Molecular Microbiology (2003) **47**(5), 1251–1263

Characterization of a novel inhibitory feedback of the anti-anti-sigma SpoIIAA on Spo0A activation during development in *Bacillus subtilis*

Ana L. Arabolaza,[1] Akira Nakamura,[2] María E. Pedrido,[1] Luciano Martelotto,[1] Lelia Orsaria[1] and Roberto R. Grau[1]*

[1]*Department of Microbiology, Rosario University School of Biochemistry and Pharmacy, and Institute for Molecular and Cellular Biology of Rosario, IBR-CONICET. Suipacha 531, Sala 9, Rosario-2000, Argentina.*

[2]*Institute of Applied Biochemistry, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305–8572, Japan.*

Summary

Compartmentalized gene expression during sporulation is initiated after asymmetric division by cell-specific activation of the transcription factors σ^F and σ^E. Synthesis of these σ factors, and their regulatory proteins, requires the activation (phosphorylation) of Spo0A by the phosphorelay signalling system. We report here a novel regulatory function of the anti-anti-σ^F SpoIIAA as inhibitor of Spo0A activation. This effect did not require σ^F activity, and it was abolished by expression of the phosphorelay-independent form Spo0A-Sad67 indicating that SpoIIAA directly interfered with Spo0A~P generation. IPTG-directed synthesis of the SpoIIE phosphatase in a strain carrying a multicopy plasmid coding for SpoIIAA and its specific inhibitory kinase SpoIIAB blocked Spo0A activation suggesting that the active form of the inhibitor was SpoIIAA and not SpoIIAA-P. Furthermore, expression of the non-phosphorylatable mutant SpoIIAAS58A (SpoIIAA-like), but not SpoIIAAS58D (SpoIIAA-P-like), completely blocked Spo0A-dependent gene expression. Importantly, SpoIIAA expressed from the chromosome under the control of its normal *spoIIA* promoter showed the same negative effect regulated not only by SpoIIAB and SpoIIE but also by septum morphogenesis. These findings are discussed in relation to the potential contribution of this novel inhibitory feedback with the proper activation of σ^F and σ^E during development.

Accepted 15 November, 2002 *For correspondence. E-mail rrgrau@infovia.com.ar; Tel. (+54) 341 4353377; Fax (+54) 341 4804601.

Introduction

The soil bacterium *Bacillus subtilis* can respond to nutrient starvation activating a developmental pathway leading to the formation of resistant dormant spores (Piggot and Coote, 1976; Losick and Stragier, 1992). In this primitive system of cell differentiation the transcription factor Spo0A is the critical regulator of the shift from vegetative growth to sporulation (Ireton *et al*., 1993). As a typical response regulator activation of Spo0A is obtained by phosphorylation, which is under the control of the phosphorelay (Burbulys *et al*., 1991). The phosphorelay is composed for at least five independently activated sensor-histidine kinases (KinA-E) that donate phosphate to Spo0F, a response regulator with no output domain. The phosphate from Spo0F~P is transferred to Spo0B and finally from Spo0B~P to Spo0A generating Spo0A~P, the active form of the regulator (Jiang *et al*., 2000a). A very small amount of Spo0A~P is high enough to repress the synthesis of AbrB, which is itself a repressor of several genes involve in alternative stationary phase responses (Perego *et al*., 1988; Jiang *et al*. 2000a; Hamon and Lazazzera, 2001). When these options failed multiple signals impact on the phosphorelay, and a little higher amount of Spo0A~P is formed, which establishes a positive autoregulatory loop activating further transcription of spo0 genes (Strauch *et al*., 1993). As a final result a threshold amount of Spo0A~P is formed, which activates the transcription of several sporulation-specific genes needed for asymmetric division and early compartmentalization of gene expression (Chung *et al*., 1994). Among these Spo0A-dependent genes are *spoIIE* and the operons *spoIIA* (coding for SpoIIAA, SpoIIAB, and SpoIIAC or σ^F), and *spoIIG* (coding for SpoIIGA, and SpoIIGB or pro-σ^E). The asymmetric process of cell division occurs well before the complete translocation of the forespore-destined chromosome and divides the developing cell (the sporangium) into a large mother cell compartment and a small forespore chamber that differ in fate from one another as well as from the vegetative progenitor cell. The transcription factors σ^F and σ^E are activated after cytokinesis and are responsible for setting in motion the compartmentalized gene expression in the forespore and mother cell compartments, respectively. A regulatory pathway involving the proteins SpoIIE, SpoIIAA, and SpoIIAB controls cell-specific activation of

the transcription factor σ^F (for reviews see Stragier and Losick, 1996; Errington, 2001). SpoIIAB is an anti-sigma factor that binds to σ^F and inhibits σ^F-directed transcription. SpoIIAA is an anti-anti-sigma factor capable of overcoming SpoIIAB-mediated inhibition of σ^F by binding to the SpoIIAB • σ^F complex and causing the release of free and active σ^F • SpoIIAA is in turn, negatively regulated by SpoIIAB, that is also a protein kinase that phosphorylates SpoIIAA on serine residue 58, thereby impairing the capacity of SpoIIAA to bind to SpoIIAB. Thus SpoIIAA is an inhibitor of SpoIIAB, and conversely, SpoIIAB is an inhibitor of SpoIIAA that inactivates the anti-anti-sigma factor by phosphorylation (Stragier and Losick, 1996; Errington, 2001). SpoIIAA is also positively regulated by the developmental phosphatase SpoIIE that soon after the onset of sporulation is recruited to the Z rings at both cell poles (for a review see Errington, 2001). After its assembly into the Z rings, SpoIIE is believed to begin to hydrolyse SpoIIAA-P, the phosphorylated form of SpoIIAA (Stragier and Losick, 1996; King *et al*., 1999; Errington, 2001). However, dephosphorylation of SpoIIAA-P is not sufficient to activate σ^F, which is exclusively detected in the cytoplasm of the forespore only after complete septum formation (Lewis *et al*., 1996; Feucht *et al*., 1999; King *et al*., 1999). The evidences indicating that SpoIIAA-P could be dephosphorylated before cytokinesis (Feucht *et al*., 1999; King *et al*., 1999) strongly suggested the existence of additional mechanism/s to prevent the inappropriate activation of σ^F before polar septation and the cell specificity of its activation (King *et al*., 1999; Errington, 2001). On the other hand, σ^E is synthesized as an inactive proprotein pro-σ^E before asymmetric division and is converted to the mature form, in a mother cell-restricted event, by the putative processing enzyme SpoIIGA (Stragier *et al*., 1988; Peters and Haldenwang, 1994). Processing of pro-σ^E is controlled by the signaling protein SpoIIR, which is produced in the forespore under the control of σ^F and is secreted, it is believed, into the space between the two membranes of the polar septum, where its interacts with SpoIIGA (Hofmeister *et al*., 1995; Karow *et al*., 1995; Londoño-Vallejo and Stragier, 1995). This intercellular signal transduction pathway is a timing device that ensures that processing does not commence until after σ^F is activated, which is in turn dependent upon the complete formation of the polar septum (Feucht *et al*., 1999; King *et al*., 1999). The mechanism by which σ^E is confined to the mother cell is not well understood. Recently experimental data have been presented supporting a model in which persistent and preferential transcription of the *spoIIG* operon in the mother cell and degradation and/or proper instability of the σ factor in the forespore contribute to the selective accumulation of σ^E in the larger compartment (Fujita and Losick, 2002).

Here we present evidences for a novel regulatory function of the dephosphorylated form of the anti-anti-σ^F factor SpoIIAA as a negative regulator of Spo0A activation. We hypothesize that one role of this negative regulatory feedback is to block the expression of Spo0A-dependent genes whose products are not longer required at that stage of development (e.g. SpoIIE). In addition we present a workable hypothetical scenario for this regulatory feedback contributing to the proper establishment of early compartmentalization of gene expression.

Results and discussion

Identification of SpoIIAA as inhibitor of the activation of Spo0A-dependent developmental genes

DNA libraries of *B. subtilis* have proven to be useful for the identification of negative regulators of spore development. In this sense several inhibitors of predivisional Spo0A activation (KipI inhibitor of histidine kinase A, RapE phosphatase of Spo0F~P, YisI phosphatase of Spo0A~P, and PepF oligopeptidase of PhrA) were identified by their property of inhibiting sporulation at stage 0 when expressed from internal promoters of the multicopy plasmid pHT315 (Wang *et al*., 1997; Jiang *et al*., 2000b; Perego, 2001; Kanamaru *et al*., 2002). With the aim to find new insights about the regulatory mechanisms restricting the activation of σ^F and σ^E we searched a large collection of pHT315-derivate sporulation-inhibitory plasmids. Levels of β-galactosidase activity were measured and used as indication of gene expression from reporter *lacZ* fusions to developmental promoters under the control of Spo0A~P, σ^F and σ^E. In this communication we report, in detail, one plasmid (pAR230) that differently affected the activities of the reporter *lacZ* fusions besides of its inhibitory effect on spore formation (Fig. 1). Strains carrying pAR230 and different Spo0A-dependent sporulation *lacZ* fusions showed a strong inhibition of accumulation of β-galactosidase activity (Fig. 1A–C). By contrast the activity of σ^F was more than 10-fold increased and σ^E-dependent gene expression severely decreased in strains carrying this multicopy sporulation-inhibitory plasmid (Fig. 1D and E). Comparisons of the nucleotide sequence of the insert with the annotations for the genome sequence of *B. subtilis* (Kunst *et al*., 1997) showed that pAR230 harboured an insert of 2.2 kb related to the *spoIIA* operon with complete copies of *spoIIAA* and *dacF* (Fig. 1H). Moreover, the dephosphorylated form of SpoIIAA might be predominant in cells carrying pAR230 as the truncated form of *spoIIAB* harboured by the multicopy plasmid (Fig. 1H) should coded for an inactive SpoIIAB protein lacking its essential ATP binding domain (Pan *et al*., 2001). This information permitted a direct explana-

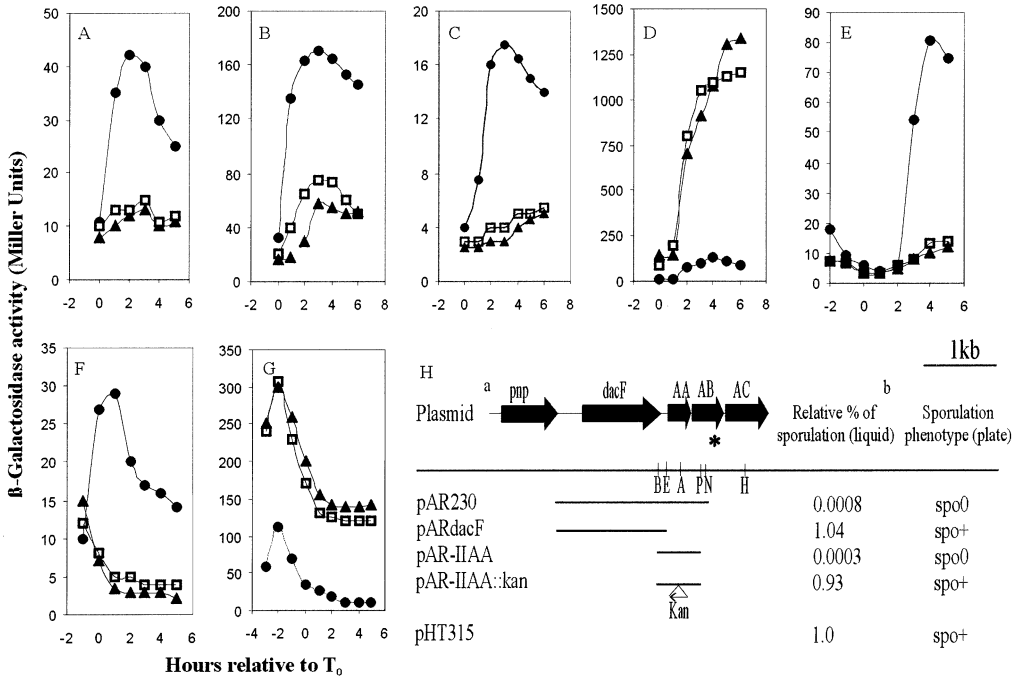


Fig. 1. Effect of sporulation-inhibitory plasmids on expression of Spo0A~P, σ^F- and σ^E-dependent genes.
A–G. Cells were grown in SSM and samples were collected at the indicated times and assayed for β-galactosidase activity expressed in Miller Units (Wang *et al*., 1997). Time 0 represents the transition from vegetative to stationary phase. Wild-type *B. subtilis* strains harboured the following β-galactosidase fusions: JH16124 *spoIIA-lacZ* (A), JH16182 *spoIIG-lacZ* (B), JH16480 *spoIIE-lacZ* (C), RG2051 *spoIIQ-lacZ* (D), RG1679 *spoIID-lacZ* (E), JH19004 *spo0F-lacZ* (F), and JH12604 *abrB-lacZ* (G). Plasmids carried for each strain were: pHT315 (–●–), pAR230 (▲), and pAR-IIAA (□). Plasmids pAR230 and pAR-IIAA also overexpressed and inhibited the expression of the σ^F-dependent *spoIIR-lacZ* (strain RG6418) and the Spo0A-dependent *spo0A-lacZ* (strain JH19005) fusions, respectively (data not shown).
H. Restriction map[a] and effect on spore formation[b] of the multicopy plasmids.[a]Arrows indicate the position and length of the various genes. Abbreviations for the main restriction enzymes used for plasmid and strain constructions are N (*Nco*I), E (*Eco*RI), B (*Bst*XI), P (*Pvu*II), A (*Ava*I), and H (*Hin*cII). (*) indicates the location of the ATP-binding coding region of *spoIIAB* not present in pAR230. [b]Cultures were grown for 36 h at 37°C in SSM supplemented with erythromycin 15 mg ml^{-1} and assayed for spore formation. The relative sporulation frequency is the spore production per millilitre relative to that of a wild-type culture under the same growth conditions. Data are representative of five independent experiments.

tion for the positive effect of pAR230 on the activity of σ^F as overexpression of *spoIIAA* from the multicopy plasmid should produce elevated levels of SpoIIAA and hence an upregulated activity of σ^F (Fig. 1D). On the other hand, the low level of σ^E activity measured from the *spoIID-lacZ* fusion (Fig. 1E) could be explained with the poor expression of the pro-σ^E coding operon *spoIIG* when pAR230 is present (Fig. 1B). By the contrary, a feasible explanation for the effect of pAR230 on the activity of the Spo0A-dependent fusions was not evident. Introduction of multicopy *dacF* into the sporulation proficient (spo$^+$) reference strain JH642 neither affected the sporulation efficiency (Fig. 1H) nor affected the expression of developmental β-galactosidase activities under the control of Spo0A~P or σ^F (data not shown). In addition, the low activity of the Spo0A-activated *spo0F-lacZ* fusion (Fig. 1F), and the higher expression of the Spo0A-repressed *abrB-lacZ* fusion (Fig. 1G) suggested that the cellular level of active Spo0A was low. In fact, the most sensitive *in vivo* indicator for the level of phosphorylated Spo0A is the transcription of the *abrB* gene that is repressed by very low amounts of Spo0A~P (Jiang *et al*., 2000a; Hamon and Lazazzera, 2001). Having discarded *dacF* we investigated the effect of *spoIIAA*, and a pHT315-derived plasmid carrying the *spoIIAA* gene (pAR-IIAA) was constructed from pAR230 (see *Experimental procedures* and Fig. 1H). This new multicopy plasmid produced the same effect on activation of sporulation *lacZ* fusions and spore formation as pAR230 (Fig. 1). Furthermore, inactivation of *spoIIAA* in pAR-IIAA (pAR-IIAA::kan) restored the expression of developmental β-galactosidase activity (data not shown) and spore formation to wild-type levels (Fig. 1H) indicating that transcription of *spoIIAA* from pAR-IIAA, probably from Spo0A-independent internal promoters of pHT315 (Wang *et al*., 1997), was needed to produce the observed negative effects on sporulation. These overall results suggested that SpoIIAA, coded from the multicopy plasmid, was responsible not only for the upregulated activity of σ^F

but surprisingly for the generation of low levels of Spo0A activity.

SpoIIAA does not require the activity of σ^F to block the expression of Spo0A~P-dependent developmental genes

The low activity of early sporulation genes could be caused by a direct negative effect of SpoIIAA on Spo0A (possibility 1 in Fig. 2A) or to an indirect effect due to a putative negative Spo0A regulator under the control of σ^F (possibility 2 in Fig. 2A; Zhang *et al.*, 1999). In order to distinguish between these two possibilities we measured the accumulation of Spo0A-dependent β-galactosidase activities in plasmid-carrying strains devoid of σ^F activity (*spoIIAC::kan*). In Fig. 2B–F it is showed that pAR230 and pAR-IIAA produced the same pattern of expression, now in a *spoIIAC*$^-$ background, on the analysed Spo0A-dependent *lacZ* fusions than originally produced in wild-type strains. This result indicated that the activity of σ^F was not needed for the negative effect of SpoIIAA on Spo0A and suggested a new regulatory function of the anti-anti-sigma apart from its essential role on σ^F activation.

The phosphorelay independent form Spo0A-Sad67 suppresses the negative effect of SpoIIAA on Spo0A activity

The poor activation of the Spo0A-dependent sporulation *lacZ* fusions, and the increased activity of the *abrB-lacZ* fusion observed in Fig. 1 could be due to an inhibitory effect on Spo0A produced before or after its activation by the phosphorelay signalling system (Fig. 3A). In the first case the activity of the phosphorelay should be affected resulting in the generation of low amounts of Spo0A~P insufficient to trigger the transcription of early sporulation genes (possibility 1 in Fig. 3A). In the second case overproduced SpoIIAA should affect the expression of Spo0A-dependent genes independently of the amount of active Spo0A formed as previously described for other stage I and II regulators (Mandic-Mulec *et al.*, 1992; Mandic-

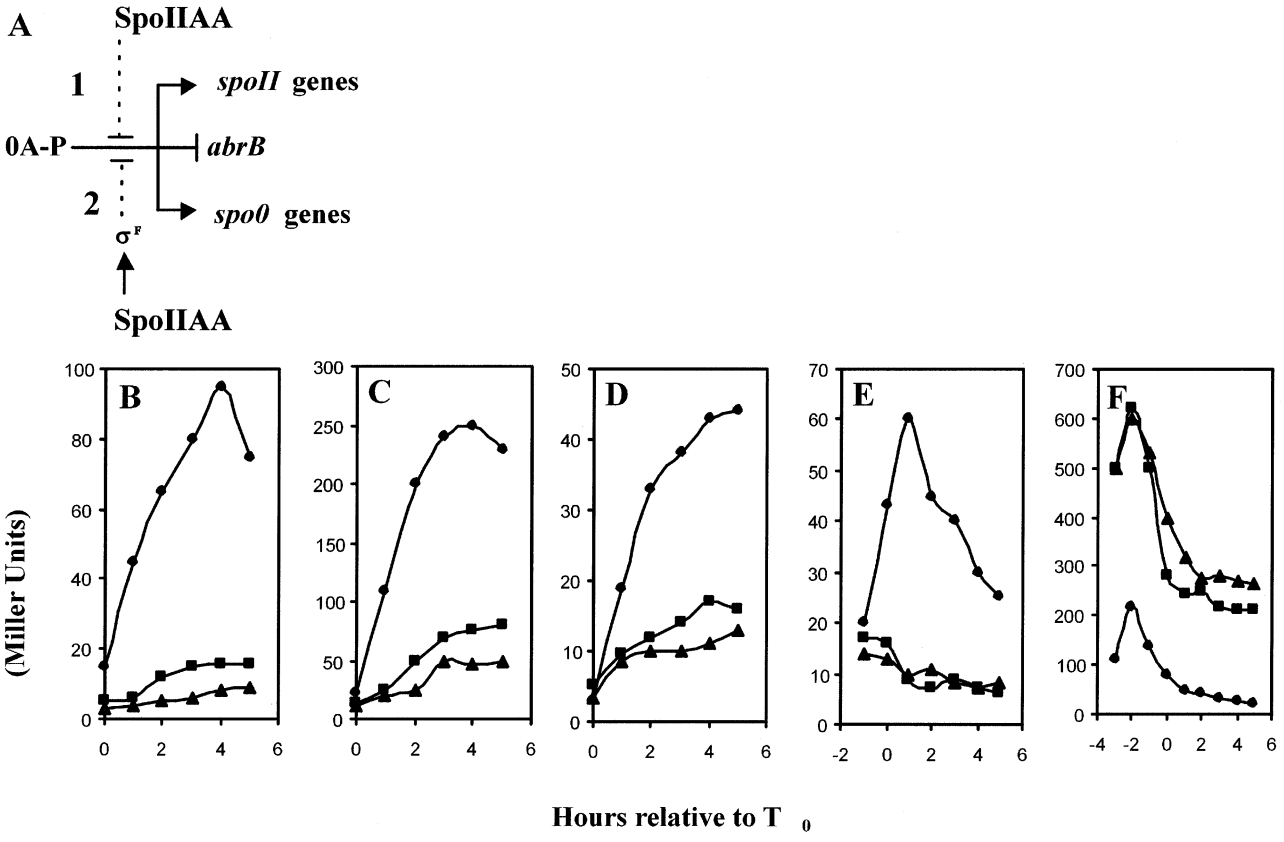
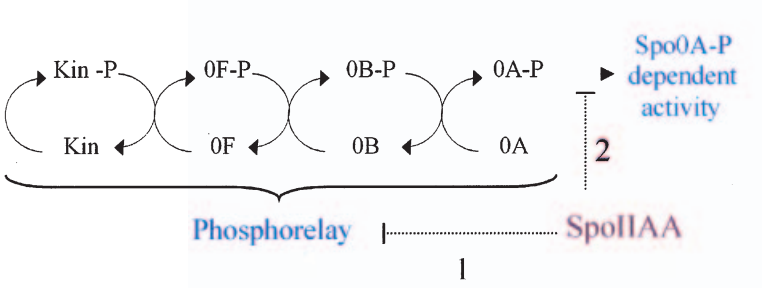
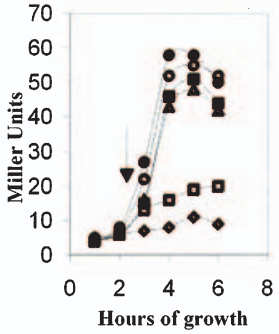


Fig. 2. The inhibitory effect of SpoIIAA on Spo0A regulation does not depend on the activity of σ^F-dependent genes.
A. The poor Spo0A~P activity detected in strains carrying multicopy *spoIIAA* could be the resulf of a direct negative effect of SpoIIAA on the generation and/or activity of Spo0A~P (possibility 1) or to an uncharacterized σ^F-dependent gene product acting as inhibitor of Spo0A~P (possibility 2).
B–F. β-galactosidase activities of the *spoIIAC*$^-$ strains RG19149 *spoIIA-lacZ* (B), RG19150 *spoIIG-lacZ* (C), RG19151 *spoIIE-lacZ* (D), RG19152 *spo0F-lacZ* (E), and RG19153 *abrB-lacZ* (F) carrying the multicopy plasmids pHT315 (●), pAR230 (▲), and pAR-IIAA (■).

A



B



C

	relevant genotype	IPTG addition	viable cells/ml	spores/ml	%
RG1117 /pHT315	*spo0A*$^+$ P$_{spac}$*sad67*$^+$	NO	7 x 10^8	6 x 10^8	85.7
	spo0A$^+$ P$_{spac}$*sad67*$^+$	YES	4.6 x 10^8	4.6 x 10^8	100.0
RG1117 /pARIIAA	*spo0A*$^+$ P$_{spac}$*sad67*$^+$	NO	4 x 10^8	2 x 10^6	0.5
	spo0A$^+$ P$_{spac}$*sad67*$^+$	YES	3 x 10^8	1.4 x 10^8	46.6
RG1114 /pHT315	*spo0A*$^+$	-	5 x 10^8	4 x 10^8	80.0
RG1114 /pARIIAA	*spo0A*$^+$	-	9 x 10^7	1 x 10^5	0.1

D

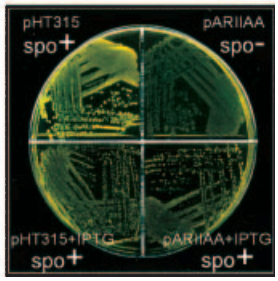


Fig. 3. Expression of *spo0A-sad67* suppresses the negative effect of multicopy *spoIIAA* on spore development.
A. This cartoon summarizes the activity of the phosphorelay signal transduction pathway leading to Spo0A activation (generation of Spo0A~P, option 1) and the activity of the transcription factor after its phosphorylation (option 2) as the possible targets for the inhibitory effect of SpoIIAA.
B. β-galactosidase activities of strains RG1114 (*spoIIE-lacZ spo0A*$^+$), and RG1117 (*spoIIE-lacZ spo0A*$^+$ *Pspac-spo0A-sad67*) carrying multicopy plasmids pHT315 and pAR-IIAA. Bacterial cultures were grown in SSM and by the time indicated by the arrow IPTG (1 mM) was added to one half of the cultures of RG1117. Cultures of RG1114 remained non-supplemented during the complete experiment. β-galactosidase activity was monitored as indicated in Fig. 1. Symbols: RG1114/pHT315 (△), RG1114/pAR-IIAA (◇), RG1117/pHT315 (○), RG1117/pAR-IIAA (□), RG1117/pHT315 + IPTG (•), and RG1117/pAR-IIAA + IPTG (■). The same pattern of accumulation of β-galactosidase activity was obtained with strain RG1116 (*spoIIA-lacZ spo0A*$^+$ *Pspac-spo0A-sad67*) carrying the multicopy plasmids in the absence or presence of IPTG (data not shown).
C. Effect of pAR-IIAA on spore formation of cultures expressing wild type and *sad67* alleles of *spo0A*. Cultures were grown and supplemented in SSM at 37°C with erythromycin 5 mg ml^{-1}. Samples were tested for spore formation after 24 h of growth. The results of a representative experiment are shown.
D. Sporulation phenotype of different RG1117 cultures carrying multicopy plasmids after growth on solid SSM with or without IPTG. The photograph was taken after 36 h of growth at 37∞C. Note that in this sporulation medium (SSM) wild-type sporulation proficient (spo$^+$) cells form opaque–brownish colonies, whereas sporulation deficient (spo$^-$) cells make diaphanous dying colonies. Also note for the IPTG-supplemented cultures carrying pHT315 and pAR-IIAA the small size of the colonies due to the toxic effect of Spo0A-Sad67 that was not affected by SpoIIAA (Ireton et al., 1993).

Mulet *et al.*, 1995, Cervin *et al.*, 1998), possibility 2 in Fig. 3A. In order to distinguish between these two possibilities we recurred to the use of a phosphorelay-independent form of Spo0A that does not require phosphorylation for its activity: Spo0A-Sad67 (Ireton *et al.*, 1993). We monitored the effect of multicopy *spoIIAA* on gene expression of cells engineered to produce Spo0A-Sad67 under the control of the IPTG-inducible P$_{spac}$ promoter (strain RG1117; Ireton *et al.*, 1993). In this way we were able to separate the integrity of the activating pathway of Spo0A (the phosphorelay) from the activity of the transcription factor. In Fig. 3B it is showed the low Spo0A-directed β-galactosidase activity accumulated by the strain RG1117/pAR-IIAA grew in sporulation medium in the absence of IPTG. By contrast, when IPTG was added to one half of that culture just before the onset of the stationary phase a strong Spo0A-Sad67-dependent β-galactosidase activity, indistinguishable from the one obtained with a control culture, was observed. In Fig. 3C it is showed the efficiency of spore formation under the different conditions. In accordance with the induction of the reporter *lacZ* fusion IPTG addition restored the ability of RG1117/pAR-IIAA to make spores at a comparable level to the control culture of RG1117/pHT315 (Fig. 3C). Moreover, in the absence of IPTG both strains harbouring the P$_{spac}$-*sad67* fusion formed on SSM plates big colonies with the classical opaque (Fig. 3D top-left panel) and translucent appearances (Fig. 3D top-right panel) produced by sporulating or non-sporulating cells, respectively. By the contrary, in the presence of IPTG the same strains formed small, pinpoint colonies that segregated sporulation deficient cells (Fig. 3D bottom panels). Because this sick phenotype was attributable to the activity of the *sad* allele after IPTG addition (Ireton *et al.*, 1993), and it was the same for both strains carrying pAR-IIAA or pHT315 it can be concluded that SpoIIAA did not affect the activity of Spo0A

after its activation (Ireton *et al*., 1993). Taken together (Fig. 3B–D) these results strongly suggested that SpoIIAA inhibited the accumulation of Spo0A~P (possibility 1 in Fig. 3A) and not its activity as transcription regulator. Something unsolved up to now is the nature of the specific target of SpoIIAA on the phosphorelay. Because the commitment to sporulation represents a tough and irreversible decision, a diverse set of extracellular and intracellular signals seems to regulate the accumulation of Spo0A~P by regulating the activity of the phosphorelay (Ireton *et al*., 1993; Chung *et al*., 1994; Wang *et al*., 1997; Jiang *et al*., 2000a,b; Burkholder *et al*., 2001; Perego, 2001). In any case the identification of the particular target of SpoIIAA on Spo0A~P formation would require *in vitro* biochemical analysis (Wang *et al*., 1997; Burkholder *et al*., 2001) and/or the isolation of suppressors of the SpoIIAA effect on Spo0A activation (Grau *et al*. unpublished).

Dephosphorylated SpoIIAA, but not SpoIIAA-P, is the active form of the inhibitor of Spo0A activation

As SpoIIAA exists under two different forms (SpoIIAA-P and SpoIIAA) during the normal development of the spore we analysed which form of the anti-anti-σ was active as

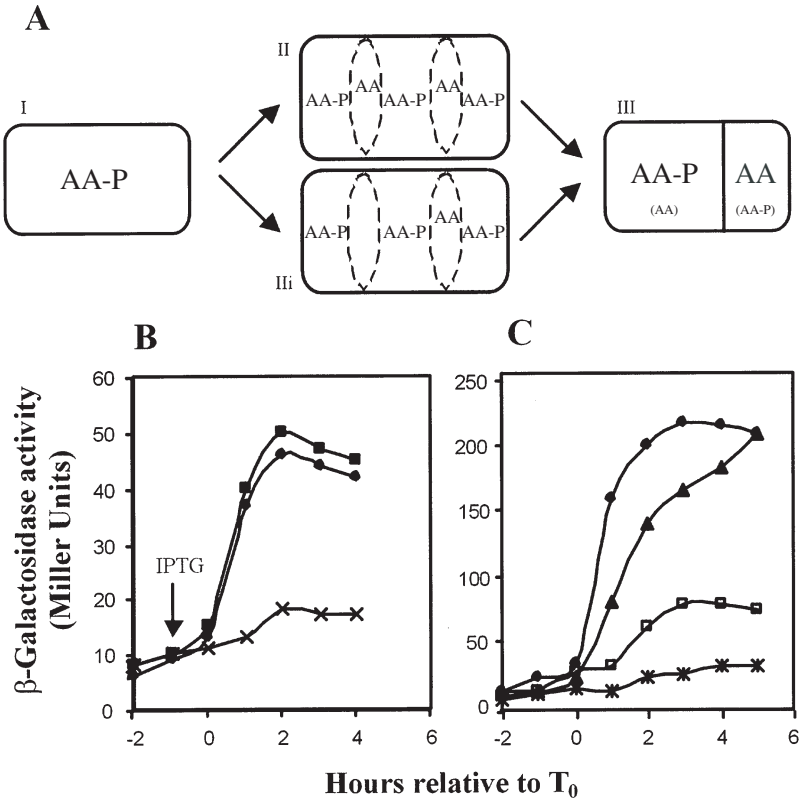


Fig. 4. Dephosphorylated SpoIIAA blocks the activation of the master transcription factor Spo0A.
A. This cartoon summarizes the different forms of the cell-fate determinant SpoIIAA and a hypothetical model based in published data for its temporal and spatial distribution during development (Lewis *et al*., 1996; Feucht *et al*., 1999; King *et al*., 1999; Errington, 2001). First, in the predivisional sporangium, before Z ring formation, only a phosphorylated inactive form (SpoIIAA-P) is detected (I). Then, after the assembling of SpoIIE into the Z rings (possibly at both cell poles) SpoIIAA-P is started to be dephosphorylated by SpoIIE. Because of the absence of σ^F activity at this stage of development it is speculated that dephosphorylated SpoIIAA would be sequestered with a not showed sporulation protein in an inactive complex within the incipient polar septa (II). Finally, shortly after asymmetric division the EZ ring at the pole distal to the septum is disassembled, while the anti-anti-sigma activity of dephosphorylated SpoIIAA is in somehow exclusively restricted to the forespore. The exquisite compartmentalization of σ^F activity strongly suggests that both forms of SpoIIAA might be also compartmentalized (III). This interpretation raises the possibility that dephosphorylated SpoIIAA sequestered in the EZ ring located on the opposite pole where the septum was formed (left ring in II) is degraded soon after asymmetric division or that SpoIIAA-P was not dephosphorylated in this incipient EZ ring due to a presumed lower concentration of SpoIIE on it (IIi).
B. Strain RG2057 (*Pspac-spoIIE spoIIA-lacZ*) carrying the multicopy plasmid pAR341 was grown in SSM until 1 h before the onset of the stationary phase when IPTG was added to one half of the culture. Both halves (with and without IPTG, x and –■–, respectively) were incubated for several hours and β-galactosidase activity was measured as indicated. The accumulation of β-galactosidase activity from a culture of RG2057 carrying the vector pHT315 is also showed as comparison (–●–).
C. β-galactosidase activity of strain AR19150 (*spoIIG-lacZ spoIIAC::kan*) carrying the multicopy plasmids pHT315 (•), pAR-IIAA (□), pAR-IIAAS58A (⋆), and pAR-IIAAS58D (▲).

inhibitor of Spo0A activation (Fig. 4A). First, we used an engineered *B. subtilis* strain (RG2057) that harboured a Spo0A-dependent *lacZ* fusion and the phosphatase-coding gene *spoIIE* under the control of the P_{spac-} promoter. In addition, the strain RG2057 carried a multicopy plasmid (pAR341) that harboured complete copies of *spoIIAA* and *spoIIAB* (see *Experimental procedures*). A culture of RG2057/pAR341 in sporulation medium without IPTG addition showed normal and temporally regulated Spo0A-directed β-galactosidase activity that was indistinguishable from the control RG2057/pHT315 (Fig. 4B). However, a significant decrease on the accumulation of β-galactosidase activity occurred when IPTG was added, before the onset of the stationary phase, to one-half of the RG2057/pAR341 culture (Fig. 4B). We interpreted that in the absence of IPTG phosphorylation of SpoIIAA by SpoIIAB (both proteins expressed from the multicopy plasmid) yielded SpoIIAA-P as the predominant form of the regulator, which was unable to inhibit the activation of the Spo0A-dependent *spoIIG-lacZ* fusion. Conversely, in the presence of IPTG, we interpreted that the SpoIIE phosphatase expressed from the P_{spac-} fusion competed with the SpoIIAB kinase expressed from the multicopy plasmid generating SpoIIAA from SpoIIAA-P. Under these circumstances a decrease on the expression of the Spo0A-dependent developmental fusion was observed, all of which suggested that the active form of the inhibitor was SpoIIAA and not SpoIIAA-P. To confirm this conclusion we designed a second experiment using strains carrying multicopy plasmids that coded for two different non-phosphorylatable mutant forms of SpoIIAA where Ala or Asp replaced the SpoIIAB-phosphorylable Ser^{58} residue of SpoIIAA (SpoIIAA-S58A and SpoIIAA-S58D, respectively). Therefore, plasmids pAK-IIAAS58A and pAK-IIAAS58D expressing those mutant forms were introduced into a *spoIIAC*⁻ strain. According to our hypothesis, multicopy pAK-IIAAS58A should produce a SpoIIAA-S58A non-phosphorylatable protein with a conformation similar to wild type SpoIIAA, and hence should inhibit the activation of Spo0A. Effectively in Fig. 4C it is showed that pAK-IIAAS58A produced a much more severe negative effect on the induction of the developmental Spo0A-dependent fusion than original pAR-IIAA. On the other hand, multicopy pAK-IIAAS58D should also produce a non-phosphorylatable SpoIIAA protein but as consequence of the nature of the PCR-modification (S→D) the conformation of the new protein (SpoIIAA-S58D) should be more similar to SpoIIAA-P than to SpoIIAA. According to this prediction, pAK-IIAAS58D did not affect the activation of the *lacZ* fusion in comparison with the control strain carrying pHT315 (Fig. 4C). These results confirmed the dephosphorylated form of SpoIIAA, and not SpoIIAA-P, as the active form of the inhibitor of Spo0A activation.

The inhibitory effect of SpoIIAA on Spo0A activation is under normal developmental control of SpoIIAB, SpoIIE and septum morphogenesis

Because SpoIIAA synthesis requires Spo0A~P, and the activity of its dephosphorylated form releasing free and active σ^F is only detected in the forespore compartment when septum formation/maturation has been completed we hypothesize that the described inhibition of Spo0A activation constitutes a negative feedback loop established by the anti-anti-sigma also when cytokinesis is completed. One prediction from this model is that the negative effect on Spo0A activation should be influenced by the natural regulators, SpoIIAB and SpoIIE, of the anti-anti-σ (Fig. 5A). In fact, the balance of the activities of the SpoIIAB kinase and the SpoIIE phosphatase should determine the amount of dephosphorylated SpoIIAA formed and hence, according to our model, the level of active Spo0A also formed after cytokinesis (Lewis *et al*., 1996; Stragier and Losick, 1996; Feucht *et al*., 1999; King *et al*., 1999; Errington, 2001). Some preliminary evidence for this regulatory circuit was obtained with the results showed in Fig. 4B where we used a construct in which the transcription of *spoIIE* was under the control of an IPTG-inducible promoter (P_{spac-}). In a strain harbouring this construct and a multicopy plasmid expressing *spoIIAA* and *spoIIAB* the addition of the IPTG inducer before the onset of stationary phase resulted in an inhibition of β-galactosidase induction under the control of Spo0A~P. This result suggested that indeed the IPTG-dependent SpoIIE phosphatase antagonized the effect of the plasmid-coded SpoIIAB kinase regenerating the active form of the inhibitor (dephosphorylated SpoIIAA) from SpoIIAA-P previously formed by SpoIIAB (Fig. 5A).

To confirm that SpoIIAA established a physiologic negative feedback on Spo0A activation no due to its overexpression from the multicopy plasmid, and to validate the predictions made in Fig. 5A we recurred to the use of strains free of plasmids that in addition harboured mutations that blocked the expression of one or both of the natural regulators of the anti-anti-sigma. First, we analysed the effect of the anti-anti-sigma expressed from the single chromosomal copy of its coding *spoIIA* operon under the control of its normal promoter. To this end we used the strains RG10382 (*spoIIAB::neo*) and RG16663 (Δ*spoIIAAAB::neo*), which also harboured a *spoIIG-lacZ* fusion as reporter of Spo0A activity. As it is showed in Fig. 5B strain RG10382, which codes under the control of the normal *spoIIA* promoter for SpoIIAA but not for SpoIIAB and SpoIIAC showed a lower accumulation of Spo0A-dependent β-galactosidase activity comparing with the isogenic strain RG16663 which did not code for any of the SpoIIA proteins. Because the only difference between

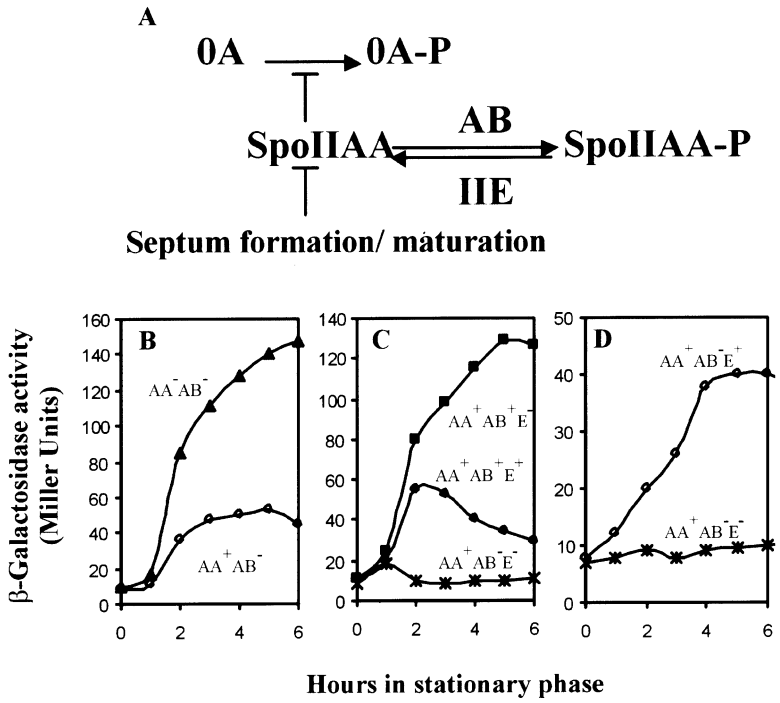


Fig. 5. Post-divisional activation of Spo0A is regulated by the opposing activities of the SpoIIAB kinase/SpoIIE phosphatase and the process of septum formation.
A. The well-characterized regulators of SpoIIAA activity as anti-anti-sigma factor (SpoIIAB, SpoIIE) and the process of septum formation/ maturation should also regulate the activity of SpoIIAA as inhibitor of Spo0A activation as the same form of the protein (dephosphorylated SpoIIAA) harboured both regulatory properties.(B–D) The panels show the accumulation of β-galactosidase activity from a *spoIIG-lacZ* fusion in wild type, *spoIIE*, and *spoIIA* mutant strains free of plasmids.
B. Strains AR10382 *spoIIAB::neo*^{r} (○), and AR16663 *ΔspoIIAAAB::neo*^{r} (▲).
C. Strains AR16182 (●), AR16183 *spoIIE::cat* (■), and AR10383 *spoIIE::cat spoIIAB::neo*^{r} (⋆).
D. Strains AR10383 (⋆), and AR10382 (○).
$AA^{+/-}$, $AB^{+/-}$ and $E^{+/-}$ denotes the absence (–) or presence (+) of the SpoIIAA, SpoIIAB, and SpoIIE developmental proteins respectively.

these two strains was the functional copy of the *spoIIAA* gene this result suggested that high enough levels of SpoIIAA are physiologically formed from the chromosomal copy of *spoIIAA* to account for a normal negative feedback effect on continue Spo0A activation after the first hours of the start of sporulation (Fig. 5B). Furthermore, this negative effect should be expected to depend, as indicated before, on the balance of the opposing activities of the anti-σ^F/kinase SpoIIAB and the septum-located SpoIIE phosphatase that favours the generation of SpoIIAA-P and SpoIIAA respectively (Fig. 5A). In fact, comparison of the accumulation of developmental β-galactosidase activity in *spoIIE*$^+$ and *spoIIE*$^-$ isogenic strains showed that in the absence of the phosphatase there was a considerately higher activity of the Spo0A-dependent *lacZ* fusion clearly evidenced after the first hours of the onset of the stationary phase (Fig. 5C). We interpreted this result considering that the inactive form of the inhibitor SpoIIAA-P was predominant, if not exclusive, under this genetic background (*spoIIE*$^-$*spoIIAB*$^+$) accounting in consequence for the higher activity of Spo0A (Fig. 5C). Furthermore, the upregulated Spo0A-dependent β-galactosidase activity accumulated in this strain decreased dramatically when *spoIIAB* was inactivated (*spoIIE*$^-$*spoIIAB*$^-$, Fig. 5C lower curve). In this situation only dephosphorylated SpoIIAA should be formed after the initial Spo0A-activated transcription of the *spoIIA* operon at the beginning of the stationary phase. Therefore, shortly after its formation and in the absence of its regulatory proteins, dephosphorylated SpoIIAA which is particularly stable, (Pan *et al*. 2001) blocked additional expression of Spo0A-dependent genes (Fig. 5C).

A second prediction that we analysed, based in the model of King *et al*. (1999), was that the process of septation should also regulate the inhibitory effect of SpoIIAA on Spo0A activation (Fig. 5A). In fact, it is believed that dephosphorylation of SpoIIAA starts before cytokinesis. However, this dephosphorylation is not enough to activate σ^F until completion of septation when dephosphorylated SpoIIAA is presumably released from its inhibitory complex (King *et al*., 1999; and Fig. 4A). According to this model we observed that the Spo0A-dependent developmental β-galactosidase activity accumulated in a *spoIIAB*$^-$ *spoIIE*$^+$ strain was considerately higher than the one accumulated in a *spoIIAB*$^-$ *spoIIE*$^-$ strain (Fig. 5D). In both strains, as a result of the *spoIIAB* mutation (*spoIIAB::neo*), only dephosphorylated SpoIIAA might be formed. However in the *spoIIE*$^+$ strain, and in accordance with the model of King *et al*. (1999), we expected (as it happened) that the hypothesized sequestration of dephosphorylated SpoIIAA during septum formation impairs the anti-anti-sigma to block Spo0A activation comparing with a strain where septum formation is prematurely blocked. In fact, in the used strain harbouring the *spoIIE* mutation (derived from SL7240, see Khvorova *et al*., 1998) septation is impaired at an early stage (before EZ-ring formation), and in accordance with the proposed model sequestration of dephosphorylated SpoIIAA should not occur (King *et al*., 1999). Under these circumstances continue generation of free and dephosphorylated SpoIIAA might explain the

extremely low activity of the Spo0A-dependent reporter fusion detected in the *spoIIAB*$^{-}$ *spoIIE*$^{-}$ strain (Fig. 5D). According to our interpretation it was previously found (Levin and Losick, 1994) that in a *divIC* mutant blocked at a late stage of septation, after EZ-ring formation, the level of expression of a Spo0A-dependent *spoIIA-lacZ* fusion reached a fivefold higher level than in the wild-type strain. Furthermore, it was demonstrated that in this mutant dephosphorylated SpoIIAA accumulated to high levels but σ^F remained completely inactive (King *et al*., 1999). To conciliate these findings it was proposed that the anti-anti-σ was permanently sequestered, and hence inactive, in the EZ-ring as consequence of the inability of this strain to complete septum formation/maturation. The presumed inability of dephosphorylated SpoIIAA to abandon in the *divIC* mutant the EZ-ring might explain now, taking into consideration our present results, the unexpected higher activity of the Spo0A-dependent *lacZ* fusion obtained in that study (Levin and Losick, 1994). Taken together these results (Fig. 5B–D) suggested, as it was predicted, that the new regulatory function of SpoIIAA (inhibition of Spo0A activation) was under the same regulatory mechanisms that its well known activity as anti-anti-sigma.

A possible scenario for the new regulatory function of SpoIIAA during spore development

The current models that explain how $\sigma^{F.}$ activation is prevented before completion of septation, taking into consideration the evidences indicating that SpoIIAA-P would be dephosphorylated in the predivisional sporangium, and how accurate activation of this σ factor is specifically restricted to the forespore after septum formation are controversial and not universally accepted (for a review see Errington, 2001). However, the fact that the activity of dephosphorylated SpoIIAA activating σ^F is only detected in the forespore after asymmetric division strongly suggests that the active form of dephosphorylated SpoIIAA would be predominantly restricted to this compartment (see also Fig. 4A-III). Taking into consideration this interpretation, and the results that indicated that both regulatory properties of SpoIIAA (activation of σ^F and inhibition of Spo0A) were similarly regulated (see Fig. 5) prompt us to hypothesize that the SpoIIAA-directed inhibition of Spo0A activation would also take place essentially into the forespore (Fig. 6A and B). So, what might be the role of this SpoIIAA-dependent regulatory feedback during the normal development of the spore? We hypothesize that this inhibition of Spo0A activation would has two immediate consequences. First, it should contribute to block the expression of Spo0A-dependent genes whose products are not longer necessary at that stage of development. One firm candidate is the product of the *spoIIE* gene: the bifunctional phosphatase and morphogenetic protein SpoIIE. In fact, it has been extensively reported that this developmental protein contributes to the proper polar localization of the Z rings at the onset of sporulation and the formation of a functional septum besides its crucial role to confine σ^F activity to the forespore compartment of the developing sporangium (for a review see Errington, 2001). All of these SpoIIE-mediated key activities have been fully achieved by the moment that σ^F activity becomes compartmentalized, and therefore SpoIIAA would prevent unnecessary *de novo* transcription of *spoIIE*. By other hand, we hypothesize that this inhibitory feedback could also contribute to the proper cell-specific activation of σ^F and σ^E. In fact, recently it was reported that σ^E continues to accumulate in the mother cell well after the formation of the polar septum (Fujita and Losick, 2002). These authors showed that this accumulation resulted from the selective and persistent transcription of the Spo0A-dependent *spoIIG* operon in the mother cell and the absence of its transcription after polar septation in the forespore. Our results are in concordance with the absence of *spoIIG* transcription into the forespore taking into consideration the inhibitory effect of SpoIIAA on Spo0A activation described here. In addition, it was showed that a $\sigma^{E.}$–GFP fusion protein disappeared more quickly in the forespore than in the mother cell (Fujita and Losick, 2002). The rapid disappearance of the $\sigma^{E.}$-GFP signal in the forespore could be explained considering that pro-σ^E/σ^E is equally unstable in both compartments but is only replenished in the mother cell as a result of the selective SpoIIAA-directed inhibition of its synthesis in the forespore (Fig. 6C and D).

Simultaneously, how this negative feedback would contribute to the mechanism/s responsible for the proper cell-specific activation of $\sigma^{F.}$? Cell-specific activation of σ^F is achieved by multiple overlapping and reinforcing pathways (Lewis *et al*., 1996; Frandsen *et al*., 1999; Losick and Dworkin, 1999; Pan *et al*., 2001). Accordingly, it was recently demonstrated that $\sigma^{F.}$ activation is partly governed by the position of the *spoIIAB* gene (Dworkin and Losick, 2001). Because the entire *spoIIA* operon is located in one of the last regions of the chromosome to enter the forespore (King *et al*., 1999; Dworkin and Losick, 2001), the gene for the SpoIIAB anti-σ^F factor is initially not present in the forespore. Whereas degradation of SpoIIAB would be expected to occur in both the mother cell and the forespore (Pan *et al*. 2001), the transient absence of *spoIIAB* would prevent the anti-σ^F factor from being replenished in the forespore (Dworkin and Losick, 2001). This would cause a decreases in SpoIIAB levels in the forespore relative to its more stable partners σ^F and SpoIIAA (Dworkin and Losick, 2001; Pan *et al*. 2001). According to this elegant model SpoIIAB is partially depleted from the forespore during the interval when the *spoIIAB*

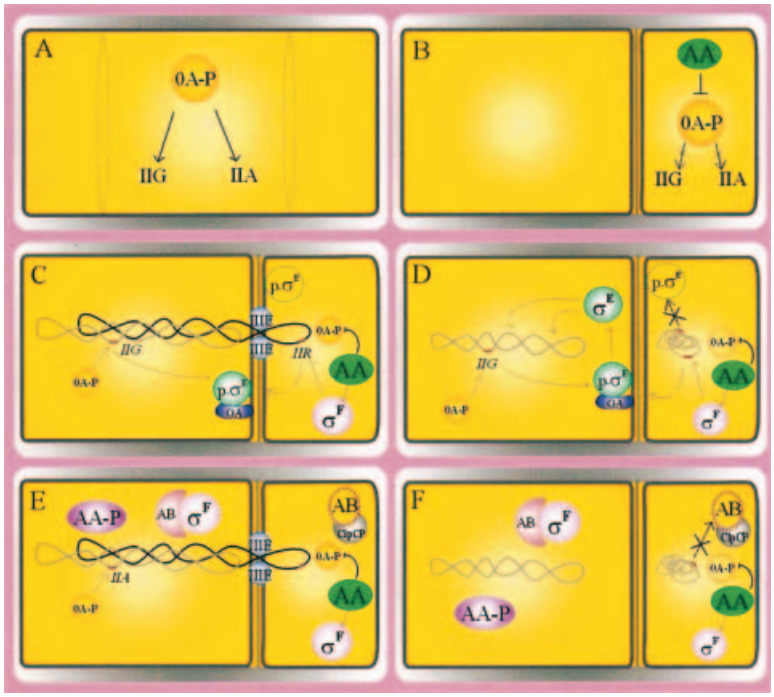


Fig. 6. A workable model for the contribution of the negative effect of SpoIIAA on expression of Spo0A-dependent genes during development.
A. In the predivisional sporangium Spo0A~P (0A-P) activates the transcription of the operons *spoIIA* and *spoIIG*, and the gene *spoIIE* (not shown).
B. In the post-divisional sporangium the proposed forespore-favoured inhibition of Spo0A activation leads to a blockage of *de novo* transcription of *spoIIE*, *spoIIA* and *spoIIG*.
C. Cell specific activation of σ^E. Following cytokinesis only about the 30% of the forespore-destined chromosome is trapped in that compartment leaving the *spoIIG* operon transiently outside of the developing endospore. Predivisional pro-σ^E disappears from this compartment because of a selective degradation by an unknown protease (not shown) or because of the intrinsic instability of the pro-protein (Fujita and Losick, 2002). Meanwhile, the anti-anti-sigma factor SpoIIAA (denoted AA) is free and active in the cytoplasm of the forespore to liberate σ^F from its complex with the anti-sigma factor SpoIIAB (AB), which now is substrate for degradation by the ClpCP protease (not shown, Pan *et al.,* 2001). Free σ^F set in motion cell type-specific gene expression, including transcription of *spoIIR*, which produces a transmembrane activating signal for pro- σ^E processing in the mother cell compartment (Hofmeister *et al.,* 1995; Karow *et al.*, 1995; Londoño-Vallejo and Stragier, 1995). This sequence of events ensure that σ^E is not activated until after the septum is formed, and rapid activation of σ^E following septation may be important in preventing further septation (Fujita and Losick, 2002). In addition, free SpoIIAA inhibits *de novo* formation of Spo0A~P (0A-P).
D. When translocation of the forespore-destined chromosome has been completed active levels of Spo0A~P might be too low to activate the transcription of the recently translocated *spoIIG* operon. This transcriptional repression along with proteolysis and/or protein-instability contributes to the absence of pro-σ^E/σ^E in the forespore. Persistent transcription of *spoIIG* under Spo0A~P control in the mother cell compartment allows constitutive synthesis of SpoIIGA/pro-σ^E (Fujita and Losick, 2002) that are activated, as said, by the forespore-signalling protein SpoIIR.
E. Cell specific activation of σ^F. After cytokinesis the *spoIIA* operon is also transiently outside the forespore. There the anti-anti-sigma (AA) activates σ^F releasing it from the complex with AB, which is degraded by ClpCP (Pan *et al.*, 2001). Also as indicated free IIAA inhibits *de novo* generation of 0A-P. Simultaneously, in the mother cell compartment SpoIIAA might be largely present in its inactive form (AA-P), and σ^F held inactive in a complex by IIAB. This complex would protect the anti-sigma factor from degradation by ClpCP (Pan *et al.*, 2001).
F. When the translocation of the *spoIIA* operon (coding for IIAB) to the forespore is completed active levels of 0A-P should be too low to activate its transcription. Therefore, IIAB is depleted in the forespore ensuring σ^F activation into that compartment.

gene is excluded from this compartment. However, because the half-life of SpoIIAB is approximately 25 min (Pan *et al.*, 2001), and the time required for complete DNA translocation could be as short as 10–15 min (Lewis *et al.*, 1994; Pogliano *et al.*, 1999) the anti-σ^F factor would undergo only a modest decrease in concentration in the forespore if its coding *spoIIAB* gene were transcribed after its translocation. This apparent drawback of the model might be solved by the model presented here, which suggests that by the time when *spoIIAB* is translocated into the forespore its transcription would be kept off because the generation of its activator (Spo0A~P) should be blocked by SpoIIAA helping to ensure the proper activation of σ^F (Fig. 6E and F).

A major challenge in developmental biology is the problem of understanding how transcription factors are activated in a cell-specific manner during differentiation considering that the proper temporal and spatial regulation of key cell-fate determinants is crucial for the progress and success of the developmental programme (Losick and Dworkin, 1999; Scott and Posakony, 2002). Accurate activation of σ^F and σ^E specifically restricted to the fore-

spore and mother cell compartments of the sporangium involves a combination of several reinforcing and overlapping mechanisms not completely understood (Lewis *et al.*, 1996; Feucht *et al.*, 1999; Frandsen *et al.*, 1999; King *et al.*, 1999; Dworkin and Losick, 2001; Pan *et al.* 2001; Fujita and Losick, 2002). The main contribution of this work are the evidences presented for a novel regulatory function of SpoIIAA on the activation of the master transcription factor Spo0A that might reinforce the models for the cell-specific activation of σ^F and σ^E.

Experimental procedures

Strains and growth conditions

The *B. subtilis* strains used in this study are JH642 derivates and are described in Table 1. For sporulation efficiency, *B. subtilis* strains were grown in Schaeffer's sporulation medium (SSM) and then treated with $CHCl_3$ 10% for 15 min before plating (Wang *et al.*, 1997). Transformation of *B. subtilis* was carried out as described (Wang *et al.*, 1997). Beta-galactosidase in *B. subtilis* strains harbouring *lacZ* fusions were assayed as described previously, and the specific activity was expressed in Miller Units (Wang *et al.*, 1997). The β-galactosidase experiments described in the figures were independently repeated five times, and a representative set of results is showed in each figure. *Escherichia coli* strain DH5α (Gibco/BRL) was used to maintain all plasmids.

Plasmid and strains constructions

Plasmids pAR230, pAR341, and pAK22 are sporulation inhibitory plasmids isolated from a *B. subtilis* JH642 chromosomal library constructed by ligating partial *Sau*3A restriction fragments in the multiple cloning site of the pHT315 shuttle vector (25–50 copies/cell) as previously described (Wang *et al.*, 1997). Plasmid pAR230 was digested with *Kpn*I (with a cut site at the multiple cloning site) and *Eco*RI that released a 1.4 kb fragment containing the complete copy of *dacF* gene (see Fig. 1H). This fragment was ligated, maintaining the original orientation, in the shuttle vector pHT315 to generate pARdacF. Plasmid pAR230 was also digested with *Pvu*II and *Bst*XI to release a 670 bp fragment containing the *spoIIAA* gene (Fig. 1H). This fragment was treated with the Klenow fragment of DNA polymerase I and ligated in pHT315 previously digested with *Sma*I to generate pAR-IIAA (Fig. 1H). Plasmid pAR-IIAA::Kan was obtained from pAR-IIAA by insertion of the 1.4 kb kanamycin resistance cassette from pJM114 in the *Ava*I internal site of *spoIIAA* (Fig. 1H). Plasmid pAR341 harbours a *B. subtilis* insert which perfectly overlaps with the *dacF* extreme of pAR230 but harbours in addition a complete copy of *spoIIAB* and a short segment of *spoIIAC* which should code for the first 13 AA of SpoIIAC (σ^F). A wild-type *B. subtilis* strain harbouring the strong σ^F-dependent *spoIIQ-lacZ* fusion and this multicopy plasmid completely lacked of β-galactosidase activity (data not shown), which suggested that SpoIIAB phosphorylated SpoIIAA and sequestered all the available molecules of σ^F. This pAR341 plasmid was used for the experiment described in Fig. 4.

Table 1. *Bacillus subtilis* strains.

Strain	Relevant genotype	Comments and/or source
JH 642	*trpC2 pheA1*	Laboratory stock (Hoch, J.A)
JH 16124	*amyE::spoIIA-lacZ cat*	Laboratory stock
JH 16182	*amyE::spoIIG-lacZ cat*	Laboratory stock
JH 16480	*amyE::spoIIE-lacZ cat*	Laboratory stock
JH 19004	*amyE::spo0F-lacZ cat*	Laboratory stock
JH 19005	*amyE::spo0A-lacZ cat*	Laboratory stock
JH 12604	*amyE::abrB-lacZ cat*	Laboratory stock
RG 2051	*amyE::spoIIQ-lacZ cat*	MO2051 (Stragier P) (→) JH642
RG 1679	*amyE::spoIID-lacZ cat*	MO1679 (Stragier P) (→) JH642
RG 6418	*amyE::spoIIR-lacZ cat*	SL6418 (Piggot P) (→) JH642
RG 19148	*spoIIAC::kan*	This study
RG 19149	*amyE::spoIIA-lacZ cat, spoIIAC::kan*	RG19148 (→) JH16124
RG 19150	*amyE::spoIIG-lacZ cat, spoIIAC::kan*	RG19148 (→) JH16182
RG 19151	*amyE::spoIIE-lacZ cat, spoIIAC::kan*	RG19148 (→) JH16480
RG19152	*amyE::spo0F-lacZ cat, spoIIAC::kan*	RG18148 (→) JH19004
RG19153	*amyE::abrB-lacZ cat, spoIIAC::kan*	RG19148 (→) JH12604
RG 1113	*spoIIA⁺@IIA-lacZ neo*	KI 1113 (Grossman A.D) (→) JH642
RG 1114	*spoIIE⁺@IIE-lacZ neo*	KI 1114 (Grossman A.D) (→) JH642
RG 1115	*amyE:: P-spac-sad67 cat*	Sik 31 (Grossman A.D) (→) JH642
RG 1116	*amyE:: P-spac-sad67 cat, spoIIA⁺@IIA-lacZ neo*	RG1115 (→) RG1113
RG 1117	*amyE:: P-spac-sad67 cat, spoIIE⁺@IIE-lacZ neo*	RG1115 (→) RG1114
RG 2057	*spoIIA⁺@IIA-lacZ neo, P-spac-spoIIE@IIE cat*	SL7243 (Piggot P) (→) RG1113
RG 16182	*amyE::spoIIG-lacZ cat::spc*	pCm::Spc (BGSC) (→) JH16182
RG 10381	*spoIIAB::neo*	This study
RG 10382	*amyE::spoIIG-lacZ cat::spc, spoIIAB::neo*	RG10381 (→) RG16182
RG 16663	*amyE::spoIIG-lacZ cat::spc,Δ spoIIAAAB::neo*	SL6663 (Piggot P) (→) RG16182
RG 16183	*amyE::spoIIG-lacZ cat::spc,spoIIE::cat*	SL7240 (Piggot P) (→) RG16182
RG 10383	*amyE::spoIIG-lacZ cat::spc, spoIIE::cat, spoIIAB::neo*	SL7243 (Piggot P) (→) RG16183

BGSC: Bacillus Genetic Stock Center, Ohio State University, Ohio, USA.

Plasmid pAK22 is similar to pAR341 but harbours in addition a complete copy of *spoIIAC*. This plasmid was used for the construction of the *B. subtilis spoIIAC*$^-$ strain RG19148 as described below.

Plasmids pAK-IIAAS58A and pAK-IIAAS58D were constructed using Quick Change site-directed mutagenesis kit from Stratagene, using pAR-IIAA as template and oligonucleotides AAS58A-F (5′-CC TTT ATG GAC < GCG > TCG GGG CTT GG-3′), AAS58A-R (5′-CC AAG CCC CGA < CGC > GTC CAT AAA GG-3′); and AAS58D-F (5′-CC TTT ATG GAC < GA > C TCG GGG CTT GG-3′), AAS58D-R (5′-CC AAG CCC CGA G <TC > GTC CAT AAA GG-3′) as primer sets for construction of pAK-IIAAS58A and pAK-IIAAS58D respectively. Bases in < > denotes mismatched bases from original pAR-IIAA sequence. The introduction of the desire mutations was verified by DNA sequencing.

For the construction of strain RG19148 (*spoIIAC::kan*) and its derivates we used the following strategy: a 2.8 kb insert from pAK22 containing *spoIIAC* was released as a *Pst*I/*Bam*HI fragment and ligated into pUC19 generating pUC19-22. The 1.4 kb *Sma*I/*Hin*cII kanamycin-resistant cassette from pJM114 was ligated into pUC19-22 at the unique *Hin*cII site located within *spoIIAC* (Fig. 1H). The new plasmid, pUC19-spoIIAC::kan, was linearized and transformed into JH642 selecting for resistance to kanamycin, which generated, by a double crossover event, the sporulation deficient strain RG19148. Chromosomal DNA of RG19148 was used to transform competent cells of *B. subtilis* strains harbouring Spo0A and σ^F-dependent *lacZ* fusions. In the case of strains harbouring σ^F-dependent *lacZ* fusions (RG2051 *spoIIQ-lacZ*, and RG6418 *spoIIR-lacZ*) the acquisition of *spoIIAC* mutation resulted in the complete loss of β-galactosidase activity (data not shown). For construction of strain RG10381 (*spoIIAB::neo*) we amplified a 370 bp internal fragment of *spoIIAB* using chromosomal DNA of JH642 as template and the oligonucleotides 5′-CTTTGTTAAGC**TT**ATCGTTGTTCCCATTTC-3′ *Hin*dIII, and 5′-CAGAATGAA**G**C**T**TTCGCCCGTGTGACAG-3′ *Hin*dIII. The amplified PCR fragment was cloned into pUC19 at the unique *Hin*dIII site generating pUC19-Δ*IIAB*. A neomycin-resistant cassette was cloned at the unique *Nco*I site internal to the *spoIIAB* fragment, which generated pUC19-Δ*IIAB::neo*. Its linearization and transformation in competent cells of JH642 resulted in integration by a double crossover event that yield the sporulation deficient strain RG10381. In this strain *spoIIAC* should also be inactivated because of a polar effect, which was confirmed by the inability of RG10381 to induce the strong SpoIIAC (σ^F)-dependent *spoIIQ-lacZ* fusion (data not shown).

Acknowledgements

We are indebted to Diego de Mendoza for his support during the beginning of this work. We thank to Patrick Piggot, David Hilbert, Alan Grossman, Richard Losick and Patrick Stragier for their critical comments and suggestions during the course of this work. We also thank to Alan Grossman, Iren Kurtser, Patrick Piggot, Patrick Stragier, Fujio Kawamura, Masaya Fujita and Daniel Zeigler (from BGSC) for providing strains and plasmids. This research was partially supported by grants awarded to R.G. from the following national agencies: CONICET (PIP-03052), FONCyT (PICT-0103379), Rosario University (PIP202-19/B135) and Fundación Antorchas (14022-57). Finally, we thank to Paul Merolla from EnviroMetric Laboratories (USA-Argentina) for critical reading of the manuscript.

References

Burbulys, D., Trach, K.A., and Hoch, J.A. (1991) The initiation of sporulation in *Bacillus subtilis* is controlled by a multicomponent phosphorelay. *Cell* **64:** 545–552.

Burkholder, W., Kurtser, I., and Grossman, A. (2001) Replication initiation proteins regulate a developmental checkpoint in *Bacillus subtilis*. *Cell* **104:** 269–279.

Cervin, M., Spiegelman, G., Raether, B., Ohlsen, K., Perego, M., and Hoch, J. (1998) A negative regulator linking chromosome segregation to developmental transcription in *Bacillus subtilis*. *Mol Microbiol* **29:** 85–95.

Chung, J.D., Stephanopolus, G., Ireton, K., and Grossman, A.D. (1994) Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism control the initiation of sporulation. *J Bacteriol* **176:** 1977–1984.

Dworkin, J., and Losick, R. (2001) Differential gene expression governed by chromosomal spatial asymmetry. *Cell* **107:** 339–346.

Errington, J. (2001) Septation and chromosome segregation during sporulation in *Bacillus subtilis*. *Current Opinion Microbiol* **4:** 660–668.

Feucht, A., Daniel, R., and Errington, J. (1999) Characterization of a morphological checkpoint coupling cell-specific transcription to septation in *Bacillus subtilis*. *Mol Microbiol* **33:** 1015–1026.

Frandsen, N., Barák, I., Karmazyn-Campelli, C., and Stragier, P. (1999) Transient gene asymmetry during sporulation and establishment of cell specificity in *Bacillus subtilis*. *Genes Dev* **13:** 394–399.

Fujita, M., and Losick, R. (2002) An investigation into the compartmentalization of the sporulation transcription factor σ^E in *Bacillus subtilis*. *Mol Microbiol* **43:** 27–38.

Hamon, M.A., and Lazazzera, B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* **42:** 1199–1209.

Hofmeister, A., Londoño-Vallejo, A., Harry, E., Stragier, P., and Losick, R. (1995) Extracellular signal protein triggering the proteolytic activation by a developmental transcription factor in *Bacillus subtilis*. *Cell* **83:** 219–226.

Ireton, K., Rudner, D.Z., Siranosian, K.J., and Grossman, A.D. (1993) Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev* **7:** 283–294.

Jiang, M., Shao, W., Perego, M., and Hoch, J.A. (2000a) Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol* **38:** 535–542.

Jiang, M., Grau, R., and Perego, M. (2000b) Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. *J Bacteriol* **182:** 303–310.

Kanamaru, K., Stephenson, S., and Perego, M. (2002) Overexpression of the PepF oligopeptidese inhibits sporulation initiation in *Bacillus subtilis*. *J Bacteriol* **184:** 43–50.

Karow, M.L., Glaser, P., and Piggot, P. (1995) Identification of a gene, *spoIIR*, which links the activation of σ^E to the transcriptional activity of σ^F during sporulation in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **92:** 2012–2016.

Khvorova, A., Zhang, L., Higgins, M., and Piggot, P. (1998) The *spoIIE* locus is involved in the Spo0A-dependent switch in the location of FtsZ rings in *Bacillus subtilis*. *J Bacteriol* **180:** 1256–1260.

King, N., Dreesen, O., Stragier, P., Pogliano, K., and Losick, R. (1999) Septation, dephosphorylation, and the activation of σ^F during sporulation in *Bacillus subtilis*. *Genes Dev* **13:** 1156–1167.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A., Alloni, G., Azevedo, V.*et al.* (1997) The complete genome sequence of the Gram-positive model organism *Bacillus subtilis* (strain 168). *Nature* **390:** 249–256.

Levin, P.A., and Losick, R. (1994) Characterization of a cell division gene from *Bacillus subtilis* that is required for vegetative and sporulation septum formation. *J Bacteriol* **176:** 1451–1459.

Lewis, P., Partridge, S.R., and Errington, J. (1994) σ factors, asymmetry and the determination of cell fate in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **91:** 3849–3853.

Lewis, P., Magnin, T., and Errington, J. (1996) Compartmentalized distribution of the proteins controlling the prespore-specific transcription factor sigma F of *Bacillus subtilis*. *Genes Cells* **10:** 881–894.

Londoño-Vallejo, A., and Stragier, P. (1995) Cell-cell signaling pathway activating a developmental transcription factor in *Bacillus subtilis*. *Genes Dev* **9:** 503–508.

Losick, R., and Dworkin, J. (1999) Linking asymmetric division to cell fate: teaching an old microbe new tricks. *Genes Dev* **13:** 377–381.

Losick, R., and Stragier, P. (1992) Crisscross regulation of cell-type-specific gene expression during development in *Bacillus subtilis*. *Nature* **355:** 601–604.

Mandic-Mulec, I., Gaur, N., Bai, U., and Smith, I. (1992) Sin, a stage-specific repressor of cellular differentiation. *J Bacteriol* **174:** 3561–3569.

Mandic-Mulet, I., Doukhan, L., and Smith, I. (1995) The *Bacillus subtilis* SinR protein is a repressor of the key sporulation gene spo0A. *J Bacteriol* **177:** 4619–4627.

Pan, Q., Garsin, D., and Losick, R. (2001) Self-reinforcing activation of a cell-specific transcription factor by proteolysis of an anti-σ factor in *B. subtilis*. *Mol Cell* **8:** 873–883.

Perego, M. (2001) A new family of aspartyl phosphate phosphatases targeting the sporulation transcription factor Spo0A of *Bacillus subtilis*. *Mol Microbiol* **42:** 133–143.

Perego, M., Spiegelman, G.B., and Hoch, J.A. (1988) Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. *Mol Microbiol* **2:** 689–699.

Peters, H.K., and Haldenwang, W.G. (1994) Isolation of a *Bacillus subtilis spoIIGA* allele that suppresses processing-negative mutations in the pro-σ^E gene (*sigE*). *J Bacteriol* **176:** 7763–7766.

Piggot, P.J., and Coote, J.G. (1976) Genetic aspects of bacterial endospore formation. *Bacteriol Rev* **40:** 908–962.

Pogliano, J., Osborne, N., Sharp, M.D., Abanes-De Mello, A., Perez, A., Sun, Y.L., and Pogliano, K. (1999) A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. *Mol Microbiol* **31:** 1149–1159.

Scott, B., and Posakony, J.W. (2002) Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev* **16:** 1167–1181.

Stragier, P., and Losick, R. (1996) Molecular genetics of sporulation in *Bacillus subtilis*. *Annu Rev Genet* **30:** 297–341.

Stragier, P., Bonamy, C., and Karmazyn-Campelli, C. (1988) Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. *Cell* **52:** 697–704.

Strauch, M.A., Wu, J.J., Jonas, R.H., and Hoch, J.A. (1993) A positive feedback loop controls transcription of the *spo0F* gene, a component of the sporulation phosphorelay in *Bacillus subtilis*. *Mol Microbiol* **7:** 967–974.

Wang, L., / Grau, R., Perego, M., and Hoch, J.A. (1997) A novel histidine kinase inhibitor regulating development in *Bacillus subtilis*. *Genes Dev* **11:** 2569–2579.

Zhang, B., Struffi, P., and Kroos, L. (1999) σ^K can negatively regulate *sigE* expression by two different mechanisms during sporulation of *Bacillus subtilis*. *J Bacteriol* **181:** 4081–4088.