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## Just-in-Time Control of Spo0A Synthesis in *Bacillus subtilis* by Multiple Regulatory Mechanisms<sup>∇</sup>§

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The response regulator Spo0A governs multiple developmental processes in *Bacillus subtilis*, including most conspicuously sporulation. Spo0A is activated by phosphorylation via a multicomponent phosphorelay. Previous work has shown that the Spo0A protein is not rate limiting for sporulation. Rather, Spo0A is present at high levels in growing cells, rapidly rising to yet higher levels under sporulation-inducing conditions, suggesting that synthesis of the response regulator is subject to a just-in-time control mechanism. Transcription of *spo0A* is governed by a promoter switching mechanism, involving a vegetative,  $\sigma^A$ -recognized promoter,  $P_v$ , and a sporulation  $\sigma^H$ -recognized promoter,  $P_s$ , that is under phosphorylated Spo0A (Spo0A~P) control. The *spo0A* regulatory region also contains four (including one identified in the present work) conserved elements that conform to the consensus binding site for Spo0A~P binding sites. These are herein designated O<sub>1</sub>, O<sub>2</sub>, O<sub>3</sub>, and O<sub>4</sub> in reverse order of their proximity to the coding sequence. Here we report that O<sub>1</sub> is responsible for repressing  $P_v$  during the transition to stationary phase, that O<sub>2</sub> is responsible for repressing  $P_s$  during growth, that O<sub>3</sub> is responsible for activating  $P_s$  at the start of sporulation, and that O<sub>4</sub> is dispensable for promoter switching. We also report that Spo0A synthesis is subject to a posttranscriptional control mechanism such that translation of mRNAs originating from  $P_v$  is impeded due to RNA secondary structure whereas mRNAs originating from  $P_s$  are fully competent for protein synthesis. We propose that the opposing actions of O<sub>2</sub> and O<sub>3</sub> and the enhanced translatability of mRNAs originating from  $P_s$  create a highly sensitive, self-reinforcing switch that is responsible for producing a burst of Spo0A synthesis at the start of sporulation.

The Gram-positive bacterium *Bacillus subtilis* has the capacity to decide among a variety of cell fates at the end of the exponential phase of growth. At the heart of the decision-making process for several of these fates is the response regulatory protein Spo0A, which governs spore formation, biofilm formation, and cannibalism and is also required for competence (21). The activity of Spo0A is controlled by phosphorylation via a multicomponent phosphorelay, at the head of which are five histidine kinases (KinA to KinE) (4). Discrimination among alternative cell fates is determined in part by the cellular levels of phosphorylated Spo0A (Spo0A~P), with the regulatory sites for genes with moderate to high affinity for Spo0A~P (e.g., genes involved in cannibalism and biofilm formation) firing at low to intermediate levels of the phosphoprotein and those with low affinity being turned on only at high levels (14, 15).

Phosphorylation induces a conformational change in Spo0A, allowing it to dimerize and bind to target sequences (2). More than 100 genes are under the direct positive or negative control of Spo0A~P (23). Genes activated by Spo0A~P include those with promoters recognized by RNA polymerase containing the housekeeping sigma factor  $\sigma^A$ , as well as genes whose promoters are recognized by RNA polymerase containing the alter-

native sigma factor  $\sigma^H$  (including the  $P_s$  promoter for *spo0A* investigated here) (25). The target sites reveal a consensus binding sequence, TTTGTCRAA, which is known as the 0A box (23). The crystal structure of the C-terminal (DNA-binding) domain of the *Bacillus stearothermophilus* ortholog in a complex with 0A box sequences has been solved (32). This structure reveals contacts of the DNA-binding domain with bases in the 0A box and with the DNA backbone and has led to the suggestion that Spo0A~P dimers are capable of forming tandem arrays in a head-to-tail arrangement along the DNA (32). It was suggested that such arrays may form in the regulatory region for genes such as *spoIIA*, *spoIIIE*, and *spoIIG* that exhibit multiple 0A boxes in tandem extending far upstream from the start site of transcription. That said, the mechanism by which Spo0A~P activates transcription remains largely unclear, with few examples that have been well characterized, two for promoters recognized by  $\sigma^A$  RNA polymerase ( $P_{spoIIG}$  and  $P_{skf}$ ) (10, 26) and one ( $P_{spo0F}$ ) for promoters recognized by  $\sigma^H$  RNA polymerase (2).

Interestingly, Spo0A is maintained at relatively high levels (~2,000 molecules/cell) during exponential phase, rapidly rising to even higher levels (~20,000 molecules/cell) under sporulation-inducing conditions (9, 13). We have attributed the high maintenance level of Spo0A and the rapid increase to yet higher levels to a just-in-time regulatory system that ensures that Spo0A molecules do not become rate limiting at both low and high rates of flux of phosphoryl groups through the phosphorelay (9).

How are Spo0A protein levels regulated during the transition to stationary phase? The *spo0A* gene is transcribed from two promoters, whose start sites are located 204 and 45 bp upstream from the start codon for the open reading frame

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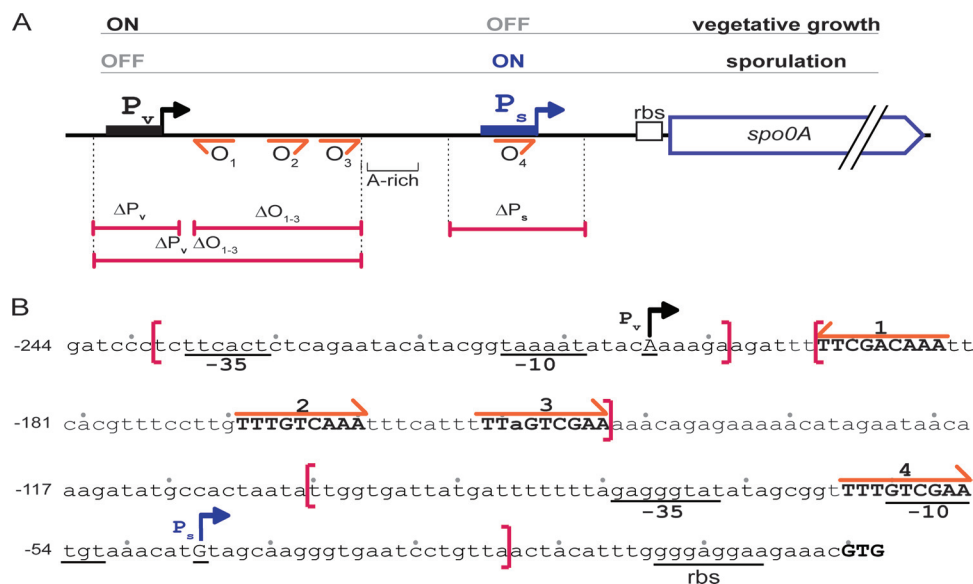


FIG. 1. The *spo0A* regulatory region. (A) Cartoon of the regulatory region.  $P_v$  and  $P_s$  are indicated by black and blue boxes with arrows, respectively. Sequences matching the Spo0A consensus binding site (0A box), which are designated operator sites  $O_1$ ,  $O_2$ ,  $O_3$ , and  $O_4$ , are labeled with orange arrows and numbered in order from 5' to 3'. The region extending from  $O_1$  to  $O_3$  (−192 to −143) is referred as  $O_{1-3}$  and the AT-rich region from position −142 to −99 (relative to the start codon) as “A-rich.” Regions deleted in mutants are indicated by pink bars with corresponding mutant names. (B) Sequence of the regulatory region. The start sites of transcription (+1) for the  $P_v$  and  $P_s$  promoters are underlined and highlighted by a vertical arrow. Positions are numbered relative to the initiation codon GTG (indicated in bold). Sequences matching 0A boxes are indicated with orange arrows and numbered as in panel A. Brackets indicate limits of the deletion in  $\Delta P_v$  (Abs894),  $\Delta O_{1-3}$  (Abs883),  $\Delta P_v \Delta O_{1-3}$  (Abs875), or  $\Delta P_s$  (Abs786).

(Fig. 1A) (12, 29)). The more upstream promoter,  $P_v$ , is expressed during the exponential phase of growth under the control of  $\sigma^A$ -RNA polymerase. This transcription fluctuates strikingly and in a manner that correlates with small changes in the growth rate (22) but then shuts off during the transition to stationary phase (12). The downstream promoter,  $P_s$ , in contrast, is induced after the end of exponential-phase growth under the control of  $\sigma^H$ -RNA polymerase and Spo0A~P. Thus, transcription of *spo0A* switches from  $P_v$  to  $P_s$  as cells exit exponential-phase growth (11, 12, 29). Transcription from  $P_s$  is required in order for Spo0A to reach the high levels needed for the activation of key sporulation genes, such as *spolIA*, *spolIE*, and *spolIG*, but not for the low levels required for efficient entry into competence (14, 18). Insights into the mechanism of promoter switching has come from the work of Strauch and coworkers, who showed that it is mediated by Spo0A~P and that the protein binds three 0A boxes in the regulatory region (29).

Here we revisit the mechanism of promoter switching, which has not been investigated since the early work of Chibazakura (11) and Strauch (29). We report the discovery of a fourth 0A box that plays a key role in promoter switching and other features of the regulatory region that are conserved among multiple *Bacillus* spp. We refer to the four 0A boxes as  $O_1$ ,  $O_2$ ,  $O_3$ , and  $O_4$  in the reverse order of their distance from the open reading frame (renaming them to accommodate the newly identified site  $O_2$ ). Our principal findings are that  $O_1$  is a negatively acting element that is responsible for repressing  $P_v$  at the end of exponential phase, that the newly identified  $O_2$  site is a negatively acting element that represses  $P_s$  during growth, that  $O_3$  is a positively acting element that activates  $P_s$

upon entry into stationary phase, and that  $O_4$  is dispensable. We also report the discovery of a translational control mechanism that impedes translation of mRNAs originating from  $P_v$  but not that originating from  $P_s$ . We also show that  $P_v$  provides a basal level of Spo0A that is required for efficient entry into the state of genetic competence and strong activation of  $P_s$ , and we suggest that  $P_v$  plays a pump-priming role in the activation of the Spo0A~P-controlled promoter. An intricate regulatory region mediates a just-in-time regulatory system that helps to ensure an adequate supply of Spo0A molecules to meet the needs of the phosphorelay.

## MATERIALS AND METHODS

**Media and general methods.** Media, culture, cloning procedures, preparation of competent cells,  $\beta$ -galactosidase assay, immunoblot analysis, and sporulation assays were carried out as previously described (7, 9, 16, 28). RNA secondary structure prediction was performed with the RNA2 prediction software program using default settings and sequence alignments with ClustalV.

**Plasmids and strain constructions.** All the *Bacillus subtilis* strains used in the present study were derivatives of the PY79 strain (31) and are listed in Table S1 in the supplemental material. Plasmids used are listed in Table S2 and oligonucleotides in Table S3. Markerless deletions of the vegetative promoter of *spo0A* ( $\Delta P_v$ ),  $O_1$  to  $O_3$  ( $\Delta O_{1-3}$ ), and both ( $\Delta P_v \Delta O_{1-3}$ ) were constructed using pMAD (1) derivative plasmids: pAC328, pAC313, and pAC306. For this, two NcoI/EcoRI and EcoRI/BamHI DNA fragments corresponding to the *spoIVB-spo0A* locus were generated by PCR using AC-739/740 and AC-741/742 and cloned between NcoI/BamHI restriction sites of pMAD to give plasmid pAC328. pAC306 was generated likewise, except that AC-744 was used instead of AC-741, whereas in addition AC-743 was also used in place of AC-740 to generate pAC313. Markerless deletion of  $P_s$  was generated as previously described by Siranosian et al. (27) using pSK5, with the exception that PY79 was used as the recipient strain. All constructions in PY79 were checked for deletion by PCR amplification and sequencing.

Translational and transcriptional fusions with *lacZ* were obtained by cloning

PCR-generated EcoRI/SalI or EcoRI/BamHI DNA fragments, respectively, into the corresponding sites of pDG1728 (see Tables S2 and S3 in the supplemental material for details). Fusions with mutated promoters were obtained either by using the QuikChange site-directed mutagenesis kit (Stratagene) using the manufacturer's protocol or by cloning fusion PCR products. Insertion mutants were obtained by adding a 5-bp (TATCT) or 11-bp (TATCTAGAGGC) sequence at position -136/-135. For construction of promoter fusions in which the SigH binding sequence of  $P_s$  was replaced by that of  $P_{spoVG}$ , TAGAGGGTATATA GCGGTTTGTGCGAATGTA was replaced by AGCAGGATTCAGAAAAA ATCGTGGAATTGA (see Fig. S4 in the supplemental material).

**Primer extension.** Primer extension was performed mainly as previously described (8), with the exception that 2  $\mu$ g RNA was incubated with 0.1 pmol of the radiolabeled oligonucleotide AC-766.

**Spo0A purification and DNase I footprinting.** Spo0A was purified by affinity chromatography, with a procedure derived from the work of Lewis et al. (20). In brief, Spo0A was extracted from an *Escherichia coli* strain harboring pAC571, cultured at 30°C. Cells resuspended in TEN buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 50 mM NaCl) were disrupted by sonication, and clarified lysate was loaded on an S column (Amersham-Pharmacia) preequilibrated with TEN.

For the footprinting assay, the radiolabeled oligonucleotide AC-470 was used with cold AC-781 to PCR amplify DNA fragments corresponding to a wild-type or mutant version of the *spo0A* promoter, using as template DNA pAC407 (wild type), pAC463 (m1), pAC458 (m2), or pAC507 (m3). Conditions of the assay were mainly as previously published (6), with the exception that after purification, resulting radiolabeled PCR products were incubated with freshly purified native Spo0A for 20 min at room temperature before DNase I treatment.

## RESULTS

**Both vegetative and sporulation promoters are required for rapid and high-level accumulation of Spo0A.** As a starting point for this investigation, we constructed a series of deletion mutations within the *spo0A* regulatory region and examined their consequences for *spo0A* transcription and for the accumulation of Spo0A. The *spo0A* gene is transcribed from an upstream, vegetative promoter,  $P_v$ , by  $\sigma^A$ -containing RNA polymerase and from a downstream sporulation promoter,  $P_s$ , by RNA polymerase containing the alternative sigma factor  $\sigma^{H1}$  (12). Located between  $P_v$  and  $P_s$  are three 0A boxes, here referred to as  $O_1$ ,  $O_2$ , and  $O_3$  (Fig. 1). Two of these elements ( $O_1$  and  $O_3$ ) had been identified previously and confirmed as Spo0A~P binding sites by Strauch and coworkers (29). As discussed below, we detected a third 0A box located between  $O_1$  and  $O_3$ , which we designate  $O_2$  in Fig. 1. Finally, the regulatory region contains a fourth binding site,  $O_4$ , which overlaps with  $P_s$  (29). Based on these considerations, we created deletions that removed  $P_v$  ( $\Delta P_v$ ),  $P_s$  ( $\Delta P_s$ ) (27),  $O_1$  to  $O_3$  ( $\Delta O_{1-3}$ ), and both  $P_v$  and  $O_1$ - $O_3$  ( $\Delta P_v \Delta O_{1-3}$ ) (Fig. 1).

We used immunoblot analysis to investigate the effect of the deletion mutations on Spo0A accumulation. Because Spo0A accumulates to high levels by hour two of sporulation (9), we compared the levels of Spo0A at the mid-exponential phase of growth ( $T_{-0.5}$ , representing 0.5 h prior to the end of exponential phase) with those at  $T_2$  (Fig. 2A; see also Fig. S1A in the supplemental material). Whereas Spo0A levels markedly increased by  $T_2$  in the wild type, little accumulation was observed at this time in the absence of either  $P_v$  or  $P_s$ . These findings are consistent with previous reports that the removal of either promoter impairs Spo0A-directed transcription (12, 27). (Note that little Spo0A could be seen at  $T_{-0.5}$  in the immunoblot of Fig. 2A but that Spo0A was readily detected during growth with longer exposure times and higher loading levels, as previously described [9] and as seen in the experiment of Fig. S1B.) Unexpectedly, however, we found that the removal of

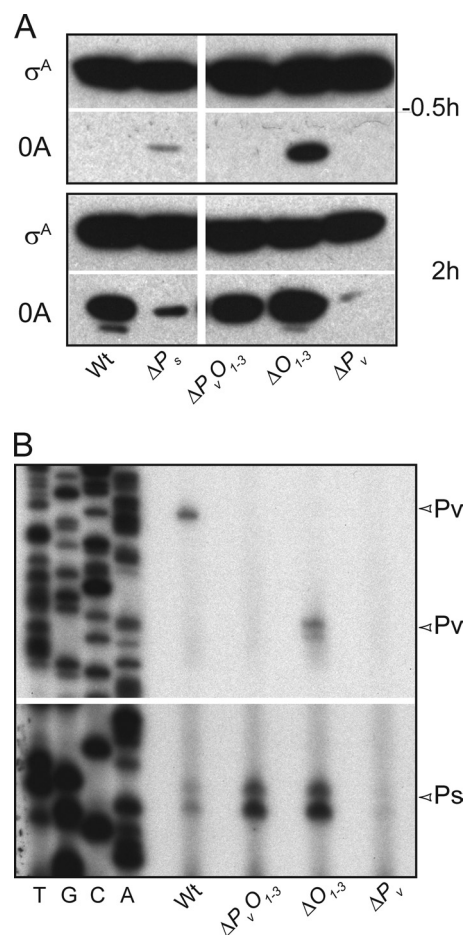


FIG. 2.  $O_1$ - $O_3$  contains a regulatory element controlling  $P_s$ . (A) Immunoblot analysis of Spo0A (0A) accumulation during sporulation. Blotting was performed using samples taken at 0.5 h before sporulation induction by resuspension (upper panel) and 2 h postinduction (bottom panel). Equal amounts of protein, as quantified by the Bradford technique, were loaded as confirmed by the control immunoblot using anti-SigA ( $\sigma^A$ ) antibodies. Samples were taken from shaking culture at 37°C of wild-type cells (Wt; PY79) or  $P_s$  ( $\Delta P_s$ ; Abs786),  $O_{1-3}$  ( $\Delta O_{1-3}$ ; Abs883),  $P_v$  ( $\Delta P_v$ ; Abs894), or  $P_v$  plus  $O_{1-3}$  ( $\Delta P_v O_{1-3}$ ; Abs875) mutant cells. (B) Primer extension analysis of *spo0A* transcripts. Analysis was performed on RNA extracted from exponential-phase wild-type cells or mutant cells as described for panel A. Start sites (+1) are indicated on the right by arrowheads. Note the shift in the site of extension products originating from  $P_v$  caused by the shortening of the transcript in the  $\Delta O_{1-3}$  deletion.

$O_1$ - $O_3$  ( $\Delta O_{1-3}$ ) boosted Spo0A to high levels as early as  $T_{-0.5}$  and bypassed the dependence of Spo0A accumulation on  $P_v$ ; that is, in a mutant in which both  $P_v$  and  $O_1$ - $O_3$  had been removed ( $\Delta P_v \Delta O_{1-3}$ ), Spo0A reached levels comparable to that seen for the wild type.

To determine which of the two promoters controlling *spo0A* was being affected by the deletion of  $O_1$ - $O_3$ , we performed primer extension analysis using RNA from cells in the exponential phase of growth (Fig. 2B). As expected,  $P_v$ -directed transcripts were seen in the wild type but were absent from a mutant lacking the vegetative promoter ( $\Delta P_v$ ) and from a mutant lacking both  $P_v$  and  $O_1$ - $O_3$  ( $\Delta P_v \Delta O_{1-3}$ ). (Note that the absence of  $P_v$  also impaired transcription from  $P_s$ , a point to





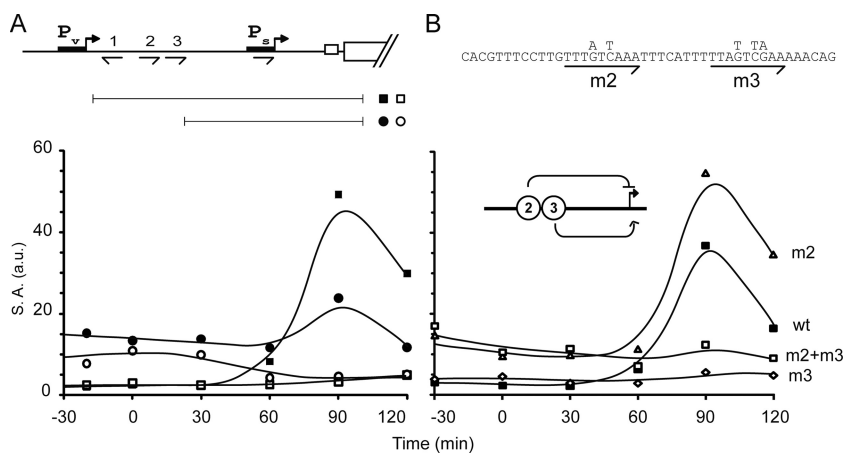


FIG. 4. *O*<sub>2</sub> and *O*<sub>3</sub> have opposing effects on *P*<sub>s</sub> expression. (A) Deletion of *O*<sub>1</sub>-*O*<sub>3</sub> increased expression of a *spo0A-lacZ* translational fusion during exponential growth phase and reduced its expression during sporulation. The fusions were integrated at *amyE*. *spo0A* sequences in the fusions extended from position +72 to position -197 (■, Abs988; □, Abs955) or position -145 (○, Abs1112; ●, Abs1149) relative to the GTG start codon. Expression was compared between the wild type (■ and ●) and *spo0B* mutant cells (□ and ○). A typical experiment is presented, in which β-galactosidase specific activity (S.A.) is plotted as a function of time (a.u. [arbitrary units]). Samples were taken every 30 min starting 30 min before induction of sporulation by resuspension and up to 120 min postinduction. (B) Expression from *spo0A-lacZ* translational fusions integrated at *amyE*. The sequences in the fusions extended from position -197 to +72 and were wild type (wt) (■; Abs988), *O*<sub>2</sub> mutant (m2) (△; Abs1055), mutant for *O*<sub>3</sub> (m3) (◇; Abs1205), or mutant for both (m2+m3) (□; Abs1172). The nature of the mutation is described above the graphs. A typical experiment is presented in which β-galactosidase specific activity (S.A.) is plotted as a function of time (a.u. [arbitrary units]). Samples were taken every 30 min starting 30 min before induction of sporulation by resuspension and up to 120 min postinduction.

similar pattern of expression was observed with a truncation that removed both the *O*<sub>1</sub>-*O*<sub>3</sub> region and the downstream AT-rich region ( $\Delta O_{1-3}$   $\Delta$ AT-rich) (see Fig. S2 in the supplemental material). Thus, consistent with the results obtained by primer extension analysis, the *O*<sub>1</sub>-*O*<sub>3</sub> region contains a regulatory element that inhibits *P*<sub>s</sub> activity during growth.

Next, we investigated the contributions of *O*<sub>1</sub>, *O*<sub>2</sub>, and *O*<sub>3</sub> individually to the activity of *P*<sub>s</sub> by separately mutating each *O*A box. Among the nine base pairs in the consensus Spo0A-binding sequence, the most critical seem to be the G and C at positions 4 and 6 (TTTGACRAA), with which Spo0A~P form hydrogen bonds (32). Less highly conserved is position 7, which is most often a G. Accordingly, we created nucleotide substitutions at positions 4 and 6 in *O*<sub>2</sub> (which has an A at position 7) and at 4, 6, and 7 in *O*<sub>3</sub> (see Materials and Methods). Mutating *O*<sub>1</sub> had little effect on expression of the fusion (see Fig. S2 in the supplemental material) and hence is not considered further. As seen in Fig. 4B, inactivation of *O*<sub>2</sub> was sufficient to allow premature expression from *P*<sub>s</sub>. In other words, mutating *O*<sub>2</sub> alone was sufficient to mimic the effect of deleting the entire *O*<sub>1</sub>-*O*<sub>3</sub> region in derepressing *P*<sub>s</sub> during growth. On the other hand, and unlike the deletion of *O*<sub>1</sub>-*O*<sub>3</sub> region, mutating *O*<sub>2</sub> mildly but reproducibly enhanced the post-exponential-phase induction of transcription from *P*<sub>s</sub>.

Conversely, mutation of *O*<sub>3</sub> almost completely abolished the expression of the reporter, an unexpected result considering that deletion of the *O*<sub>1</sub>-*O*<sub>3</sub> region was not blocked in *P*<sub>s</sub> activity. To investigate this observation further, we built a double mutant in which both *O*<sub>2</sub> and *O*<sub>3</sub> were mutated. The pattern of expression from the double mutant resembled that of the *O*<sub>1</sub>-*O*<sub>3</sub> deletion mutant.

We interpret these results as follows. *O*<sub>2</sub> is a negative element that inhibits *P*<sub>s</sub>, whereas *O*<sub>3</sub> is a positively acting element that is required for activation of *P*<sub>s</sub> during sporulation. When

both *O*<sub>2</sub> and *O*<sub>3</sub> are mutated, a high level of transcription from *P*<sub>s</sub> is seen during growth and sporulation, while induction of *P*<sub>s</sub> during sporulation is prevented.

**Evidence for DNA looping.** The large gap between *O*<sub>2</sub> and *O*<sub>3</sub> and the promoter (70 and 90 bp, respectively) raised the question of how Spo0A molecules bound so far upstream are able to control the activity of *P*<sub>s</sub>. Two models, which are not mutually exclusive, are that the intervening sequence forms a loop and/or that Spo0A bound at *O*<sub>2</sub> or *O*<sub>3</sub> spreads to the promoter region along the intervening DNA. In an effort to distinguish between these models, we introduced insertions of 5 and 11 bp in the intervening DNA with the expectation that increasing the spacing by about half a turn of the helix but not by approximately a full turn would impair function to a greater extent if the intervening DNA forms a loop. Indeed, as seen in Fig. 5, an 11-bp insertion had little effect on the pattern of expression whereas the 5-bp insertion had a marked effect, creating a phenocopy of a double mutant of *O*<sub>2</sub> and *O*<sub>3</sub>. The simplest interpretation of these results is that Spo0A~P bound at *O*<sub>2</sub> or *O*<sub>3</sub> interacts with the promoter region via the formation of a loop. Reinforcing this model is the observation that, as noted above, the intervening DNA is rich in A·T base pairs, which is known to enhance the flexibility of DNA (30). At the same time, our results do not exclude the possibility that spreading also occurs. Indeed, DNase I footprinting experiments revealed a region of protection that extended downstream of *O*<sub>3</sub> (see Fig. S3 in the supplemental material), a point we will discuss further.

***O*<sub>4</sub> is dispensable.** Next, we investigated the function of the fourth *O*A box, *O*<sub>4</sub>, which is embedded in *P*<sub>s</sub>. Because *O*<sub>4</sub> (TTTGTGCGAA) overlaps the -10 sequence of *P*<sub>s</sub> (GTCGAA TGT, in which the bold letters indicate bases shared with *O*<sub>4</sub>), *O*A box positions 4 and 7 are the R and G at positions 1 and 4 of the -10 sequence, which are conserved in  $\sigma^H$ -recognized

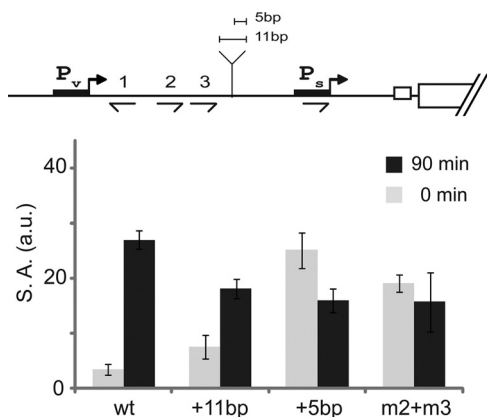


FIG. 5. Efficient regulation by  $O_2$  and  $O_3$  depends on their position relative to the face of the helix. Shown is expression from *spo0A-lacZ* translational fusions integrated at *amyE*. Bars represent the average values of three independent experiments. Sequences in the fusions extended from position  $-197$  to  $+72$  and were wild type (wt) (Abs988) or  $O_2$  and  $O_3$  mutant (m2+m3) (Abs1172) or contained a 5-bp insert (+5bp) (Abs1034) or an 11-bp insert (+11bp) (Abs1035). The inserts were at position  $-64$ . Samples were taken just before induction of sporulation by resuspension (0 min; gray bars) and at 1.5 h postinduction (90 min; black bars).

promoters (RnnGAATww) (3). Consequently, it was not possible to replace the conserved bases at positions 4, 6, and 7 of the 0A box without mutating the  $\sigma^H$ -recognized,  $-10$  sequence of  $P_s$ . Thus, we decided to make a chimeric regulatory region in which a 31-bp stretch of sequence that included the  $-35$  and  $-10$  sequences of  $P_s$  was replaced with the corresponding region of the *spoVG* promoter ( $P_{spoVG}$ ) (see Fig. S4 in the supplemental material).  $P_{spoVG}$  is a  $\sigma^H$ -controlled promoter that is not directly activated by Spo0A (Fig. 6A) (33).

Remarkably, the pattern of expression with the chimeric regulatory region was strikingly similar to that of the parental regulatory region. That is,  $P_{spoVG}$  was silent during growth and markedly induced by  $T_{1.5}$  (Fig. 6A). Next, we further modified the chimeric regulatory region by introducing mutations in  $O_2$ ,  $O_3$ , or both. As we observed with  $P_s$ , induction of the chimera was strongly dependent on  $O_3$  when  $O_2$  was intact (but not when both  $O_2$  and  $O_3$  were mutants), and the basal level of expression was increased 5-fold during exponential-phase growth in the absence of  $O_2$ . We infer that neither the function of  $O_2$  nor that of  $O_3$  requires the presence of  $O_4$ . We further conclude that  $O_2$  and  $O_3$  are sufficient to confer a Spo0A-dependent mode of expression on a promoter that lacks a 0A box.

#### $O_1$ is responsible for repression of $P_v$ during sporulation.

Finally, we returned to the question of the function of  $O_1$ , a mutant of which had little detectable effect on  $P_s$  expression (see Fig. S2 in the supplemental material). It was previously reported that promoter switching occurs during the transition to stationary phase, with  $P_v$  being turned off and  $P_s$  turned on (12). Because  $O_1$  is proximal to  $P_v$ , we postulated that it may be responsible for repression of  $P_v$ . To test this hypothesis, transcriptional fusions between  $P_v$  and *lacZ* (this time excluding  $P_s$ ) were constructed. Again, we compared the wild-type sequence or sequences mutant for  $O_1$ ,  $O_2$ , or  $O_3$  (Fig. 6B). Whereas the wild-type fusion and mutants of  $O_2$  or  $O_3$  exhibited decreased

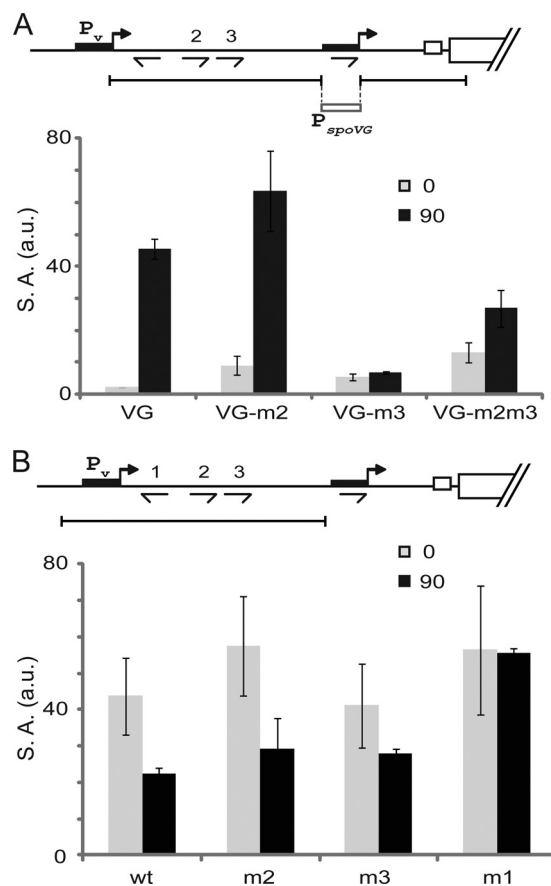


FIG. 6. Functional replacement of  $P_s$  with a  $\sigma^H$ -controlled promoter lacking an 0A box and repression of  $P_v$  by  $O_1$ . (A) Expression from *spo0A-lacZ* translational fusions integrated at *amyE*. The fusions contained sequences extending from position  $-197$  to  $+72$  relative to the initiating codon (and hence excluding  $P_v$ ) except that the 31-bp sequence from  $-6$  to  $-36$  containing  $P_s$  was replaced with a 31-bp sequence containing the *spoVG* promoter extending from  $-7$  to  $-37$  (relative to their start sites). In addition, the fusions were either wild type (VG) (Abs1223) or  $O_2$  mutant (VG-m2) (Abs1224),  $O_3$  mutant (VG-m3) (Abs1226), or both (VG-m2m3) (Abs1227). Samples were taken just before induction of sporulation by resuspension (0 min; gray bars) and 1.5 h postinduction (90 min; black bars). Values are the averages of data from two independent experiments. (B) Expression from *spo0A-lacZ* translational fusions containing sequences extending from position  $-305$  to  $-78$  (and hence excluding  $P_s$ ). In addition, the fusions either wild type (wt) (Abs1229),  $O_2$  mutant (m2) (Abs1230),  $O_3$  mutant (m3) (Abs1231), or  $O_1$  mutant (m1) (Abs1234) are analyzed. Samples were taken just before induction of sporulation by resuspension (0 min; gray bars) and 1.5 h postinduction (90 min; black bars). Values are the averages of data from three independent experiments.

levels of  $\beta$ -galactosidase during sporulation, the enzyme level in cells containing a fusion with a mutant of  $O_1$  were maintained at a high level. The results are consistent with the idea that  $O_1$  contributes to the repression of  $P_v$  at the start of sporulation.

**Translational control of Spo0A synthesis.** Finally, we noticed that transcripts originating from  $P_v$  exhibited an extensive secondary structure, including in particular complementarity between the region containing the ribosome binding site and start codon for the *spo0A* open reading frame and the region downstream of the  $O_1$  sequence and including the  $O_2$  sequence

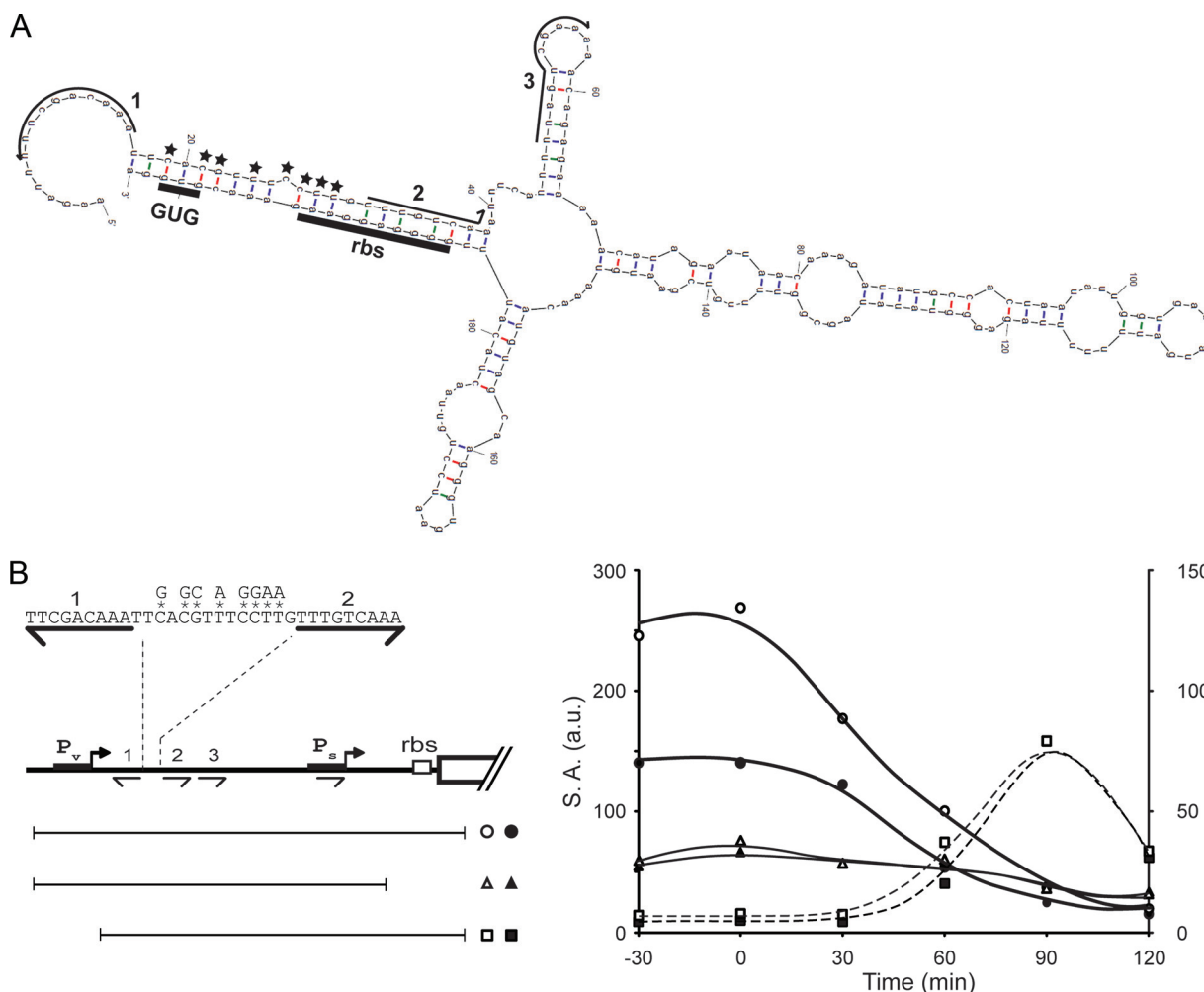


FIG. 7. RNA secondary structure impedes translation mRNAs originating from  $P_v$ . (A) Secondary structure prediction for the 5' region of transcripts originating from  $P_v$ . RNA sequences corresponding to  $O_1$ ,  $O_2$ , and  $O_3$  are indicated with arrows. The ribosome-binding site (RBS) and initiation codon (GUG) are underlined. Positions of nucleotide substitution mutations are indicated with stars. (B) Expression of *spo0A-lacZ* fusions integrated at *amyE*. A typical experiment is presented. The fusions were either translational, containing the full regulatory region (from -305 to +72) (circles) (Abs1019 and Abs1021) or lacking  $P_v$  (from -197 to +72) (squares) (Abs988 and Abs957) or were transcriptional, containing the full regulatory region (from -305 to -16) (triangles) (Abs1049 and Abs1050). The fusions either contained the wild-type sequence (black symbols) or were mutants, with the eight nucleotide substitutions indicated in the left panel. Expression of fusions containing the full regulatory region (circles and triangles) were determined in mutant cells lacking  $\sigma^H$  (*sigH*) to prevent expression from  $P_s$ . Dotted lines correspond to the right axis and continuous lines to the left axis.

(Fig. 7A). We postulated that this secondary structure would be responsible for reducing the translational efficiency of mRNAs originating from  $P_v$  but not mRNAs originating from  $P_s$ . To test this possibility, we mutated 8 bases in the sequence between sites 1 and 2 (Fig. 7B, left panel) so as to weaken its interaction with the ribosome binding site and the start codon GUG without perturbing either 0A box. As shown in Fig. 7B, the presence of the mutations (open symbols) significantly increased the expression level of a *lacZ* translational fusion (circles) but had little effect on a transcriptional fusion (triangles). (The above-described experiment was carried out in the presence of a *sigH* mutation to limit transcription to  $P_v$ .) As a control, little or no effect on expression levels was observed when the same mutations were introduced into a translational fusion that lacked  $P_v$  (squares). (In this case the cells were wild type for *sigH* so as to allow transcription from  $P_s$ .) We conclude

that the switch from  $P_v$  to  $P_s$  is accompanied by an increase in mRNA translation efficiency, thereby enhancing the rate of Spo0A synthesis at the start of sporulation. (Note that this also suggests that the high level of Spo0A observed in the  $\Delta O_{1-3}$  strain at the mid-exponential phase [Fig. 2A] reflects both derepression of  $P_s$  due to the absence of  $O_2$  and enhanced translation of transcripts originating from  $P_v$ , given that the translation-inhibiting sequences are absent in the deletion mutant.)

## DISCUSSION

Building on the early work of Chibazakura et al. (11) and Strauch et al. (29) and our recent findings on the accumulation of Spo0A (9, 15), we propose a pump-priming model for the regulation of *spo0A* that involves transcriptional, translational, and posttranslational mechanisms. At the heart of the model is



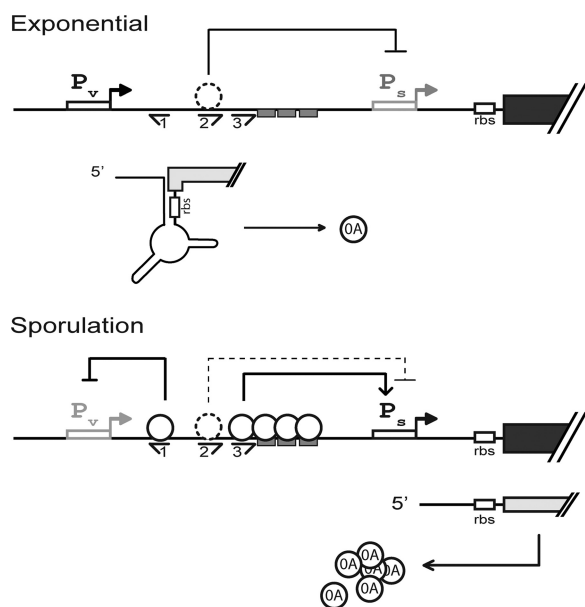


FIG. 8. Model for the control of *spo0A* expression. The upper panel shows that during the exponential phase of growth,  $O_2$  represses transcription from  $P_s$ . Thus, transcripts originating from  $P_v$  are the chief source of mRNA, which due to secondary structure is impaired in translation. The dotted circle conveys that the identity of the molecule bound at  $O_2$  has not been confirmed as being Spo0A~P biochemically. The lower panel shows that during the transition to stationary phase in sporulation-inducing medium,  $P_v$  is repressed by Spo0A~P bound at  $O_1$ , and Spo0A~P molecules bound at  $O_3$  and downstream activate  $P_s$ , overriding repression by  $O_2$ . Cooperative binding of Spo0A~P molecules renders the switch from  $P_v$  to  $P_s$  highly sensitive to small changes in Spo0A~P levels. The rate of Spo0A synthesis is enhanced both by the switch to the strong  $P_s$  promoter and the enhanced translatability of mRNA originating from  $P_s$ .

promoter switching from the vegetative promoter  $P_v$  to the  $\sigma^H$ -controlled, Spo0A~P-dependent promoter  $P_s$  during the transition to stationary phase and the 0A boxes  $O_1$ ,  $O_2$ , and  $O_3$ . We propose (Fig. 8) that early during the exponential phase of growth, synthesis of Spo0A is principally if not exclusively driven by  $P_v$ . This synthesis is maintained at a relatively low level by a translational control mechanism that sequesters the start codon and ribosome-binding site for *spo0A* in the secondary structure of transcripts originating from the upstream promoter. Meanwhile, the downstream promoter,  $P_s$ , is silent due to the absence of both Spo0A~P and  $\sigma^H$ . Then, at the mid-exponential phase of growth, the gene (*sigH*) for  $\sigma^H$  is derepressed (9) and Spo0A~P begins to accumulate due to activation of kinases at the head of the phosphorelay other than KinA (5, 9). Nonetheless, Spo0A synthesis continues to be maintained at a basal (although significant) level due to repression of  $P_s$  via  $O_2$ . Finally, during the transition to stationary phase, increasing levels of KinA lead to a surge in Spo0A~P levels (13), overpowering  $O_2$ -mediated repression of  $P_s$  and activating  $P_s$  via the binding of Spo0A~P to  $O_3$ . Meanwhile, the binding of Spo0A~P to  $O_1$  represses  $P_v$ , effecting the switch from the vegetative to the sporulation promoter. The switch results in yet higher rates of Spo0A synthesis as a consequence of unimpeded translation from mRNAs originating from the downstream promoter. Thus,  $P_v$  primes the

pump by maintaining a pool of Spo0A molecules in growing cells, which upon phosphorylation activates  $P_s$ , leading to yet higher levels of Spo0A and downregulation of  $P_v$ .

Our model raises several questions about the mechanism of action of the upstream 0A boxes as we now consider the following.

**$O_1$ .** Because  $O_1$  is close to and downstream of the start site for  $P_v$ , the binding of Spo0A~P to  $O_1$  likely represses  $P_v$  simply by interfering with the binding of RNA polymerase to the promoter.

**$O_2$ .** We presume that  $O_2$ , which is a perfect match to an 0A box, functions via the binding of Spo0A~P, but neither we (see Fig. S3 in the supplemental material) nor Strauch and collaborators observed protection of  $O_2$  in DNase I footprinting experiments (29). Assuming that Spo0A~P does bind to  $O_2$  *in vivo* despite the failure to detect such an interaction *in vitro*, an appealing possibility would be that Spo0A~P bound at  $O_2$  forms a loop with an unidentified downstream sequence that occludes the binding of RNA polymerase to  $P_s$ . An alternative possibility is that a regulatory protein other than Spo0A~P that functions during the transition to stationary phase binds to  $O_2$ . If so, evidence indicates that the protein is not SinR, CodY, or Hpr (ScoC) (data not shown).

**$O_3$ .**  $O_3$  is a positively acting 0A box that governs the Spo0A~P-dependent activation of  $P_s$ . Binding of Spo0A~P to  $O_3$  has been confirmed biochemically. We presume that  $O_3$  works via the direct interaction of Spo0A~P bound at this site with  $\sigma^H$ -RNA polymerase, facilitating the binding of the transcription enzyme to  $P_s$ . Evidence presented in Results is consistent with the idea that this interaction occurs via looping of the intervening sequence. We further suggest that loop formation is facilitated by the observed binding and oligomerization of Spo0A~P toward  $P_s$ . Zhao et al. (32) propose that dimers of Spo0A~P oligomerize in a head-to-tail manner. In this oligomeric state, only one Spo0A~P molecule in each dimer can interact with the Spo0A-binding sequence (chiefly with Gs), the other molecule being tilted and making interactions with the phosphate backbone (32). Thus, it is tempting to speculate that in curved DNA both Spo0A~P molecules of a dimer contact bases, possibly Gs, thereby stabilizing or changing the degree of curvature. In support of this idea, the AT richness of the intervening DNA would be expected to impart flexibility and/or natural bending to the intervening DNA, and the G in the repeated, 10-bp motifs (AAAWNNDAGA) downstream of  $O_3$  (see Results) could be a contact site for Spo0A~P molecules. If so, cooperative binding of multiple Spo0A~P molecules to the intervening DNA could render activation of  $P_s$  sensitive to small changes in Spo0A~P levels, creating a high-sensitivity switch and counteracting the repressive effect of  $O_2$ .

**$O_4$ .** The promoter-proximal, Spo0A~P binding site  $O_4$  evidently does not play an important role in promoter switching given the demonstration that we could functionally replace  $P_s$  with another  $\sigma^H$ -controlled promoter ( $P_{spoVG}$ ) that is Spo0A~P independent without apparently impeding the function of  $O_2$  or  $O_3$ . Thus, in our view  $P_s$  is intrinsically simply a  $\sigma^H$ -controlled promoter, and the function of an embedded 0A box in  $P_s$  remains mysterious.

What is the biological significance of such a complicated and intricate regulatory region? We suggest that the regulatory region allows the cell to produce extremely large amounts of

Spo0A (rising from ~2,000 to ~20,000 molecules per cell) on demand as the phosphorelay is activated such that the Spo0A protein never becomes rate limiting for the generation of Spo0A~P. In this just-in-time scenario,  $P_v$  generates a basal level of Spo0A (~2,000 molecules/cell) that can prime the pump for rapidly producing Spo0A when the phosphorelay is activated. Thus, in its absence, Spo0A accumulation is greatly delayed and sporulation efficiency is slightly impaired (see Fig. S1 and S5 in the supplemental material) (12). We have also discovered that entry into the state of genetic competence is impaired in a mutant lacking  $P_v$  (see Fig. S5) (but not in a mutant lacking  $P_s$  [27]). It was known that genetic competence requires Spo0A, and our present results indicate that  $P_v$  is responsible for providing enough Spo0A molecules for entry into this state. This is in agreement with the view that Spo0A from  $P_v$ -directed transcription is limiting for development of competence (22). Nonetheless, we are left with the puzzling finding that  $P_v$  is dispensable when  $O_1$ ,  $O_2$ , and  $O_3$  are removed. That is, the pattern of Spo0A accumulation when both  $P_v$  and  $O_1$ - $O_3$  were removed was similar to that seen when the regulatory region was left intact (Fig. 2A). Thus, a truncated regulatory region, simply representing a  $\sigma^H$ -controlled promoter ( $P_s$ ), was sufficient to mimic, at least at a coarse level, the post-exponential-phase induction of Spo0A synthesis seen in the wild type. We presume that  $P_s$  alone is not as tightly regulated as the entire intact regulatory region in a manner that confers a fitness advantage to the cell. For example, the intact regulatory region may be needed to suppress noise and minimize cell-to-cell variation in Spo0A levels during growth. If so, additional detailed experiments will be required to uncover differences between the behavior of  $P_s$  and the intact regulatory region.

In summary, we propose that  $P_v$  and  $O_2$  maintain Spo0A at a high, basal level (~2,000 molecules/cell) during growth. As a result, the cells are poised to respond rapidly to signals triggering activation of the phosphorelay. Phosphorylation of Spo0A as a result of flux through the relay sets up a self-reinforcing cycle that rapidly amplifies Spo0A production to extremely high levels (~20,000 molecules/cell), preventing Spo0A from becoming limiting for the accumulation of Spo0A~P. Whereas the basal level of Spo0A may be relatively constant from cell to cell (as we propose), Spo0A~P levels vary considerably from cell to cell at the start of sporulation (9). This heterogeneity likely originates from noise in the phosphorelay and is the basis for the diversification of cell types during the transition to stationary phase, resulting in cannibals, biofilm formers, and spore formers (17, 21).

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