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Postexponential Regulation of *sin* Operon Expression in *Bacillus subtilis*

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The expression of many gene products required during the early stages of *Bacillus subtilis* sporulation is regulated by *sinIR* operon proteins. Transcription of *sinIR* from the P1 promoter is induced at the end of exponential growth. In vivo transcription studies suggest that P1 induction is repressed by the transition-state regulatory protein Hpr and is induced by the phosphorylated form of Spo0A. In vitro DNase I footprinting studies confirmed that Hpr, AbrB, and Spo0A are *trans*-acting transcriptional factors that bind to the P1 promoter region of *sinIR*. We have also determined that the P1 promoter is transcribed in vitro by the major vegetative sigma factor, σ^A, form of RNA polymerase.

Natural environments are oligotrophic (35). Organisms such as the common soil bacterium *Bacillus subtilis* frequently exist in slow- or nongrowing physiological states. The rich diversity of *B. subtilis* transition-state regulatory systems (50, 55, 56) confirms the biological importance of managing the transition from rapid- to slow- to nongrowing cell states. Depending on the environmental cues present, *B. subtilis* transition-state regulation can channel a cell toward motility, nutrient scavenging through the production of extracellular enzymes, competence, or sporulation cell fates (for a review, see references 12 and 53). The best-characterized *B. subtilis* transition-state regulators are the AbrB, Hpr, Spo0A, and SinR DNA-binding proteins.

Recent structural studies have shown that the AbrB protein is a tetramer of 10,500-Da subunits that interacts with a variety of specific nucleotide sequences, presumably by recognizing a particular three-dimensional DNA architecture (54, 59, 62). AbrB can function as a repressor of genes such as *spo0E*, *spo0H*, *spoVG*, and *aprE* (14, 34, 43, 64) and as an activator of genes such as *hpr* and the *rbs* operon (52, 53). Transcription of *abrB* is controlled by negative autoregulation and repression by Spo0A (53, 55). The *hpr* gene product is a 23,718-Da protein, which was originally identified as a locus (*hpr*, *scoC*, and *catA*) for mutations causing protease overproduction and catabolite-resistant sporulation (10, 21, 39). Hpr binds to a consensus DNA sequence RATAnTATY (25, 53). Hpr represses the expression of the protease genes *aprE* and *nprE* and oligopeptide permease operons (20, 26) and when present on a multicopy plasmid can inhibit sporulation in an as-yet-undetermined manner (39). The Spo0A 29,691-Da protein is the master controller of early developmental events (55, 56). Metabolic and environmental signals cause the autophosphorylation of sensor kinases such as KinA, KinB, and KinC (1, 24, 27, 41), which transfer phosphate groups through a phosphorelay (Spo0F and Spo0B) to generate Spo0A~P (24, 55, 57). Spo0A~P recognizes a 0A box DNA sequence, TGNCGAA (51). Spo0A~P is a repressor of *abrB* transcription and an activator of *spoIIA*, *spoIIG*, and *spoIIE* operon expression (4, 45, 51, 57, 63). *spo0A* expression is controlled by σ^A and σ^H promoters (9, 44, 49). Vegetative *spo0A* expression originates from the σ^A promoter, and catabolite-regulated postexponential expression is controlled from the σ^H promoter.

The dicistronic *sin* operon was originally identified as a clone which could inhibit sporulation and protease production when present on a high-copy-number plasmid (16, 17). The first gene in the operon encodes a 57-amino-acid protein, SinI, that posttranslationally antagonizes the activity of SinR, the product of the second gene in the operon (3). SinR is a 111-amino-acid protein, which is a repressor of *aprE*, *amyE*, *sacB*, *spo0A*, *spoIIA*, *spoIIG*, and *spoIIE* (17, 18, 31, 32, 33, 38, 50) and binds to a DNA sequence whose consensus appears to be GNCNC GAAATACA. The crystal structure of SinR has revealed that the DNA-binding domain is similar to that of the bacteriophage 434 repressor proteins, C_I and Cro (30). The SinR tetramer represses transcription of the *spoIIG* promoter by inducing DNA conformational changes, preventing activation of transcription by Spo0A~P (7). SinR is an activator of competence, motility, and autolysin production (15, 46). The *sin* operon is expressed from three differentially regulated promoters (17); promoters P1 and P2 precede *sinI* and produce RNAs which span the operon and terminate at two rho-independent terminators; the P3 promoter abuts the *sinR* gene and produces a transcript which starts 15 nucleotides (nt) upstream of the first *sinR* codon and terminates at the rho-independent termination sites. P1 expression is downregulated in vegetative growth and increases dramatically at the onset of stationary phase (17). P3-derived RNAs are expressed in vegetative growth and during the first 2 h of stationary phase. Synthesis of *sin* operon mRNA originating from P2 commences 2 h after

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TABLE 1. Bacterial strains

Strain	Description (relevant genotype)[a]	Source or reference[b]
1A180	*hpr16*	BGSC
RS1000	*168*	This study
RS1001	*metC2 lys-1*	This study
RS1004	*spo0A12*	This study
EE1000	*spo0E11*	This study
EE1001	*spo0F221*	This study
EE1002	*spo0H116*	This study
EE1003	*spo0K141*	This study
EE1004	*kinA*::Tn*917*	This study
EE1005	*abrB*::Tn*917*	This study
SWV119	*abrB*::*Tet trpC2 phe-1*	This study
SWV185	*abrB*::*Tet trpC2 phe-1 spo0E*::*lacZ*	This study
RS5101	*rvtA11*	This study
SS11	*metC2 lys-1 sinI*::*lacZ*	This study
SS12	*metC2 lys-1 sinR*::*lacZ*	This study
SS13	*sinI*::*lacZ*	This study
SS14	*sinR*::*lacZ*	This study
SS15	*spo0A12 sinI*::*lacZ*	SS13→RS1004 (Cm^r selection)
SS16	*spo0A12 sinR*::*lacZ*	SS14→RS1004 (Cm^r selection)
SS17	*spo0E11 sinI*::*lacZ*	SS13→EE1000 (Cm^r selection)
SS18	*spo0E11 sinR*::*lacZ*	SS14→EE1000 (Cm^r selection)
SS19	*spo0F221 sinI*::*lacZ*	SS13→EE1001 (Cm^r selection)
SS20	*spo0F221 sinR*::*lacZ*	SS14→EE1001 (Cm^r selection)
SS21	*spo0H116 sinI*::*lacZ*	SS13→EE1002 (Cm^r selection)
SS22	*spo0H116 sinR*::*lacZ*	SS14→SS1750 (Cm^r selection)
SS23	*spo0K141 sinI*::*lacZ*	SS13→EE1003 (Cm^r selection)
SS24	*spo0K141 sinR*::*lacZ*	SS14→EE1003 (Cm^r selection)
SS25	*kinA*::Tn*917 sinI*::*lacZ*	SS13→EE1004 (Cm^r selection)
SS26	*kinA*::Tn*917 sinR*::*lacZ*	SS14→EE1003 (Cm^r selection)
SS27	*rvtA11 sinI*::*lacZ*	SS13→RS5101 (Cm^r selection)
SS28	*rvtA11 sinR*::*lacZ*	SS14→RS5101 (Cm^r selection)
SS29	*hpr16 sinI*::*lacZ*	SS13→1A180 (Cm^r selection)
SS30	*hpr16 sinR*::*lacZ*	SS14→1A180 (Cm^r selection)
SS33	*abrB*::Tn*917 sinI*::*lacZ*	SS13→EE1005 (Cm^r selection)
SS34	*abrB*::Tn*917 sinR*::*lacZ*	SS14→EE1005 (Cm^r selection)
SS35	*abrB*::*Tet sinI*::*lacZ*	SS13→SS43 (Cm^r selection)
SS37	*abrB*::Tn*917 rvtA11 sinI*::*lacZ*	SS43→SS27 (Tet^r selection)
SS38	*abrB*::Tn*917 spo0A12 sinI*::*lacZ*	EE1005→SS15 (MLS^r selection)
SS40	*spo0F221 rvtA11 sinI*::*lacZ*	SS27→EE1001 (Cm^r selection, Tet^r screening)
SS43	*abrB*::*Tet*	SWV119→RS1000 (Tet^r selection)
SS44	*abrB*::*Tet spo0E*::*lacZ*	SWV185→RS1000 (Cm^r selection and congression)
SS46	*hpr*Ω*pMTL20EC*	Linearized pSS60→RS1000 (Em^r selection)
SS62	*hpr*Ω*pMTL20EC sinI*::*lacZ*	SS46→SS13 (Em^r selection)
SS63	*hpr*::*pMTL20EC spo0A12 sinI*::*lacZ*	Linearized pSS60→SS30 (Em^r selection)
SS955	*spo0A*::*Em sinI*::*lacZ*	Linearized pSS950→SS13 (Em^r selection)
SS1750	*spo0H*::*Em sinI*::*lacZ*	Linearized pSS1750→SS13 (Em^r selection)

[a] For clarity, the auxotrophic genotypes have been omitted.
[b] BGSC, *Bacillus* Genetics Stock Center. Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.

entry into stationary phase. *sinR* gene disruptions suppress the effects of sporulation defects caused by null mutations in *kinA* and missense mutations in *ftsA* and *spo0K* (31). These effects are consistent with findings (33) that *sinR* deletions relieve repression of *spo0A* and would be expected to increase the concentration of Spo0A~P, thus bypassing the effects of *kinA* and *spo0K* mutations. *sinR* deletions also cause derepression of the *sinIR* operon (16, 32), suggesting that SinR may autogenously regulate *sinIR* expression.

In the studies described here, we have investigated the interplay of these genetic factors in controlling the activity of the *sinIR* operon. We describe in vitro transcription studies suggesting that the *sinIR* P1 and P3 promoters are transcribed by σ^A RNA polymerase. To elucidate the genetic factors that regulate expression from these promoters, we have examined the role of transition-state and *spo* genes in governing the in vivo expression of *sinIR*. These data establish that *sin* operon expression is regulated by the phosphorelay and Hpr.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used, their genotypes, and their sources are listed in Table 1. Plasmids containing the *sinIR* operon have been described (16, 17).

β-Galactosidase synthesis with *B. subtilis lacZ* fusion strains. The following *lacZ* fusions were used in these studies: *sinI*::*lacZ* and *sinR*::*lacZ* (17) are translational fusions and were introduced into the *amyE* locus as described earlier (31). The β-galactosidase expression from *lacZ* fusions was determined as described previously (11). Specific activity is expressed as nanomoles of *o*-nitrophenyl hydrolyzed per milligram of cellular protein per minute.

Cell growth, induction of sporulation, and sporulation quantitation. Cell growth, induction of sporulation in Schaeffer's medium, and sporulation quantitation were performed as described elsewhere (48).

FIG. 1. Mapping of in vivo and in vitro transcription start sites for *sinIR* promoters. Start sites were determined by reverse transcriptase extension of ^{32}P-end-labeled primers by using in vivo RNA isolated from IS75 (16), grown in NSM, and harvested at T_0 and in vitro RNA isolated from transcription reaction mixtures. *B. subtilis* RNA polymerase holoenzyme containing σ^A was allowed to transcribe pSM109 (16), a plasmid containing *sinIR* P1 and P3, with nonradioactive ribonucleoside triphosphates, and RNA was isolated from the reaction mixtures. After radioactive primers were annealed to the RNAs and after extension with reverse transcriptase, the samples were separated by electrophoresis on 6% polyacrylamide gels, followed by autoradiography. Dideoxy sequencing gel lanes 1 and 2 show the primer extension products obtained from in vitro RNA (lane 1) and in vivo RNA (lane 2) using primer OSIN-1. The dideoxy sequencing ladder adjacent to lane 2 was obtained with the same primer and pSM179. The nucleotide sequence with the arrow pointing to the derived transcriptional start sites, also indicated by asterisks, is presented in the adjacent lanes. Lanes 3 (in vitro RNA) and 4 (in vivo RNA) show the equivalent primer extension products with primer P3-1, which corresponds to a region ca. 60 nt downstream from P3. Adjacent to lane 4 is the sequencing ladder obtained from pSM109 (16) with primer P3-1. The DNA sequence and derived transcription start sites are also presented.

DNase I footprinting analysis of the *sinIR* 5′ regulatory region. DNase I footprinting experiments were performed utilizing a fragment containing the first 395 bp (*Xba*I-*Aha*III) of the *sinIR* operon (16, 17). The transcribed strand was labeled at its 3′ end by using the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) and [α-^{32}P]dATP (Amersham). Protein binding and footprinting reactions were performed as described elsewhere (25, 51, 54). The labeled fragment was also subjected to Maxam-Gilbert A+G and C+T sequencing reactions (36) to generate a reference ladder. AbrB was purified as described previously (54). Spo0A was a gift from Jim Hoch. Each protein was purified separately from different *Escherichia coli* strains harboring expression vectors as described previously (25). Each protein preparation was >95% homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Spo0A was not phosphorylated.

***sinIR* in vitro transcription analysis.** To map the in vivo and in vitro transcriptional start sites of the *sinIR* operon P1 and P3 promoters, primer extension with 5′ ^{32}P-end-labeled primers and reverse transcriptase was performed as described previously, as was the purification of RNA polymerase (44). For in vivo primer extension reactions, 60 μg of RNA was harvested from cells grown in nutrient sporulation medium (NSM) at the T_0 stage of growth. The in vitro start sites were determined with RNA prepared from four-times-normal-size transcription reaction mixtures. The sequence of the primer used for the P1 promoter mapping, OSIN-1 (5′-CAG CCA GTC CGG CCA TGA C-3′), corresponds to nucleotides −37 to −56 with respect to the start of the *sinI* coding sequence. The equivalent primer for P3 mapping, P3-1 (5′-CAG CTA GTT CTG ATA GTG AGT-3′), corresponds to nucleotide positions +270 to +249, also with respect to the start of the *sinI* coding sequence. The plasmid used for the in vitro primer extensions and for the dideoxy DNA sequencing reactions was constructed by subcloning the *Xba*I-*Nru*I fragment, containing P1 and P3 from pIS74 (16), into a *lacZ* shuttle plasmid. This insert contains the *Msc*I-*Nru*I fragment that was removed from pIS109.

Plasmids. (i) pIS109. Plasmid pIS109 was constructed from excision of the *Xba*I-*Hin*DIII fragment from pIS90 (16, 17) and ligation into *Xba*I-*Hin*DIII-restricted pUC19. In this construct, a 240-bp internal *Msc*I (*Bal*I)-*Nru*I fragment was deleted. This plasmid contains the *sinIR* operon P1, P2, and P3 promoters and the T2 and T3 terminators 5′ to the *Hin*DIII site. Predicted transcript sizes are as follows: P1, 455 nt when terminated at T2 and 497 nt when terminated at T3; and P3, 142 nt when terminated at T2 and 184 nt when terminated at T3.

(ii) pSS60. Plasmid pSS60 carries a 256-bp internal sequence, +108 to +364 with respect to the start codon, from the *hpr* (*scoC*) coding region. This plasmid was used to inactivate *hpr* by Campbell integration.

(iii) pSS950. Plasmid pSS950 carries a null mutation in *spo0A*. A 64-bp internal fragment from the *spo0A*-coding region, +535 to +599 with respect to the start codon, was replaced with the erythromycin-resistance (Em^r) gene from pHP13 (19) and cloned into a Topo vector (Invitrogen). The plasmid was linearized with *Sma*I restriction enzyme and used to inactivate *spo0A* by gene replacement.

(iv) pSS1750. Plasmid pSS1750 carries a null mutation in *spo0H*. A 30-bp internal fragment from the *spo0H*-coding region, +532 to +562 with respect to the start codon, was replaced with the Em^r gene from pHP13 (19) and cloned into a Topo vector (Invitrogen). The plasmid was linearized with *Sma*I restriction enzyme and used to inactivate *spo0H* by gene replacement.

RESULTS

Transcriptional analysis of the *sinIR* promoters. Previous in vivo studies (17) had identified three separate and individually regulated promoters involved in the transcription of the *sinIR* operon. Among these promoters, P1 is the most important in regulating *sinIR* expression at the onset of sporulation and in determining the ratio of SinI and SinR proteins (3). Although P1 has typical σ^A promoter motifs, it had not been directly demonstrated that P1 could be transcribed in vitro by σ^A RNA polymerase. To more accurately establish the transcriptional start sites, we performed RNA mapping studies, utilizing reverse transcriptase-based primer extension assays. Total RNA prepared from IS75 (16) cells harvested at T_0 and RNA isolated from in vitro transcription reaction mixtures with *B. subtilis* RNA polymerase holoenzyme containing σ^A were used for these experiments. The in vivo and the in vitro extension products obtained for each promoter were similar (Fig. 1, lanes 1 and 2 for P1 and lanes 3 and 4 for P2), indicating that the in vivo and the in vitro start sites for P1 and P3 are identical. A minor extension product was observed, however, in the P1 in vitro reaction (lane 1) that was not present in the in vivo RNA reaction (lane 2). Its significance (alternative start site, degra-

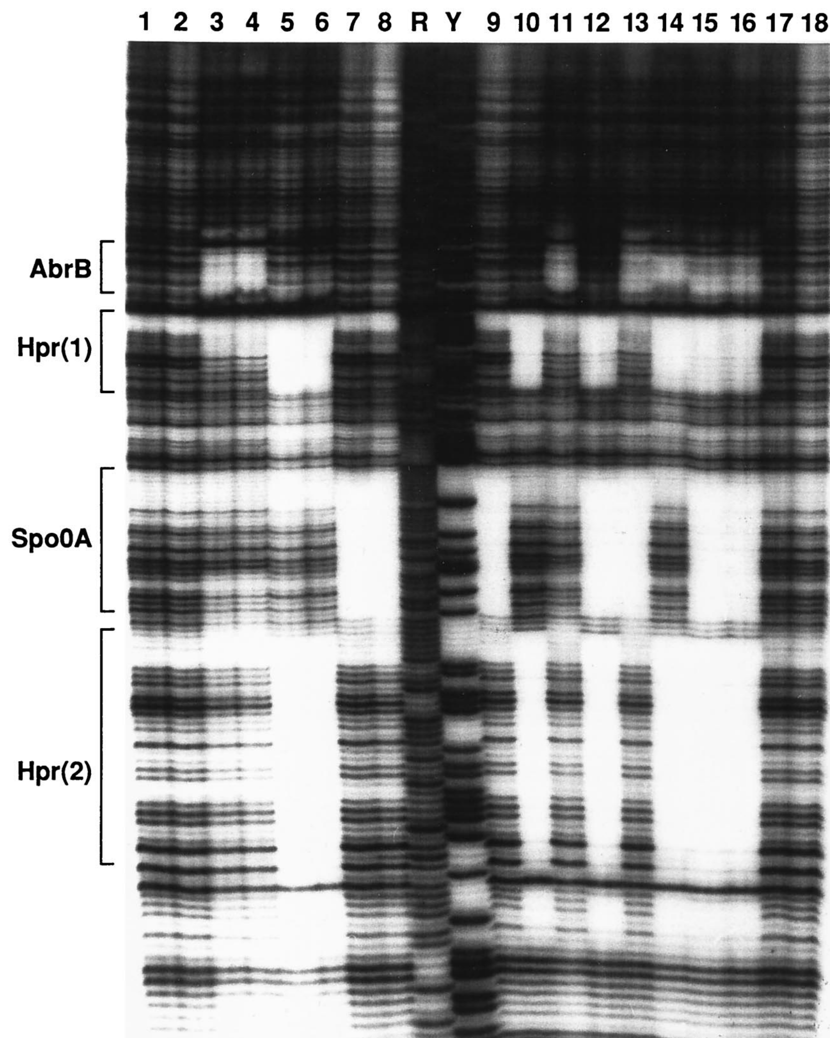


FIG. 2. DNase I footprinting of the *sinIR* promoter region. The regions bound by the indicated proteins are indicated in brackets at the left (also see Fig. 3). Lanes 1, 2, 17, and 18, no binding protein; lanes 3, 11, and 13 to 16, 1.5 μM AbrB; lane 4, 3 μM AbrB; lanes 5, 10, 12, and 14 to 16, 0.4 μM Hpr; lane 6, 0.8 μM Hpr; lanes 7, 9, 12, 13, 15, and 16, 2.5 μM Spo0A; lane 8, 5 μM Spo0A. Maxam-Gilbert A+G (R) and C+T (Y) sequencing ladders are shown for reference. AbrB binds the *sinIR* region with a K_d in the range of 40 nM (52); Hpr binds with a K_d of less than 20 nM (Strauch, unpublished); the K_d for Spo0A binding has not been determined, in part because of the recalcitrance of Spo0A-DNA complexes to be resolved by gel mobility shift assays (Strauch, unpublished).

dation product, or an artifact of in vitro transcription) is not known. The mapping of the P3 start is ambiguous, in that the start site could be either an A or the adjacent G (Fig. 1, lanes 3 and 4). The DNA sequences in the P1 and P3 promoter regions are presented below, with the initiating nucleotide(s) in boldface: P1, GATTATAATAAAGGT**A**TATT; and P3, TGCTATAATATCACA**AG**GA. These results confirm a previous transcriptional mapping of the P1 promoter, which had been obtained by the S1 nuclease protection method, to the nucleotide, but the results with P3 presented here indicate that the actual initiating nucleotide is 3 nt downstream of the one previously described (17).

Spo0A, Hpr, and AbrB in vitro binding to the *sinIR* P1 promoter region. Previous studies had shown that *sinI* expression was regulated by Spo0A (17) and had suggested a possible regulatory role for Hpr (25). The negative effects of *spo0A* mutations on *sinI* expression could be due to a direct activation

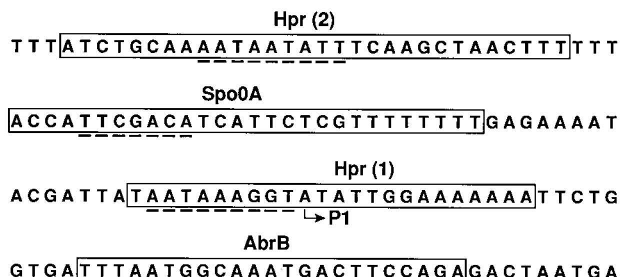
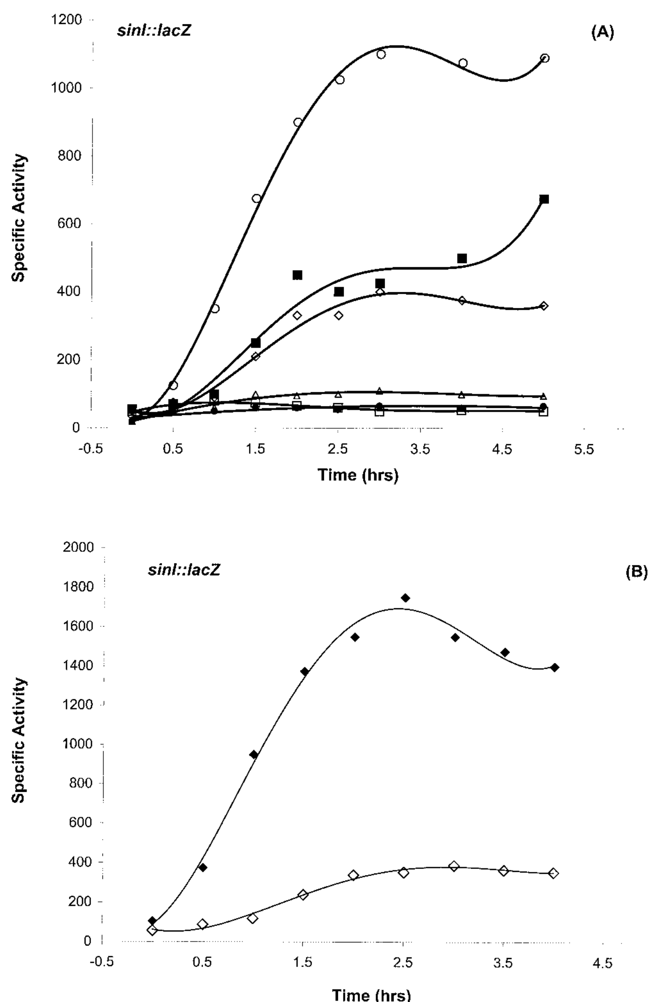


FIG. 3. Localization of protein binding sites within the *sinIR* upstream region. Boxes represent the binding regions of the indicated proteins on the nontemplate strand that corresponds to the DNase I footprinting regions identified for the template strand (Fig. 2). Dashed lines beneath the Hpr binding regions are sequences with homology to the putative Hpr recognition element. The dashed line beneath the Spo0A binding region is a sequence similar to the 0A box (TGTCGAA on the strand shown). P1 indicates the transcriptional start site of the *sinIR* promoter, which is postexponentially activated (17).



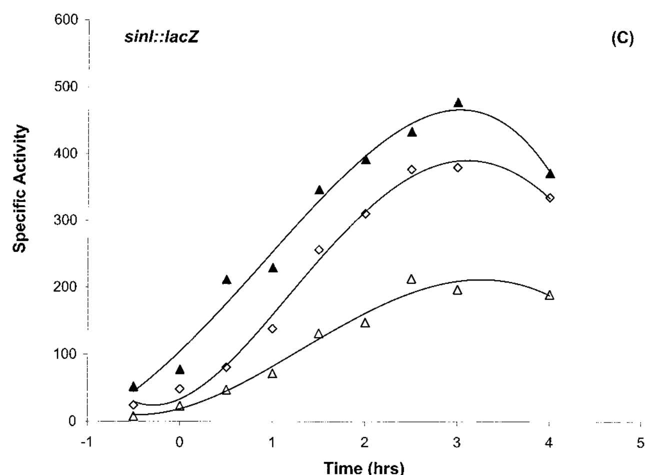


FIG. 4. Differential regulation of *sinI*::*lacZ* expression. (A) Dependence of *sinI*::*lacZ* expression on the phosphorelay. The indicated strains were grown in Schaeffer's sporulation medium and analyzed as described previously (11). T_0 denotes the end of exponential growth. (A) β-Galactosidase expression of *sinI*::*lacZ* in SS13 (wild type) (◇), SS15 (*spo0A12*) (●), SS17 (*spo0E11*) (△), SS19 (*spo0F221*) (□), SS40 (*spo0F221 rvtA11*) (■), and SS27 (*rvtA11*) (○) strains. (B) The effect of a loss-of-function mutation in *hpr* on *sinI*::*lacZ* expression. β-Galactosidase expression of *sinI*::*lacZ* in SS13 (wild type) (◇) and in SS30 (*hpr16*) (◆) strains. (C) The negative effect of a loss-of-function mutation in *abrB* on *sinI*::*lacZ* expression is suppressed by *rvtA11* mutation. β-Galactosidase expression of *sinI*::*lacZ* in SS13 (wild type) (◇), SS33 (*abrB*::Tn*917*) (△), and SS37 (*abrB*::Tn*917 rvtA11*) (▲) strains. (D) The epistatic relationship between Spo0A and Hpr in the regulation of *sinI* expression. β-Galactosidase expression of *sinI*::*lacZ* in SS13 (wild type) (◇), SS15 (*spo0A12*) (●), and SS63 (*hpr*Ω*pMTL20EC spo0A12*) (□) strains.

of *sinI* transcription by Spo0A (4, 58, 63), to an indirect effect of *spo0A* through control of *hpr* (39) or *abrB* (4), or to both. We examined the direct binding of these proteins by a DNase I protection assay. We found that Spo0A and AbrB bound to discrete sites at or near the *sinIR* P1 promoter (Fig. 2 and 3). In agreement with a previous report (25), we also found two Hpr binding sites in the *sinIR* upstream regulatory region (Fig. 2 and 3). P1 promoter binding by each of these proteins was independent and noncompetitive. We did not detect binding of any of these proteins to the P3 promoter region (data not shown).

The phosphorylated form of Spo0A regulates *sinI* expression. DNase I footprinting results (Fig. 2 and 3) established that Hpr, AbrB, and Spo0A can bind to the *sinIR* upstream regulatory region. The region protected by Spo0A contained a perfect match to the 0A box consensus sequence (51). The location (−43 to −49) of the 0A box relative to P1 is similar to cases in which binding of Spo0A~P has been shown to activate transcription (4, 45, 57, 63). These data suggested a dual regulatory mechanism for P1 transcription: activation by Spo0A~P and repression by Hpr. We examined *sinI*::*lacZ* expression in *spo0A* mutant strains and in a number of other *spo* gene mutations that are known to prevent phosphorylation of the Spo0A protein. Postexponential induction of *sinI* was not observed in *spo0A12*, *spo0A*::*Em*, *spo0F221*, *spo0H*::*Em*, or *spo0K141* mutant backgrounds and was substantially reduced in a *kinA* mutant background (Fig. 4A and data not shown). The *rvtA11* allele of *spo0A* bypasses the requirement for many of the normal phosphorelay gene products and can suppress sporulation defects such as *spo0F* mutations (48). The *rvtA11* allele restored *sinI* expression in a *spo0F* mutant background and elevated *sinI* expression in a wild-type background (Fig. 4A). The *spo0E11* gain-of-function mutation encodes an overactive phosphatase that inhibits sporulation by specifically dephosphorylating Spo0A~P (37, 43). *sinI* expression was also substantially reduced in this mutant background (Fig. 4A). These results indicate that the phosphorylated form of Spo0A is required for *sinI* induction.

Hpr is a negative regulator of *sinI* expression. P1 expression is downregulated in vegetative growth and increases dramatically at the onset of stationary phase (17). We have examined whether the binding of Hpr near P1 can account for repression of this promoter. Loss-of-function mutations in *hpr* (*hpr16* and *hpr*Ω *pMTL20EC*) caused substantial overexpression of *sinI*::*lacZ* (Fig. 4B and data not shown), indicating that Hpr binding near the P1 promoter (Fig. 2 and 3) is repressive in nature. We have characterized the nature of the *hpr16* mutation by DNA sequencing. This mutation is caused by a mis-

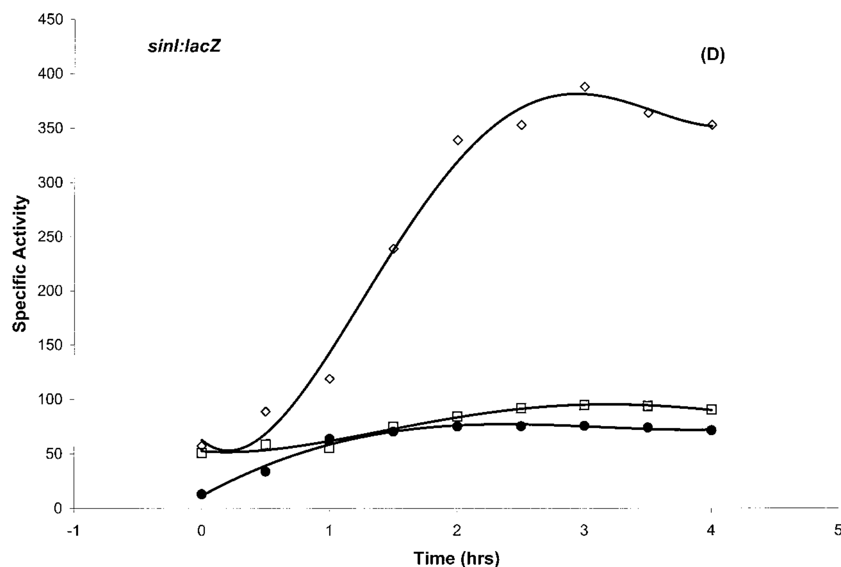


FIG. 4—*Continued.*

sense mutation (T→A) at position +205, with respect to the start codon, resulting in a Phe69-Ile amino acid substitution. *sinI*::*lacZ* expression remained inhibited in a *spo0A hpr* double-mutant background (Fig. 4D), indicating that Spo0A is epistatic to Hpr in regulating *sinI* gene expression.

Role of AbrB in the regulation of *sinI* and sporulation. *sinI*::*lacZ* expression is unexpectedly decreased in the *abrB*::Tn*917* or *abrB*::*Tet* null mutant backgrounds (Fig. 4C and data not shown). Expression was restored to wild-type levels when the *rvtA11* mutation was introduced into these *abrB* mutant backgrounds (Fig. 4C and data not shown).

DISCUSSION

Inactivation of *spo0A*, *spo0F*, *spo0H*, *spo0K*, or *kinA* substantially reduced the postexponential expression of *sinI*::*lacZ*. These results suggest that *sinI* expression is controlled by the *spo0A* phosphorelay. Two other lines of genetic evidence support this interpretation. The *spo0E11* mutation, a gain-of-function mutation which causes increased dephosphorylation of Spo0A~P (37, 43), also diminished *sinI*::*lacZ* expression (Fig. 4A). On the other hand, the *rvtA11* mutation in *spo0A*, which bypasses the requirement for other phosphorelay gene products (27), restored expression of *sinI* in a *spo0F* mutant background and elevated *sinI* expression in a wild-type background (Fig. 4A). Loss-of-function mutations in *hpr* (*hpr16* and *hpr*Ω*pMTL20EC*) caused substantial overexpression of *sinI*::*lacZ* (Fig. 4B and data not shown). *sinI* expression remained low in the *hpr* mutant background during vegetative growth (although reproducibly two- to threefold higher than the corresponding time points in the wild-type background) but was substantially derepressed as the cells entered postexponential growth phase. These data suggest that the absence of high-level expression from the P1 promoter during vegetative growth time points might be due to the absence of the phosphorylated form of Spo0A. This interpretation is supported by the finding that postexponential derepression of *sinI*, observed in an *hpr* mutant background (Fig. 4B), is abolished in *hpr spo0A* double-mutant backgrounds (Fig. 4D), indicating that Spo0A~P binding may be essential for expression from the P1 promoter. Moreover, the −35 region of the P1 promoter is significantly different from the σ^A RNA polymerase consensus sequence, supporting the interpretation that the P1 promoter may be weakly transcribed in the absence of its activator, Spo0A~P.

sinI::*lacZ* expression was diminished in both *abrB*::Tn*917* and *abrB*::*Tet* loss-of-function mutations (Fig. 4C and data not shown). The downregulation of *sinI*::*lacZ* expression in *abrB* mutants could be due to AbrB functioning as an activator of *sinIR* or as a repressor of another gene(s), such as *spo0E*, whose product directly or indirectly inhibits *sinI*::*lacZ* expression. Spo0E phosphatase inhibits sporulation by removing the phosphate group from Spo0A~P (37, 43). Moreover, *spo0E* expression is inhibited by AbrB during the vegetative phase and dramatically increases during transition into stationary phase (43). The expression of *sinI* was fully restored in an *abrB rvtA11* double-mutant background (Fig. 4C), suggesting that the in vivo levels of Spo0A~P may be reduced in *abrB* null mutant backgrounds. The exact role of AbrB in regulation of the *sinIR* operon, however, cannot be unambiguously determined from these results and is currently under investigation.

Expression of the *sinR*::*lacZ* fusion driven from the P3 promoter was not affected by any of the mutations (*spo*, *abrB*, *hpr*, and *rvtA*) shown here to regulate *sinI*::*lacZ* expression (data not shown).

In vitro DNase I footprinting experiments (Fig. 2 and 3) confirmed that the proteins implicated by in vivo genetic studies of *sinIR* regulation (Spo0A, AbrB, and Hpr) were able to bind to regions upstream of the P1 promoter. The binding motifs for Hpr and Spo0A are typical of sites where negative (Hpr) (25; M. A. Strauch, unpublished results) and positive (Spo0A) (4, 45, 57, 63) regulation is exerted. In vitro primer extension transcription studies with *sinIR* templates and σ^A RNA polymerase established that the RNA transcripts seen in vivo originated from the P1 (*sinI* proximate) and P3 (*sinR*

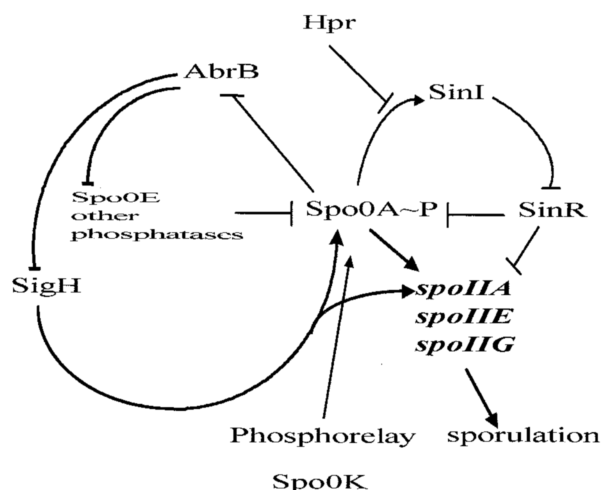


FIG. 5. A diagram depicting the *sinIR* transcriptional control circuit. The arrows indicate a positive interaction at either a transcriptional or later step; the T bars denote a negative interaction. For clarity, several proteins and regulatory pathways affecting the phosphorelay have been omitted.

proximate) promoters (Fig. 1). The P2 promoter site, which resembles a σ^E sequence (17, 50), was not used in vitro by σ^A RNA polymerase (data not shown).

Analysis of *sin* operon transcription provides an opportunity to investigate the genetic strategies used to control complex postexponential regulons. A diagram summarizing these interlocking control circuits is shown in Fig. 5. The combinatorial alternative states provided by positive and negative regulator interactions are more than sufficient to account for the effects of the *sin* operon on postexponential cell state regulation.

The balance of Hpr and Spo0A~P effects on *sin* operon transcription may determine the set point of the system in terms of selecting sporulation cell fate. Hpr would appear to be a checkpoint for the commitment to sporulation. If insufficient levels of Spo0A~P are present, Hpr repression will downregulate *sinI* transcription, allowing SinR to repress *spo0A*, *spoIIA*, *spoIIG*, and *spoIIE* expression (18, 31, 32, 33, 50) and to upregulate genes involved in motility and competence (15, 46). The finding that a *sinR* deletion can suppress the sporulation phenotypes caused by mutations in *spo0K* and the cell cycle control gene *ftsA* (31) is consistent with this interpretation. The principal regulatory interplay dominating the expression of the *sin* operon is thus determined by Spo0A~P and Hpr. As critical nutrients become limiting, a sequence of transition state interlocks guide the cells to a "postexponential soft landing." Exponential-phase cells can enter motility, nutrient-scavenging, or competence postexponential differentiation pathways (for a review, see references 12 and 53). Cells committed to sporulation traverse these interlock pathways by inactivation of the Hpr system and culminate in the activation of the SinI interlock, which in turn relieves SinR repressive effects on sporulation, allowing subsequent progression to stage II and irreversible commitment to sporulation.

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