Surfaces of Spo0A and RNA Polymerase Sigma Factor A That Interact at the spoIIG Promoter in *Bacillus subtilis*

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In *Bacillus subtilis*, the DNA binding protein Spo0A activates transcription from two classes of promoters, those used by RNA polymerase containing the primary sigma factor, $\sigma^A$ (e.g., *spoIIG*), and those used by RNA polymerase containing the secondary sigma factor, $\sigma^{HI}$ (e.g., *spoIJA*). Several single amino acid substitutions in region 4 of $\sigma^A$ define positions in $\sigma^A$ that are specifically required for Spo0A-dependent promoter activation. Similarly, several single amino acid substitutions in Spo0A define positions in Spo0A that are required for $\sigma^{HI}$-dependent promoter activation but not for other functions of Spo0A. It is unknown whether these amino acids in Spo0A interact directly with those in region 4 of $\sigma^A$ or whether they interact with another subunit of RNA polymerase to effect promoter activation. Here we report the identification of a new amino acid in region 4 of $\sigma^A$, arginine at position 355 (R355), that is involved in Spo0A-dependent promoter activation. To further investigate the role of R355, we used the coordinates of Spo0A and sigma region 4, each in complex with DNA, to build a model for the interaction of $\sigma^A$ and Spo0A at the *spoIIG* promoter. We tested the model by examining the effects of amino acid substitutions in the putative interacting surfaces of these molecules. As predicted by the model, we found genetic evidence for interaction of R355 of $\sigma^A$ with glutamine at position 221 of Spo0A. These results appear to define the surfaces of Spo0A and $\sigma^A$ that directly interact during activation of the *spoIIG* promoter.

Spo0A from *Bacillus subtilis* is a member of the response regulator class of two-component signal transducing systems (reviewed in reference 13). Activation of Spo0A by phosphorylation triggers the onset of endospore development. Active Spo0A binds to DNA at a 7-bp sequence, referred to as the 0A box (2, 26), to positively or negatively regulate the expression of a large number of genes (7). Spo0A activates transcription from two classes of promoters, those used by RNA polymerase containing the primary sigma factor, $\sigma^A$ (e.g., *spoIIG* and *spoIIE* promoters [22, 30]), and those used by RNA polymerase containing the secondary sigma factor, $\sigma^{HI}$ (e.g., the *spoIJA* promoter [19, 29]). Spo0A may activate the $\sigma^{HI}$-dependent promoters by interacting directly with the $\sigma^{HI}$ subunit of the RNA polymerase. Baldus et al. (1) found that *spoIIG* and *spoIIE* promoter activity was reduced in mutants of *B. subtilis* in which $\sigma^A$ contained one of two single amino acid substitutions in region 4, with the lysine at position 356 replaced by glutamate (K356E) or the histidine at 359 replaced by arginine (H359R). However, these substitutions did not affect the utilization of $\sigma^{HI}$-dependent, Spo0A-independent promoters or $\sigma^{HI}$-dependent, Spo0A-dependent promoters. Alanine substitutions at these sites in $\sigma^{HI}$ produced similar effects (1). Therefore, these substitutions define positions in region 4 of $\sigma^{HI}$ that are essential specifically for Spo0A-dependent activation of the *spoIIG* and *spoIIE* promoters.

A specific region of Spo0A is required to activate the $\sigma^A$-dependent promoters, such as *spoIIG*. This region has been defined by several single amino acid substitutions (4, 11, 14).

For example, substitution of alanine for isoleucine at position 229 of Spo0A (4) or for threonine at position 239 (14) reduces activation of the $\sigma^A$-dependent *spoIIG* promoter, but does not severely affect activation of the $\sigma^{HI}$-dependent *spoIJA* promoter. The structure of the Spo0A DNA binding domain has been determined by X-ray crystallography for both free Spo0A and Spo0A in complex with DNA (15, 31). Interestingly, all mutations in Spo0A specifically affecting the ability of $\sigma^{HI}$-dependent RNA polymerase to activate transcription cluster in $\alpha$-helix E, a flexible helix in the C terminus of the protein that is positioned away from the core structure of the protein.

A simple model to account for the requirements for specific amino acids in region 4 of $\sigma^A$ and amino acids of $\alpha$-helix E of Spo0A suggests that these surfaces interact directly during Spo0A-dependent promoter activation. Some support for this model comes from the finding that a single amino acid substitution in $\alpha$-helix E of Spo0A, with a serine at position 231 replaced by phenylalanine (S231F), partially suppresses the effect of the $\sigma^A$ H359R substitution (4). However, the Spo0A S231F mutation also partially suppresses the effects of the H359A and K356E substitutions in $\sigma^A$ and the S231F Spo0A mutant efficiently stimulates wild-type $\sigma^A$ RNA polymerase. Because the Spo0A suppressor mutation S231F does not display an allele-specific suppression of the sporulation defect caused by the H359R mutation in $\sigma^A$, it is unlikely that the phenylalanine at position 231 in the mutant Spo0A interacts directly with the arginine at 359 in the mutant $\sigma^A$. Perhaps the Spo0A S231F mutant creates a form of Spo0A that interacts with another position of $\sigma^A$ or in another subunit of RNA polymerase. Therefore, whether region 4 of $\sigma^A$ interacts directly with $\alpha$-helix E of Spo0A is unknown.

Here we report genetic evidence for an interaction between region 4 of $\sigma^A$ and Spo0A. We found an intragenic sig4 sup-
pressor mutation resulting in substitution of tryptophan for arginine at position 355 (R355W) that almost fully suppresses the sporulation defect due to both the H359R and H359A substitutions in σ^A. We used the coordinates of structures for the C-terminal domain of Spo0A (C-Spo0A) (15) and the C-Spo0A bound to DNA (5) to model the interactions of Spo0A and σ^A at the spoIIG promoter. The resulting model predicts that R355 in region 4 of σ^A interacts with the glutamine at position 221 of Spo0A. The effects of alanine substitutions in σ^A and Spo0A on spoIIG promoter activity were consistent with this model.

**MATTERIAls AND METHODS**

**Bacterial strains and culture media.** Routine microbiological manipulations and procedures were carried out by standard techniques as described previously (6). The concentrations of antibiotics used for selection in Luria-Bertani (LB) broth or Difco sporulation medium (DSM) were 5 μg/ml for chloramphenicol, 100 μg/ml for spectinomycin, 100 μg/ml for ampicillin, and 10 μg/ml for kanamycin. Cultures were grown in Luria-Bertani broth, and sporulation was induced by nutrient exhaustion in DSM. Competent cells were prepared and transformed by the two-step method as described previously (6).

The *B. subtilis* strains used are listed in Table 1. Plasmids derived from pCB2 (4) were used for inserting various mutations at the wild-type spo0A locus as described by Kumar et al. (14). Plasmids derived from pJB2 (1) were used for inserting various mutations at the wild-type sigA locus. For creating substitutions at the sigA locus, pJB2-derived plasmids were digested with ScaI and transformed into strain JH642. Chromosomal DNA was prepared from kanamycin-resistant colonies and subjected to PCR with primer sets SA104AFOR-Kan60REV and SA3REV-Kan741FOR to indicate that recombination occurred in the correct location on the chromosome. The resulting PCR fragment was then sequenced with primer SAREV to confirm the presence of the desired mutation.

The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) was used to create mutations in spo0A or sigA that resulted in single alanine substitutions. The combinations of forward and reverse primers listed in Table 2 were used to make the single amino acid substitutions in pCB2 or pJB2. The resulting new plasmid for each mutation was subjected to sequencing (Emory University DNA Sequencing Facility) to confirm the presence of the mutations.

**Sporulation assay.** Sporulation was induced by medium exhaustion in DSM (24) as described previously (6). Sporulation efficiency was determined in 36-h cultures as the total number of heat-resistant (80°C for 20 min) CFU compared with the total number of CFU before heat treatment. Data presented are averages of two independent experiments.

**EMS mutagenesis and suppressor screen.** The mutagenesis procedure was adapted from the procedure described by Green et al. (9). Strain EUB9401 (sigA H359R) was grown in 50 ml of LB liquid with 10 μg of kanamycin per ml until reaching an optical density of 600 nm of 0.6. The cells were spun down and resuspended in 1 ml of LB broth, and various dilutions were plated onto DSM agar and allowed to dry. A paper disk, to which 3 drops of ethyl methane sulfonate (EMS) (1.7 g/ml; Sigma) were added, was placed in the center of the plate, and the cells were incubated at 42°C for 2 days. At that time, the plates were inverted twice over 400-μl pools of chloroform for 20 min and then removed from the chloroform exposure for 20 min. The cells were returned to growing at 42°C overnight. Six sporulation-proficient (Spo^+^) survivors, which were able to form colonies by using nutrients released from the lysed, nonsporulating cells, were picked off the plates and restreaked, and single colonies were used to inoculate 10 ml of LB broth. These cultures were harvested at an optical density at 600 nm of 0.8, and chromosomal DNA was extracted by use of the Quick Procedure kit (6). We determined the nucleotide sequences of the spo0A and sigA regions for these six strains and found a single base pair substitution, a transition, that changed codon 355 (TGG) of the sigA gene, encoding arginine, to CGG, which encodes tryptophan. This allele was reconstructed in vitro by the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif.).

**Bacteriophages**

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<td>SPb abrB-lacZ</td>
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**TABLE 1.** *B. subtilis*-ACTIVATED TRANSCRIPTION

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TABLE 2. Oligonucleotides used for PCR, sequencing, and mutagenesis

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<td>Kan741FOR</td>
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<td>SA2REV</td>
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complex (protein database [PDB] no. 1KU7) on the nons模板strand of the Spo0A region sequence (5’-TTCGACA-3’) from the 2.3-A resolution crystal structure of the C-Spo0A-DNA complex (PDB no. 1LQ1) and then examined the predicted positions of the two proteins. Additional models were tested by moving the −35 DNA element (5’-TTCGACA-3’) from the ααα-DNA complex (PDB no. 1KU7) downstream at 1-bp intervals from the Spo0A recognition sequence (5’-TTCGACA-3’) in the C-Spo0A-DNA complex (PDB no. 1LQ1). The DNA in the final model was extended with B-form DNA such that the final model comprises B-form DNA (−63 to −46). DNA from sequence IQL1 (−45 to −33), with the Spo0A binding sequence centered at −37 (as in spoIIG). DNA from sequence KU7 (−32 to −28), and further downstream B-form double-stranded DNA (−27 to +1). All modeling was done with the molecular modeling program Sybyl 6.1 (Tripos, Inc.).

β-Galactosidase activity. Cultures were grown in duplicate in DSM with antibiotic (5 mg of chloramphenicol per ml) to initiate sporulation (l). Two 300-μl aliquots of each culture were collected, one to measure the optical density and the other to assay β-galactosidase activity. Enzymatic activity was averaged from three independent experiments and expressed in Miller units (12).

RESULTS

An intragenic suppressor of the sporulation-defective phenotype caused by the H359R substitution in ααα. During a search for suppressors of the sporulation defect observed in the strain encoding the ααα H359R substitution, we found an intragenic suppressor. This allele contained a second site mutation in the sigA gene, resulting in replacement of the arginine at 355 with a tryptophan (R355W). We examined the effect of the R355W substitution on the efficiency of endospore formation in strains in which the sigA allele contained, in addition to the R355W substitution, the H359R or H359A substitution or an otherwise wild-type sigA allele. Strains EUB9401 and EUC9604 express the H359R and H359A forms of ααα, respectively, and produced <50 or 1 × 107 spores per ml, respectively. Strains EUC9703 (sigAR355W H359R) and EUC9704 (sigAR355W H359A) both produced approximately 107 spores per ml. Strain EUC9702, with the singly substituted R355W allele of sigA, also produced approximately 107 spores per ml. These results indicate that R355 may lie in close proximity to Spo0A and, like K356 and H359 of ααα, may play an important role in Spo0A-dependent promoter activation. The role of R355 was investigated as described below.

A structure-based model of ααα region 4-Spo0A interaction predicts a role for ααα R355. To determine if the surface of ααα defined by R355 interacts directly with Spo0A, we used the published coordinates derived from crystal structures of a Spo0A-DNA complex (31) and region 4 of T. aquaticus ααα in complex with its cognate −35 DNA sequence (5) to model potential interactions between Spo0A and ααα region 4 on the spoIIG promoter. We made several assumptions in building the model. We expected that ααα region 4 of T. aquaticus would be a good model for ααα region 4 of B. subtilis because their amino acid sequences are 66% identical over the 73 amino acid residues present in the crystal structure. We also expected that Spo0A bound at the site centered at position −37 of the spoIIG promoter would interact with ααα. The sites on the spoIIG promoter at which Spo0A binds are known (Fig. 1) (12). Moreover, we know that position −38 in the spoIIG promoter corresponds to the C in the Spo0A target sequence TTCGACNA (oriented 5’ to 3’ on the nontranscribed strand of spoIIG) because a substitution of C at this position in the spoIIG promoter increases the affinity of Spo0A for this site and increases spoIIG promoter activity (2, 23). There are also additional Spo0A binding sites near position −90 of the spoIIG promoter. However, since binding to the site at position −37 determines the level of spoIIG promoter activity (2, 23), we ignored the upstream site in our modeling. Therefore, all models examined included Spo0A positioned at the site centered at −37. Finally, we assumed that ααα region 4 is positioned near the DNA helix at the spoIIG promoter in a manner similar to that of ααα at Spo0A-independent promoters.

We began by examining a model in which ααα region 4 interacts with the TTCGACNA sequence centered between positions −37 and −36 on the spoIIG promoter (Fig. 1). However, the model showed that Spo0A and ααα region 4 could not simultaneously occupy this site due to steric hindrance (data not shown). Subsequently, we examined models in which ααα region 4 was moved by 1-bp intervals to various positions on the promoter DNA, keeping the position of Spo0A centered at −37. The ααα helix E of Spo0A and region 4 of ααα appeared to lie in close proximity without steric hindrance at only one
position, with region 4 of \( \alpha^4 \) positioned at the sequence centered between positions –33 and –32 of \( spoIIG \).

Before we examined the model for putative interactions between \( spo0A \) and region 4 of \( \alpha^4 \), one additional modeling step was necessary. The structure of the \( spo0A \)-DNA complex produced by Zhao et al. (31) does not include all amino acid residues of \( \alpha \)-helix E, presumably because of disorder in the crystal. However, the structure of \( Bacillus stearothermophilus \) C-\( spo0A \) produced without DNA by Lewis et al. (15) includes all of \( \alpha \)-helix E. Superimposition of the two \( spo0A \) structures revealed that the two structures are nearly identical, with a root mean square deviation of 0.43 Å (31). Therefore, we superimposed the structure of \( spo0A \) from the work of Lewis et al. onto our model to estimate the position of all residues in \( \alpha \)-helix E.

**Allele-specific interaction of \( sigA \) \((R355A) \) and \( spo0A \) \((E221A) \).** Examination of our model for the interacting surfaces of \( spo0A \) and \( \alpha^4 \) revealed that the side chain of R355 in \( \alpha^4 \) may lie in close proximity to the side chain of glutamate at position 221 in \( spo0A \) (Fig. 2). We hypothesized that the opposite charges on these residues may form the basis for an important interaction between the surfaces of these proteins at the \( spoIIG \) promoter. To test this hypothesis and to screen for other interacting residues, we examined the effects of several single alanine substitutions in \( spo0A \) and in \( \alpha^4 \) on utilization of the \( spoIIG \) promoter in vivo. We examined the effects of the amino acid substitutions, both singly and in pair-wise combinations, on expression of a \( spoIIG \) promoter-lacZ fusion. The mutant alleles of \( sigA \) tested included those encoding single substitutions R355A, R358A, S361A, and R362A. We examined the effects of these alleles on \( spoIIG \)-lacZ expression in strains that contained the wild-type \( spo0A \) allele and in strains encoding \( spo0A \) derivatives in which alanine is substituted for glutamate at position 221 (E221A), which we predicted to interact with R355 of \( \alpha^4 \), or in which alanine was substituted for isoleucine at position 229, which was previously shown to affect \( spoIIG \) promoter activation (4).

We harvested samples from cultures of each strain at hourly intervals after the end of the exponential-growth phase (i.e., the onset of sporulation) and measured the level of \( \beta \)-galactosidase activity (reporter of \( spoIIG \)-lacZ expression). We compared the level of \( spoIIG \)-lacZ expression of each strain to that of the isogenic strain containing the wild-type allele of \( sigA \), the structural gene for \( \alpha^4 \). Except for S361A, which had little effect, each amino acid substitution in \( \alpha^4 \) resulted in reduced expression of \( spoIIG \)-lacZ in strains containing the wild-type allele of \( spo0A \) (Fig. 3 and data not shown). In most cases, the amino acid substitutions in \( \alpha^4 \) also resulted in reduced expression of \( spoIIG \)-lacZ in strains containing the mutant alleles of \( spo0A \). The greatest exception was seen for the strain in which the E221A substitution in \( spo0A \) was combined with the R355A substitution in \( \alpha^4 \). In this case, the levels of \( \beta \)-galactosidase accumulation indicated that in the strains containing the E221A mutant of \( spo0A \) the \( spoIIG \) promoter was used more efficiently by RNA polymerase containing the R355A mutant \( \alpha^4 \) than by RNA polymerase containing wild-type \( \alpha^4 \).

**DISCUSSION**

We used structure-based molecular modeling in an attempt to define the interacting surfaces of \( spo0A \) and \( \alpha^4 \) during activation of the \( spoIIG \) promoter. Our model predicts interaction of glutamate at position 221 of \( spo0A \) with arginine at position 355 of \( \alpha^4 \). We tested this model by examination of the effects of amino acid substitutions in \( spo0A \) and \( \alpha^4 \) on \( spoIIG \) promoter activity in vivo. One problem with this type of analysis is that regulation of \( spo0A \) synthesis and activity is com-
plex; Spo0A synthesis is autoregulated (27) and Spo0A regulates synthesis of proteins that control Spo0A activity by controlling its phosphorylation (28). Amino acid substitutions in Spo0A may also reduce its stability or prevent DNA binding. To circumvent the complications of indirect effects on Spo0A activity, we compared the ratio of spoIIG promoter activities produced by wild-type and mutant forms of σA in the presence of a single form of Spo0A to the ratio of these activities in strains containing a different form of Spo0A. In these genetic experiments, activation of the spoIIG promoter by E221A mutant Spo0A was more efficient in combination with the R355A mutant σA than with the wild-type or R358A, R362A, or S361A mutant forms of σA (Fig. 3). Therefore, regardless of any indirect effects that the E221A substitution in Spo0A may have had on its function, the allele specificity of this effect is consistent with the model that R355 of σA interacts directly with E221 of Spo0A during spoIIG promoter activation.

To explain the effects of the E221A and R355A substitutions, we note that an amino acid substitution in a position that normally interacts directly with a ligand may have two types of effects. First, the substitution may remove a favorable interaction with the ligand, and second, the substitution may introduce, directly or indirectly, an interfering or unfavorable interaction. We suggest that the E221 substitution in Spo0A does both. It eliminates a favorable interaction with R355 in σA. In addition, the E221A substitution in Spo0A produces an interfering or interfering effect on Spo0A-σA interaction because the positive charge of R355 in σA cannot be neutralized. Substitution of alanine for R355 in σA eliminates the interfering effect created by the E221A substitution in Spo0A, partially restoring Spo0A-σA interaction at the spoIIG promoter. However, the R355A substitution does not restore the favorable interaction found between R355 of wild-type σA and E221 of wild-type Spo0A. Comparison of the absolute values of spoIIG promoter activity in mutant and wild-type cells shows that the R355A substitution does not fully suppress the effect of the E221A substitution on promoter activity because it results in only 40% of the promoter activity in wild-type cells (not shown). Therefore, we suggest that the negative charge of E221 in wild-type Spo0A is neutralized by R355 in wild-type σA during their interaction at the spoIIG promoter. There appears to be little or no interaction of Spo0A E221 and the residues R358, R362, and S361 of σA. These genetic results support our structure-based model for the interacting surfaces of Spo0A and σA at the spoIIG promoter.

Our discovery of a role for E221 in Spo0A in promoter activation is somewhat surprising. All amino acid substitutions in Spo0A that had previously been shown to play a role in spoIIG promoter activation lie within α-helix E of Spo0A. E221 is located near but outside of α-helix E in the C terminus of the DNA recognition helix. The recognition helix of the helix-turn-helix region in Spo0A is unusually long (3, 15). The N-terminal amino acids of the recognition helix bind in the major groove of the DNA (31). The C-terminal end of the helix, including position 221, extends away from the DNA where it could interact with region 4 of σA. Therefore, this position of E221 makes it unlikely that the E221A substitution affects DNA binding by Spo0A. Furthermore, the E221A mutant Spo0A repressed expression of an abrB-lacZ fusion (data not shown), an activity that requires DNA binding by Spo0A.

The glutamate at position 221 in Spo0A is strictly conserved among other spore-forming gram-positive bacteria (data not shown), suggesting that this acidic residue plays a critical role in these organisms. Acidic regions also comprise the activation regions of several bacterial and eukaryotic transcription factors. These include charge interactions between activators and region 4 of σ. For example, R596 (homologous to R355 in B. subtilis α) and R588 in σ70 are thought to interact with D38 and E34, respectively, of lambda cI (16, 18).

Our identification of the interacting surfaces of Spo0A and σA during activation of the spoIIG promoter may have revealed important insights into the mechanism of spoIIG promoter activation. The spoIIG promoter contains a consensus −35-like promoter element centered between positions −37 and −36 (Fig. 1). Binding of σA region 4 to this element would separate region 4 from the −10 promoter element by 22 bp (Fig. 4A), whereas 17 to 18 bp is the optimal spacing between −35 and −10 promoter elements in σA-dependent, Spo0A-independent promoters (17). This −35-like sequence is overlapped by a Spo0A binding site (Fig. 1) (2). Our modeling showed that Spo0A and σA region 4 are not likely to simultaneously occupy these sites (data not shown). Therefore, one aspect of the mechanism of spoIIG promoter activation by
Spo0A may be occlusion of this inappropriately spaced –35-like element by Spo0A to prevent interaction of σ^A region 4 with this DNA (Fig. 4B). However, this cannot be the only or most important role for Spo0A because most base substitutions that change this –35-like element to look less similar to a consensus –35 element reduce spoIIG promoter activity (22) rather than relieving dependence of promoter activity on Spo0A, as predicted by a simple occlusion model. A more important role for Spo0A binding to the spoIIG promoter is illuminated by our model’s prediction that the specific interactions between Spo0A and σ^A that have been demonstrated genetically occur when σ^A region 4 is located near a sequence centered between positions –33 and –32 of the spoIIG promoter (Fig. 4B). This sequence centered at –33 and –32 is only 50% identical to the consensus σ^A region 4 recognition sequence (Fig. 1 and 4), and there is no evidence that the sigma factor binds in a sequence-specific manner to this region. Therefore, it is likely that positioning of σ^A region 4 at this site would require stabilizing, specific interactions with Spo0A. This interaction between Spo0A and σ^A places region 4 of σ^A 18 bp upstream from the –10 region of the spoIIG promoter. Therefore, an important aspect of the mechanism of spoIIG promoter activation by Spo0A may include stabilization of σ^A region 4 binding to the promoter at a position that optimizes spacing between σ^A region 4 and the –10 element of the promoter. This model requires further investigation; however, the model seems consistent with the conclusions of Spiegelman and his colleagues that showed that Spo0A stimulates spoIIG promoter DNA strand separation, a step that follows the initial dissociation of the positive and negative regulatory properties of the Spo0A RNA polymerase alpha subunit. J. Bacteriol. 178:5005–5008.

It is likely that Spo0A activates other σ^A-dependent promoters by a similar mechanism. The sequence of another Spo0A-activated promoter, spoIIE, is similar to spoIIG in that it contains a –35-like sequence separated by 21 bp from the –10 region sequence (10). Moreover, its –35-like sequence overlaps with a Spo0A binding site (30). Other σ^A-dependent, Spo0A-dependent promoters have not been thoroughly characterized; therefore, we cannot speculate on the mechanism of their activation by Spo0A. Spo0A also activates σ^F-dependent promoters; however, this mechanism is even less well understood (14).

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REFERENCES


ERRATUM

Surfaces of Spo0A and RNA Polymerase Sigma Factor A That Interact at the spoIIG Promoter in Bacillus subtilis

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Volume 186, no. 1, p. 200–206, 2004. Page 203, Fig. 2: The labels at the N terminus and C terminus of Spo0A (labeled N and C, respectively) should be reversed.