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Just-in-Time Control of Spo0A Synthesis in *Bacillus subtilis* by Multiple Regulatory Mechanisms▽§

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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, σ^A-recognized promoter, P_v, and a sporulation σ^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_1, O_2, O_3, and O_4 in reverse order of their proximity to the coding sequence. Here we report that O_1 is responsible for repressing P_v during the transition to stationary phase, that O_2 is responsible for repressing P_s during growth, that O_3 is responsible for activating P_s at the start of sporulation, and that O_4 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_2 and O_3 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 σ^A, as well as genes whose promoters are recognized by RNA polymerase containing the alternative sigma factor σ^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*, *spoIIE*, 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 σ^A RNA polymerase (P_{spoIIG} and P_{skf}) (10, 26) and one (P_{spo0F}) for promoters recognized by σ^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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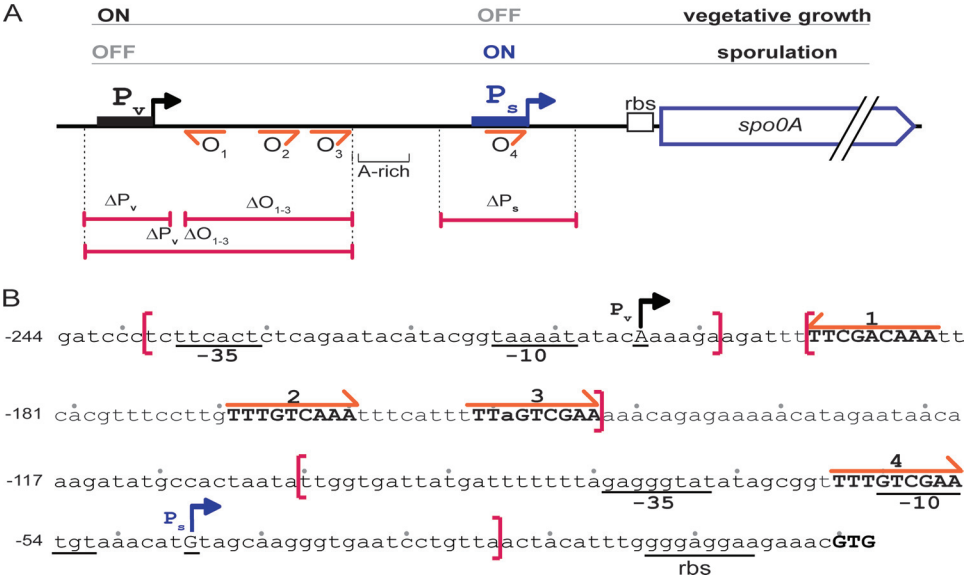

FIG. 1. The *spo0A* regulatory region. (A) Cartoon of the regulatory region. P_v and P_s are indicated by black and blue boxes with arrows, respectively. Sequences matching the Spo0A consensus binding site (0A box), which are designated operator sites O_1, O_2, O_3, and O_4, are labeled with orange arrows 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 ΔP_v (Abs894), $\Delta O_{1–3}$ (Abs883), $\Delta P_v\ \Delta O_{1–3}$ (Abs875), or ΔP_s (Abs786).

(Fig. 1A) (12, 29)). The more upstream promoter, P_v, is expressed during the exponential phase of growth under the control of σ^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 σ^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 *spoIIA*, *spoIIE*, and *spoIIG*, 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, β-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* (ΔP_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 GCGGTTTTGTCGAATGTA was replaced by AGCAGGATTTCAGAAAAA ATCGTGGAATTGA (see Fig. S4 in the supplemental material).

Primer extension. Primer extension was performed mainly as previously described (8), with the exception that 2 μg 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 σ^A-containing RNA polymerase and from a downstream sporulation promoter, P_s, by RNA polymerase containing the alternative sigma factor σ^H (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 (ΔP_v), P_s (ΔP_s) (27), O_1 to O_3 (ΔO_{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

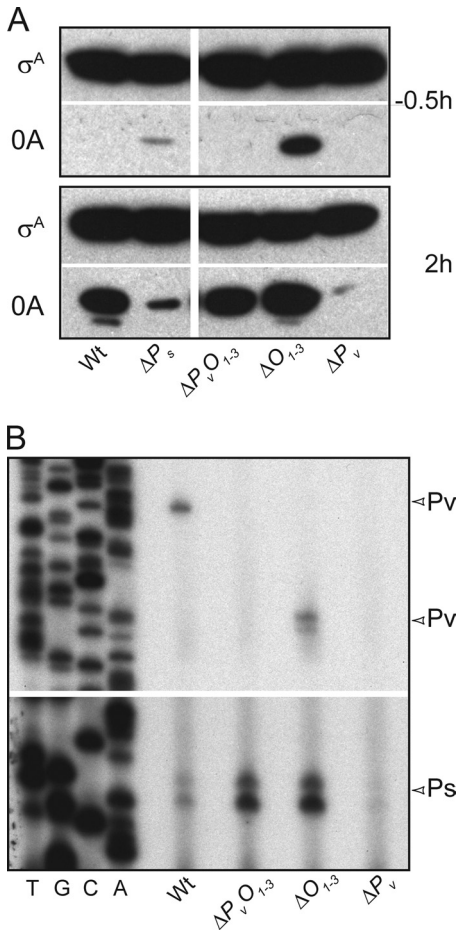

FIG. 2. O_1-O_3 contains a regulatory element controlling P_s. (A) Immunoblot analysis of Spo0A (0A) accumulation during sporulation. Blotting was performed using samples taken at 0.5 h before sporulation induction by resuspension (upper panel) and 2 h postinduction (bottom panel). Equal amounts of protein, as quantified by the Bradford technique, were loaded as confirmed by the control immunoblot using anti-SigA (σ^A) antibodies. Samples were taken from shaking culture at 37°C of wild-type cells (Wt; PY79) or P_s (ΔP_s; Abs786), O_{1-3} (ΔO_{1-3}; Abs883), P_v (ΔP_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 ΔO_{1-3} deletion.

O_1-O_3 (ΔO_{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 (ΔP_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

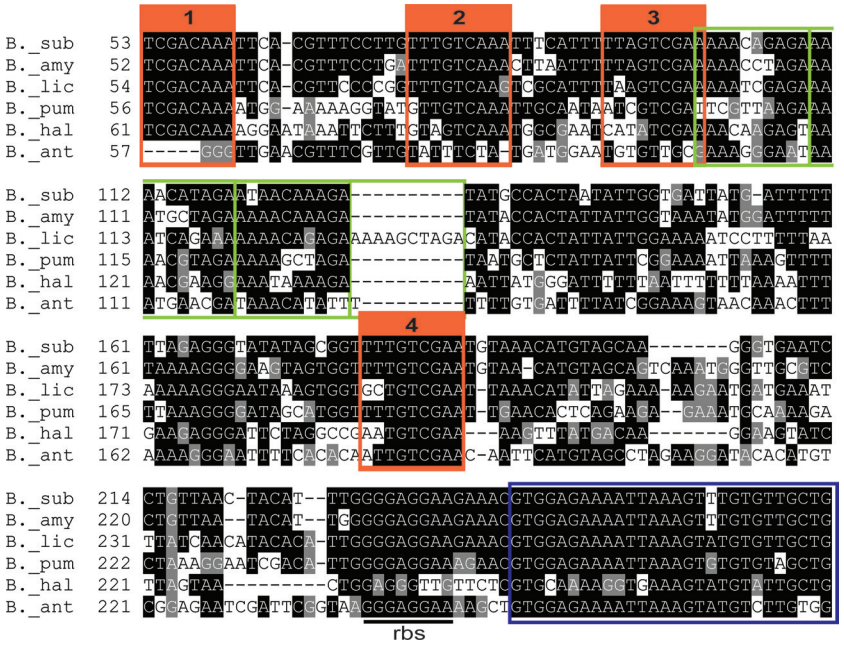

FIG. 3. The regulatory region of *spo0A* is conserved among *Bacillus* spp. Shown is a sequence alignment performed using *spo0A* promoter sequences from *Bacillus subtilis* (B._sub), *Bacillus amyloliquefaciens* (B._amy), *Bacillus licheniformis* (B._lic), *Bacillus pumilis* (B._pum), *Bacillus halodurans* (B._hal), and *Bacillus anthracis* (B._ant). Shown are alignments extending from the first 0A box to the 9th codon of the open reading frame. O_1 to O_4 are boxed in orange. The beginning of the open reading frames is boxed in blue. The conserved repeated motif AAAWNDAGA is highlighted in green.

which we will return.) However, little or no effect was seen on transcription from P_v by the simple removal of the O_1-O_3 sequence (ΔO_{1-3}). (Note the shorter length of the extension products due to the absence of the downstream O_1-O_3 sequence.) In sharp contrast, removal of O_1-O_3 markedly increased the level of transcription originating from the sporulation promoter P_s, both in the presence and the absence of P_v. We conclude that the accumulation of Spo0A to high levels in the absence of O_1-O_3 was due to enhanced transcription from P_s.

The results so far are consistent with a pump-priming model in which P_v allows enough Spo0A molecules to be produced to activate the Spo0A-dependent P_s promoter. Transcription from P_s, in turn, leads to the production of additional Spo0A molecules, thereby setting up a self-reinforcing cycle. Thus, in the absence of P_v, insufficient Spo0A is produced to activate P_s, as we had seen in Fig. 2B. We further surmise that the O_1-O_3 region contains a regulatory element that impedes transcription from P_s during the exponential phase of growth. In our model, removal of O_1-O_3 elevates transcription from P_s during growth, allowing enough Spo0A to accumulate to prime the pump even when P_v is absent.

The regulatory region for *spo0A* is conserved. As noted above, the regulatory region for *spo0A* contains four 0A boxes, three of which have been described previously and a fourth, the newly identified O_2 site, being a perfect match to the consensus sequence TTTGTCRAA (23). We wondered whether all four 0A boxes were conserved among *Bacillus* spp. Accordingly, we performed an alignment of the *spo0A* promoter region among six *Bacillus* species (Fig. 3). Strikingly, not only were all four 0A boxes conserved, but the entire regulatory region exhibited features that were conserved among all of the species examined with the exception of *Bacillus anthracis*. One such feature was the presence of an AT-rich region (80% in *B. subtilis*) located just downstream of O_3. AT richness is known to enhance DNA flexibility and to cause intrinsic DNA bending, which can facilitate promoter activation (19, 24, 30), a point we consider further below. Another conserved feature is the 10-bp motif AAAWNNDAGA, which was directly repeated three times in several of the promoter regions and four times in that of *Bacillus licheniformis*.

O_2 and O_3 have opposing effects on P_s. To further investigate the influence of the O_1-O_3 region on P_s, we created an in-frame fusion of *lacZ* to *spo0A* that extended from 23 codons downstream of the start codon to the 5′ end of the O_1-O_3 region (and hence lacked P_v). This fusion and mutant derivatives of it were introduced into the chromosome at *amyE* in cells that had a wild-type copy of *spo0A* at its normal location. (Thus, in contrast to the experiment of Fig. 2, the results with the gene fusions were carried out under conditions in which Spo0A was being produced from the wild-type copy of *spo0A*.) The results in Fig. 4A show that the full-length fusion (extending upstream through the O_1-O_3 region) was silent at $T_{-0.5}$ and sharply induced between $T_{0.5}$ and $T_{1.5}$. As expected, induction was blocked in a mutant lacking the phosphorelay protein Spo0B (□), consistent with the idea that P_s is strongly dependent on Spo0A~P.

We then investigated the contribution of the O_1-O_3 region to this pattern of expression using a truncation mutant (Fig. 4A). The results show that the pattern of expression was markedly altered, with P_s exhibiting a high basal level of activity during growth and impaired induction during sporulation. A

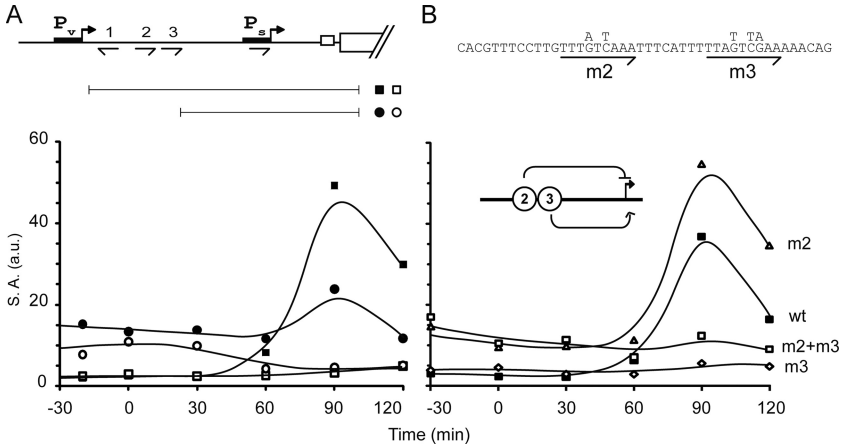

FIG. 4. O_2 and O_3 have opposing effects on P_s expression. (A) Deletion of O_1-O_3 increased expression of a *spo0A-lacZ* translational fusion during exponential growth phase and reduced its expression during sporulation. The fusions were integrated at *amyE*. *spo0A* sequences in the fusions extended 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_2 mutant (m2) (△; Abs1055), mutant for O_3 (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_1-O_3 region and the downstream AT-rich region (ΔO_{1-3} ΔAT-rich) (see Fig. S2 in the supplemental material). Thus, consistent with the results obtained by primer extension analysis, the O_1-O_3 region contains a regulatory element that inhibits P_s activity during growth.

Next, we investigated the contributions of O_1, O_2, and O_3 individually to the activity of P_s by separately mutating each 0A 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_2 (which has an A at position 7) and at 4, 6, and 7 in O_3 (see Materials and Methods). Mutating O_1 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_2 was sufficient to allow premature expression from P_s. In other words, mutating O_2 alone was sufficient to mimic the effect of deleting the entire O_1-O_3 region in derepressing P_s during growth. On the other hand, and unlike the deletion of O_1-O_3 region, mutating O_2 mildly but reproducibly enhanced the post-exponential-phase induction of transcription from P_s.

Conversely, mutation of O_3 almost completely abolished the expression of the reporter, an unexpected result considering that deletion of the O_1-O_3 region was not blocked in P_s activity. To investigate this observation further, we built a double mutant in which both O_2 and O_3 were mutated. The pattern of expression from the double mutant resembled that of the O_1-O_3 deletion mutant.

We interpret these results as follows. O_2 is a negative element that inhibits P_s, whereas O_3 is a positively acting element that is required for activation of P_s during sporulation. When both O_2 and O_3 are mutated, a high level of transcription from P_s is seen during growth and sporulation, while induction of P_s during sporulation is prevented.

Evidence for DNA looping. The large gap between O_2 and O_3 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_s. Two models, which are not mutually exclusive, are that the intervening sequence forms a loop and/or that Spo0A bound at O_2 or O_3 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_2 and O_3. The simplest interpretation of these results is that Spo0A~P bound at O_2 or O_3 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_3 (see Fig. S3 in the supplemental material), a point we will discuss further.

O_4 is dispensable. Next, we investigated the function of the fourth 0A box, O_4, which is embedded in P_s. Because O_4 (TTTGTCGAA) overlaps the −10 sequence of P_s (**GTCGAA** TGT, in which the bold letters indicate bases shared with O_4), 0A box positions 4 and 7 are the R and G at positions 1 and 4 of the −10 sequence, which are conserved in σ^H-recognized

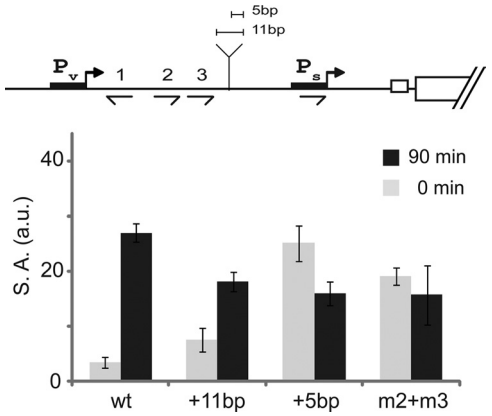

FIG. 5. Efficient regulation by O_2 and O_3 depends on their position relative to the face of the helix. Shown is expression from *spo0A-lacZ* translational fusions integrated at *amyE*. Bars represent the average values of three independent experiments. Sequences in the fusions extended from position −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 (**R**nn**G**AATww) (3). Consequently, it was not possible to replace the conserved bases at positions 4, 6, and 7 of the 0A box without mutating the σ^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 σ^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

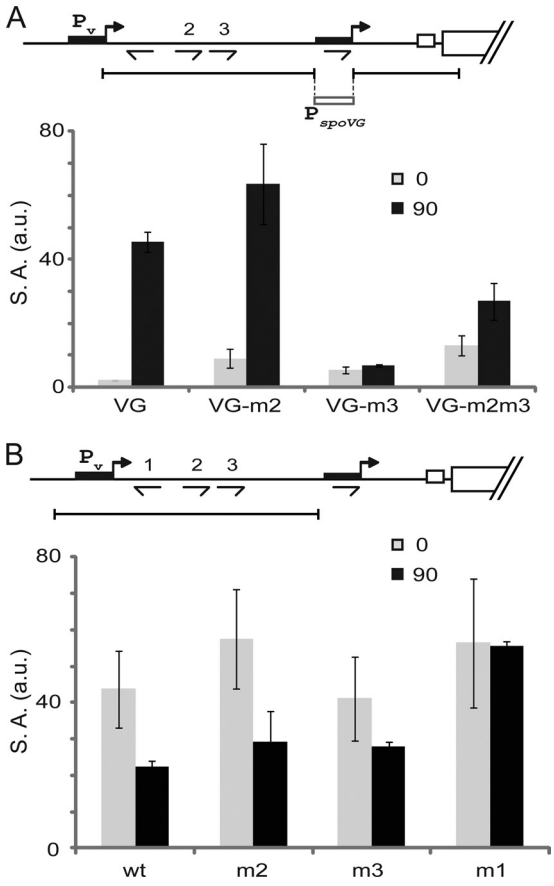

FIG. 6. Functional replacement of P_s with a σ^H-controlled promoter lacking an 0A box and repression of P_v by O_1. (A) Expression from *spo0A-lacZ* translational fusions integrated at *amyE*. The fusions contained sequences extending from position −197 to +72 relative to the initiating codon (and hence 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 β-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

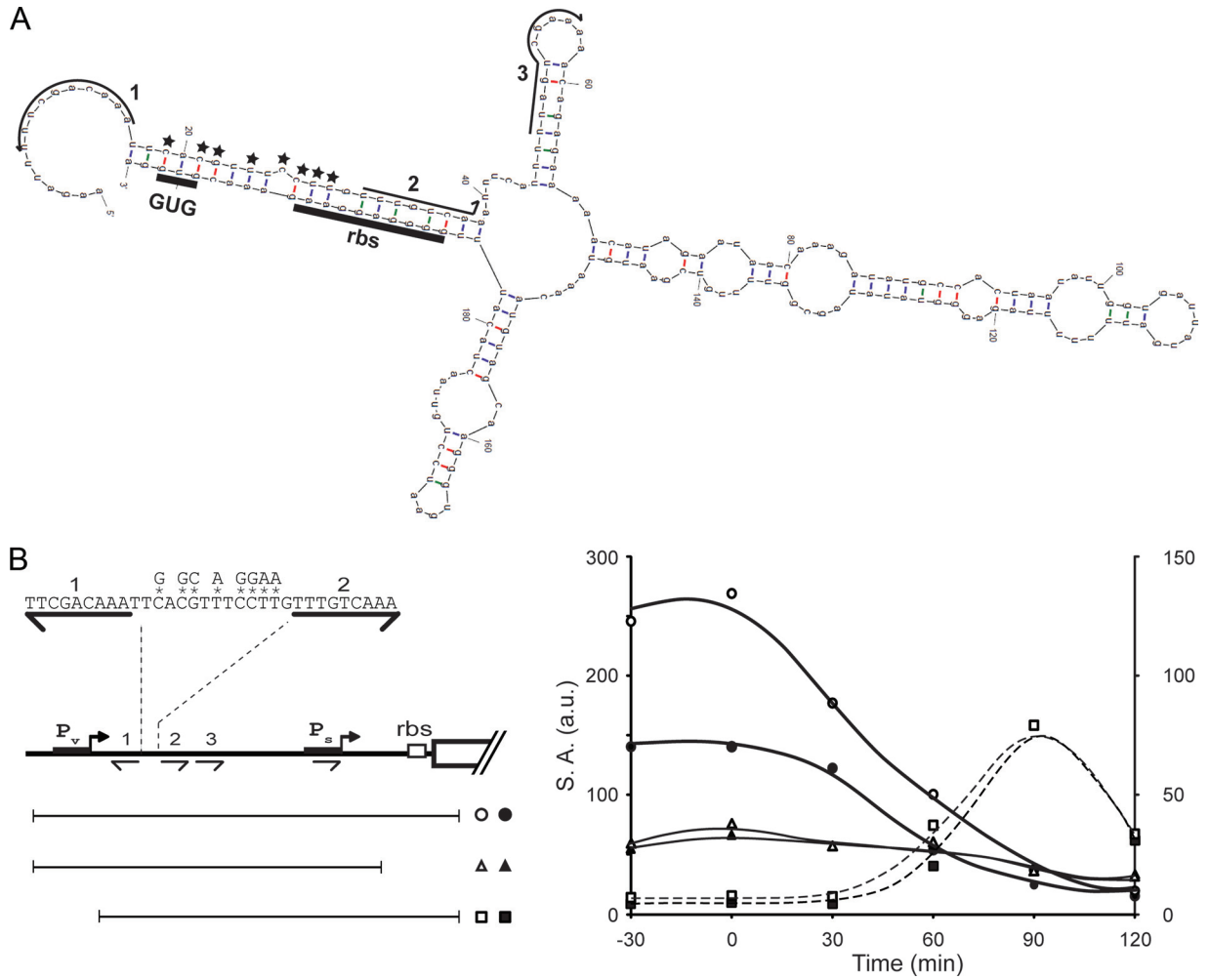

FIG. 7. RNA secondary structure impedes translation mRNAs originating from P_v. (A) Secondary structure prediction for the 5′ region of transcripts originating from P_v. RNA sequences corresponding to O_1, O_2, and O_3 are indicated with arrows. The ribosome-binding site (RBS) and initiation codon (GUG) 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 σ^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 ΔO_{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

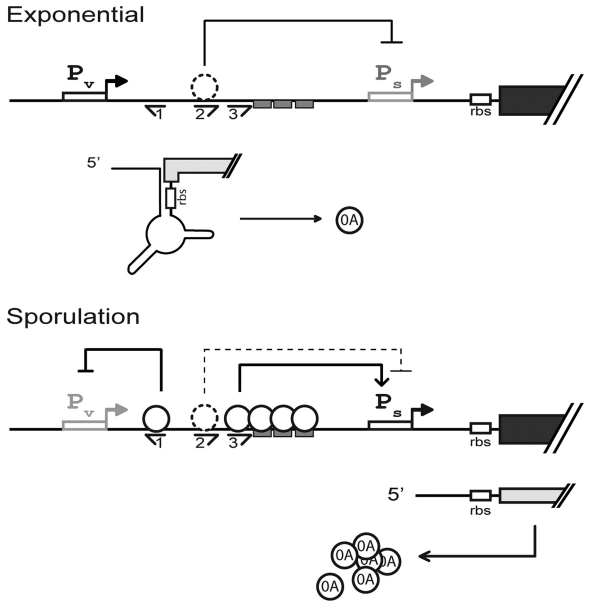

FIG. 8. Model for the control of *spo0A* expression. The upper panel shows that during the exponential phase of growth, O_2 represses transcription from P_s. Thus, transcripts originating from P_v are the chief source of mRNA, which due to secondary structure is impaired in translation. The dotted circle 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 σ^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 σ^H. Then, at the mid-exponential phase of growth, the gene (*sigH*) for σ^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 σ^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 σ^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 σ^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 σ^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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