein with the C10A substitution was expressed in IPTG-treated cells, *srf-lacZ* was repressed to nearly the same level as observed in cells expressing the parental *spxLDD* construct (Fig. 7A). An attempt was made to express a C13A mutant form of SpxLDD in *srf-lacZ* cells, but the product was unstable in *B. subtilis*, and only low levels of protein were detected (data not shown).

The activity of Spx(C10A) and Spx(C13A) in vitro was examined in transcription reactions and by DNase I footprinting (Fig. 7B and C). In vitro transcription reaction mixtures containing *srf* promoter DNA, ComA~P, RNAP, and Spx were assembled to examine Spx-dependent repression. The WT Spx repressed transcription from the *srf* promoter, while the negative control, Spx[cxs-16], showed reduced repressing activity. Both Spx(C10A) and Spx(C13A) repressed transcription nearly to the level of WT Spx (Fig. 7B). Analysis of RNAP-ComA binding to the *srf* promoter in footprinting reactions showed that the mutant Spx(C10A) had a reduced ability to displace RNAP and ComA compared to WT Spx (Fig. 7C, compare lanes 5 and 6 with lanes 9 and 10). It was concluded that the CXXC motif, while enhancing repression, is not essential for the repressor activity of Spx.

DISCUSSION

Among the genes repressed by Spx, the ComA regulon genes, particularly those of the *srf* operon, were found to un-

dergo the greatest reduction in transcript levels when Spx interacted with RNAP in cells overexpressing Spx (24). ComA activates transcription of the *srf* operon by interacting with two regions of dyad symmetry residing upstream from the *srf* promoter −35 sequence. Transcription requires these interactions as well as RNAP contact with the −35 region, which is assisted by ComA~P. Our data suggest that ComA interacts with the RNAP αCTD in a region previously shown to contact Spx (27). Thus, Spx blocks productive interaction between ComA and RNAP at the *srf* promoter by occupying an overlapping site on αCTD.

The Y263C mutation of αCTD reduced *rpsD* (ribosomal S4) and ComA-dependent *srf* transcription to nearly the same extent (Fig. 3). This residue is also necessary for functional Spx-RNAP interaction and in response regulator ResD-stimulated transcription (Geng and Nakano, unpublished), which induces anaerobic-specific gene transcription in response to oxygen limitation (22). As detailed previously, this residue is highly conserved in low-GC gram-positive bacteria that also carry *spx*. These observations reinforce the view that the Y263 residue is an important feature of RNAP in gram-positive organisms.

The footprinting and SPPR data indicate that ComA-RNAP interaction is necessary for ComA-assisted recruitment of RNAP to the *srf* promoter to form a stable promoter complex. ComA is capable of binding to the *srf* promoter without RNAP, as observed in previous studies, but RNAP-promoter binding appears to solidify ComA-*srf* promoter interaction by interaction with ComA. Spx interferes with ComA-RNAP interaction, since addition of Spx to the EMSA (25), footprinting, and SPPR reaction mixtures weakens both RNAP-promoter and ComA-promoter complexes. Footprinting shows protection in the −35 region, particularly nucleotide −30, that is attributable to ComA-assisted RNAP binding. Despite little change in DNase I protection in the −10 region, the interactions observed in the footprinting result represent a productive complex, as shown by in vitro transcription data.

C265 and K267, along with Y263, reside in the α1 helix of αCTD (Fig. 8). The C and K residues correspond to residues C269 and K271 in the *E. coli* RNAP αCTD. The two residues are located C-terminal to the α1 residues that constitute part of the DNA-binding "265 determinant" of αCTD (2, 7, 31), which includes R265 (R261 in *B. subtilis*), V264 (V260), and N268 (N264). The conservation of the three residues in *B. subtilis* and the overall structural similarity between *E. coli* and *B. subtilis* αCTD (27) suggests that the *B. subtilis* αCTD also contains the analogous 265 determinant that binds to extended promoter DNA. The residues required for ComA and Spx interaction with RNAP lie adjacent to the 265 determinant sequences of *B. subtilis* αCTD. The residues N264, K294, and S295 in *B. subtilis* αCTD, which correspond to the 265 determinant residues of *E. coli* αCTD, are required for optimal *srf* and have a modest effect on *rpsD* expression. These confer a 40 to 70% reduction in *srf-lacZ* expression. These data suggest that upstream promoter binding by αCTD is necessary for ComA-activated *srf* transcription. Alanine substitutions of residues G292 and R261 in *B. subtilis* αCTD were not recovered in our screen after 20 attempts to obtain mutant *rpoA* recombinants, raising the possibility that these substitutions were lethal.

Crystal structure analysis of Spx reveals the two-domain

structure of the ArsC homolog (27), a central domain that interacts with RNAP α and the redox domain formed by the N- and C-terminal sequences of Spx and containing the CXXC motif. Two peptide coils connect the central domain with the redox domain. The central domain contacts αCTD at the α1 region and involves the participation of helices α2 and α5 of the Spx central domain. The fact that the CXXC motif is necessary for positive transcriptional control, yet is some distance from the αCTD binding surface of Spx, suggests that Spx may contact other components of RNAP holoenzyme. The transcription factor AsiA of phage T4 contacts σ^{70} of *E. coli* RNAP holoenzyme and the flap domain of the β subunit. In doing so, the distance between regions 4 and 2 of σ^{70} is altered, and σ^{70} region 4 is now in a position to contact MotA, which is bound to phage T4-specific promoters (8, 28, 32). It is possible that the oxidized form of Spx also contacts σ^A and, perhaps, β as part of the mechanism of positive transcription control. Preliminary studies indicate that residues in σ^A region 4.2 are important for Spx-dependent transcriptional activation and repression (Y. Zhang and P. Zuber, unpublished data).

Our results show that the redox disulfide center of Spx, while enhancing the repressor activity, is not essential for negative control. This finding highlights the importance of control mechanisms affecting Spx concentration, which would seem to determine in large part when and under what conditions Spx negative control is exerted. An increase in Spx concentration is observed upon oxidative stress (24), which is the result of increased *spx* gene transcription (M. Leelakriangsak and P. Zuber, unpublished data) and enhanced Spx stability (24).

ACKNOWLEDGMENTS

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