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REGULATION OF THE PHOSPHORELAY AND THE INITIATION OF SPORULATION IN *BACILLUS SUBTILIS*

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ABSTRACT

The initiation of sporulation of bacteria is a complex cellular event controlled by an extensive network of regulatory proteins that serve to ensure that a cell embarks on this differentiation process only when appropriate conditions are met. The major signal-transduction pathway for the initiation of sporulation is the phosphorelay, which responds to environmental, cell cycle, and metabolic signals, and phosphorylates the Spo0A transcription factor activating its function. Signal input into the phosphorelay occurs through activation of kinases to phosphorylate a secondary-messenger protein, Spo0F. Spo0F~P serves as a substrate for phosphoprotein phosphotransferase, Spo0B, which phosphorylates Spo0A. The pathway is regulated by transcriptional control of its component proteins and by regulating phosphate flux through the pathway. This is accomplished by several regulatory proteins, and by activated

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Spo0A, which regulates transcription of genes for its own synthesis. Spo0A~P indirectly controls the transcription of numerous genes by regulating the level of other transcription regulators and directly activates the transcription of several regulatory proteins and sigma factors required for progression to the second stage of sporulation. Although the pathway and regulatory proteins have been identified, the signals and effectors for these regulators remain a mystery.

Introduction

Spore formation is one of the more interesting types of morphological differentiation in bacteria. This process is energy-intensive, involves the activation of numerous specific genes in a temporal sequence, and requires several hours to complete. Hence, sporulation is subject to an extensive control network that serves to ensure that a cell only embarks on this differentiation process when appropriate conditions are met. What are these conditions? What environmental and metabolic factors provide input into this network to induce sporulation? How does a cell monitor these signals and convert that information into a decision to either divide or sporulate? These are some of the questions that were asked when studies were begun of the control of the initiation process in *Bacillus subtilis*. Figure 1 graphically presents the problem.

The initiation of sporulation can only occur during a certain time period in

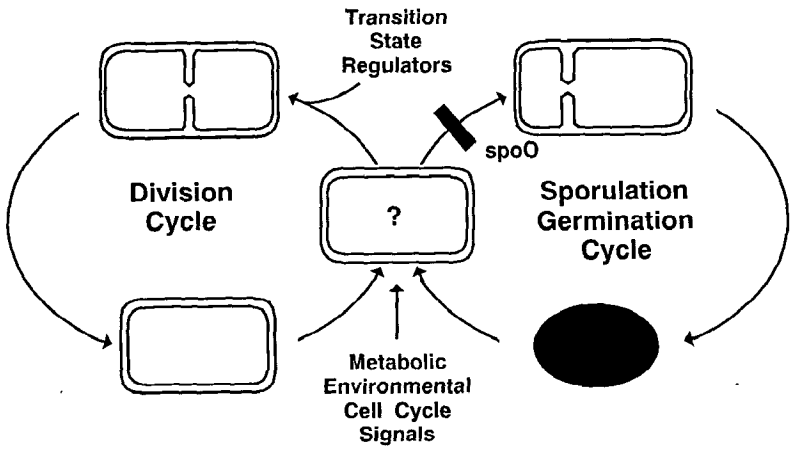

Figure 1 Factors in the initiation of sporulation. At a certain time in the cell cycle, the cell must monitor its metabolic and environmental signals and determine whether to divide or initiate sporulation. Transition-state regulators serve to direct the cell toward division rather than sporulation and respond to unknown signals. The *spo0* mutants are blocked at the very early stages of sporulation and prevent all sporulation-specific transcription.

the cell's cycle (26). In some regards, sporulation and division differ only by the location of the septum in the dividing cell. In a vegetatively growing cell, the septum is placed in the middle of the cell and two cells result, whereas in sporulation, the septum that divides the two chromosomes is placed at one end of the cell, ultimately resulting in two cells within a single cell. Regardless of the outcome, the decision to initiate sporulation cannot be made at just any time during the division cycle, but rather must be made during a distinct period or window within the cell cycle. Because sporulation normally occurs under conditions of nutritional deprivation, the cell is presumed to monitor certain environmental and metabolic signals in order to help it make this decision. Clearly, under conditions of nutritional excess and low population density, growth is favored over survival because competition depends upon increasing cell number. On the other hand, survival at high population densities, when competition for nutrients is intense, is an appropriate cause to initiate sporulation and proceed with this complex process. Thus, both nutritional signals from the environment or from the metabolism of the cell, as well as cell cycle– and density–dependent signals, might very well be involved in these initial events.

One approach to identifying the mechanism of sporulation initiation has been the isolation of mutants blocked in the initiation process (18). These mutants are termed *spo0,* or stage 0, mutants and are most commonly distinguished by their inability to produce any of the characteristic morphological structures of sporulation, as well as by defects in their expression of many enzymes, such as subtilisin, that are coincidentally produced at the very earliest stages of sporulation and/or stationary phase. The *spo0* mutants are thought to be locked in exponential growth in that they continue to grow under nutritional conditions that would normally induce sporulation, and they appear to maintain growth until the nutrients are exhausted, whereupon cell lysis occurs. Clearly, the regulation of sporulation is inexorably coupled with processes that are characteristic of stationary phase but not necessarily of sporulation. Thus, in a normal culture grown in the laboratory, sporulation occurs during the stationary phase, and many of the processes, alternate pathways, and enzymes formed during the early part of stationary phase are controlled along with sporulation because the cell controls sporulation and many of the stationary-phase processes by a single transcription factor, Spo0A. The Spo0A transcription factor and the control of its activation are the subject of this review.

spo0 *Genes and the Initiation Mechanism*

Many of the early studies of sporulation genetics dealt with the isolation of mutants deficient in the sporulation process, characterization of such mutants as to the stage of sporulation in which they stopped, and genetic mapping to

differentiate and to characterize the various genetic loci involved in the sporulation process (38). Among the mutations were the *spo0* alleles thought to define the central processing unit that received the sporulation signals and transduced this information into transcriptional activation of the sporulation process (18). Genetics alone was not sufficient to characterize the products of these genes, but the cloning and identification of the size and structure of the protein product of such genes was more informative. Furthermore, molecular cloning allowed the large-scale expression of the products of the *spo0* genes. Cloning also allowed purification of the products so that in vitro biochemistry could be undertaken to investigate their functions that previously were unknown.

The first *spo0* gene to be identified as to function was the product of the *spo0H* gene (8). Sequencing studies led to a deduced structure for the *spo0H* gene product, which had some homology to bacterial sigma factors. In vitro studies of the *spo0H* gene product led to its confirmation as a sigma factor, now known as σ^H (9). This sigma factor is very important in sporulation because it is necessary, but not sufficient, for high-level transcription of the *spo0A*, *spo0F*, and the *kinA* genes, along with several other genes that have no relevance to the sporulation process (39). The *spo0H* gene codes for a sigma factor that regulates sporulation genes and probably other genes that are expressed and function in early stationary phase (20). These studies eliminated the *spo0H* gene product as a regulatory component of the central processing unit and focused attention onto the other *spo0* genes.

Sequence studies of the *spo0A* and *spo0F* genes were exceptionally informative as to the function of their deduced gene products. Both Spo0A and Spo0F proteins had homology to an emerging class of regulators called two-component regulatory systems (30, 46). Two-component systems are thought to be simple signal transducing switches by which an environmental signal of one kind or another activates the sensor kinase, which then activates a response-regulator protein to promote the transcription of genes specific for the particular environmental change that activated the kinase. Activation results in autophosphorylation of the kinase, which then can transfer this phosphate to the response regulator, activating its transcriptional activities. These two-component switches are widespread in bacteria, and cells are thought to contain many different pairs of these switches, enabling them to respond to a wide variety of different environmental situations. The finding of two-component switches in *spo0* genes was particularly satisfying because the environment was thought to be one of the major inducers of the sporulation process.

The *spo0A* gene coded for a protein of 29,691 M_r with the typical structure of a transcription factor of two-component systems (11, 23). The amino terminal half of the protein was homologous to response regulators, whereas

the carboxyl half of the protein was unique and distinct from any other known response-regulator proteins. The product of the *spo0F* gene is a protein of 14,286 M_r without the carboxy terminal domain characteristic of transcription factors (59). At that time, the only other protein known to consist only of a response-regulator homologous domain was the CheY protein of *Salmonella typhimurium* (45). This protein acts as a vital component of the chemotaxic response of this organism. Spo0F, on the other hand, was known to not affect chemotaxis in an analogous manner, and therefore, its function remained obscure. The most curious aspect of *spo0* mutants was that none of the genes sequenced for any of the loci produced a sensor kinase molecule. Because it was thought that all response regulators were activated by phosphorylation from a sensor kinase (28), the absence of such a protein from the repertoire of genes known to be involved in sporulation initiation was particularly mystifying.

Ultimately, an important kinase for sporulation was unveiled serendipitously from the routine sequencing of a gene that gave a stage II phenotype, *spoIIJ*. The gene encoded a protein homologous to sensor kinases but without a gene for a response regulator in the same transcription unit (1, 31). Expression of this gene in *Escherichia coli* with subsequent purification of the gene product showed that the *spoIIJ* locus did indeed code for a kinase that functioned to phosphorylate both the Spo0F and the Spo0A molecules, although the relative activity on Spo0F was much higher than that observed for Spo0A (31). Therefore, a sensor kinase had been identified that phosphorylated the Spo0F protein well, yet neither the environmental stimulus for this kinase nor the function of the phosphorylated Spo0F was known.

Genetic studies had strongly indicated that the key factor in sporulation initiation was the Spo0A protein because certain mutations in Spo0A were found to suppress the need for the *spo0F* and *spo0B* gene products (22), among others. This led to the conclusion that Spo0A represented the end product that was activated by the other *spo0* gene products (19). This proposed role for Spo0A fit with its presumed transcriptional role based on homology to two-component regulatory systems.

The impasse as to how Spo0A was phosphorylated was eventually solved through in vitro biochemical studies. The first *spo0* gene to be cloned and sequenced was the *spo0B* gene (3, 10). Genetic data on *spo0B* mutants indicated that this gene product was essential for the initiation of sporulation and, therefore, must play an important role in the ultimate activation of Spo0A. The deduced amino acid sequence had no homology to any proteins of known function, and thus, its role in the process of initiation was unknown. Expression and purification of the Spo0B protein allowed its inclusion in biochemical studies of the kinase reactions with KinA, Spo0F, and Spo0A. When purified Spo0B was added to reaction mixtures with KinA, Spo0F, and

Spo0A, the Spo0B protein facilitated the phosphorylation of Spo0A. These experiments revealed a series of reactions that are now termed a *phosphorelay* (4), in which KinA phosphorylates Spo0F to yield Spo0F~P, and the phosphate group from Spo0F~P is transferred to Spo0A by the Spo0B protein, which serves here as a kinase for Spo0A that uses Spo0F~P rather than ATP as a substrate. Thus a protein that played the same role as a kinase was identified from the *spo0* genes but was simply not recognized as such because the homology between kinase proteins is in the ATP-binding domain.

The phosphorelay (Figure 2) consists of four basic reactions; the initial event is the signal transduction that causes KinA to autophosphorylate, although the nature of this signal has not been ascertained. The remainder of the phosphorelay consists of three phosphotransfer reactions in which the phosphate group is transferred first from KinA~P to Spo0F to produce Spo0F~P. Spo0B mediates the transfer of phosphate from Spo0F~P to Spo0A via a phosphorylated enzyme intermediate. Both Spo0F and Spo0A are phosphorylated as a mixed anhydride of an aspartic acid residue, which is characteristic of the phosphorylation of response regulator proteins (41). The enzyme-bound phosphorylated intermediates of KinA and Spo0B have the properties of phosphoramidates, and probably both of these are histidine-phosphate intermediates (4).

When the deduced sequences of the *spo0* gene products became available and clearly none of the genes coded for a kinase, it should have been obvious that more than one kinase could carry out this function. An experiment in which kinase genes were cloned and the effect of their mutations in combination with a *kinA*-gene mutation was tested has identified a second sporulation-specific kinase (61). The data from Table 1 indicate that *kinA*

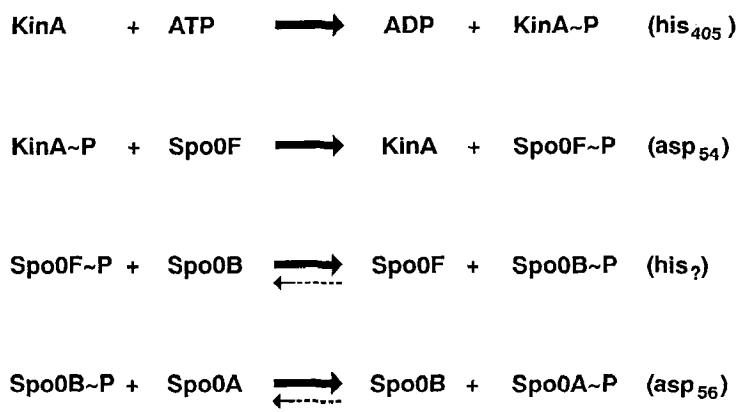

Figure 2 Component reactions of the phosphorelay. The phosphorylated amino acid residue in each product is shown in parentheses.

Table 1 Sporulation frequencies in kinase mutants

Strain background	Relevant genotype	Cells per ml	Spores per ml	Spores (%)
168	Prototroph	1.8×10^8	1.9×10^8	100
168	*kinA::pJM8115*	4.3×10^8	1.1×10^7	2.6
168	*kinB::pJH4906*	4.7×10^8	4.8×10^8	100
168	*kinA::pJM8115 kinB::pJH4906*	6.5×10^7	<10	0

mutations depress sporulation significantly, although this is simply delayed sporulation and eventually most of the cells will sporulate (31). The mutations in *kinB*, on the other hand, have very little effect on sporulation by themselves, but in combination with *kinA* mutations they depress sporulation to almost zero. Thus, both KinA and KinB can act as initiators for the sporulation event, although in vitro studies have not absolutely proven that KinB phosphorylates Spo0F as its primary target. The *kinB* gene is encoded in an operon with another gene, *kapB*, whose gene product is required for the functioning of

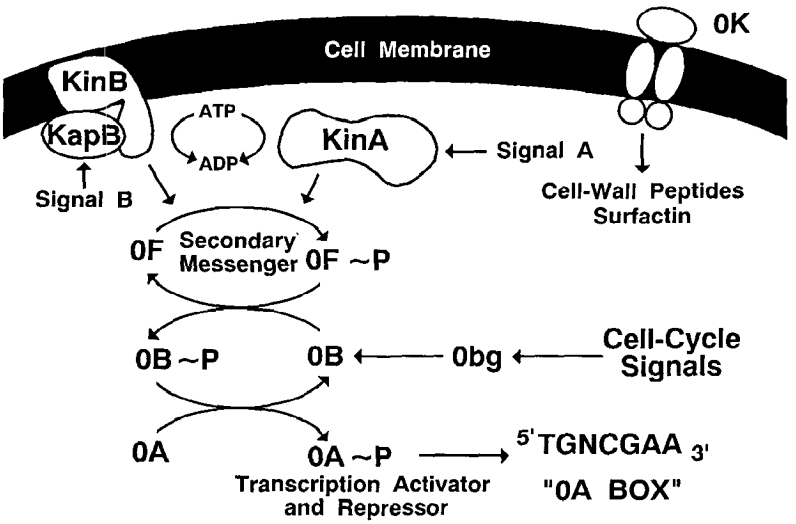

Figure 3 The role of the phosphorelay in sporulation. Two kinases, KinA and KinB-KapB, provide phosphate input to the *spo0F* secondary messenger. The Spo0F~P formed is a substrate for Spo0B and is transferred to Spo0A. Spo0A~P is a transcription factor that recognizes the 0A box in promoters it controls. Cell-cycle signals may be interpreted through Obg, a GTP-binding protein that may influence the activity of Spo0B. The Spo0K locus encodes an oligopeptide-transport system required for the transport of some peptides required for sporulation initiation.

KinB. KapB may be responsible for activation of KinB by facilitating effector ligand binding or KinB-Spo0F interaction.

The phosphorelay, as presently envisaged in Figure 3, is consistent with both the biochemical and genetic data. Mutations in the *spo0F*, *spo0B*, or *spo0A* genes are absolutely sporulation defective, indicating that both KinA and KinB must work through Spo0F to activate Spo0A. Additional kinases working directly on Spo0A must not exist or mutations in either *spo0F* and *spo0B* would not have the defective phenotype. The only substrate for Spo0B to phosphorylate Spo0A must be Spo0F~P. If other response regulators could serve as a substrate for this enzyme, *spo0F* mutations would not give a sporulation-defective phenotype. The ability of *sof* mutations of *spo0A* to bypass both *spo0F* and *spo0B* indicates that both *spo0F* and *spo0B* gene products modify Spo0A. This does not rule out that other kinases could function to phosphorylate Spo0A under nonphysiological conditions, or that response regulators other than Spo0F~P could serve as substrates for Spo0B under some unusual circumstances.

What Are the Functions of the Other spo0 *Genes?*

If the *kinA* or *kinB* and *spo0F*, *spo0B*, and *spo0A* gene products are sufficient for signal transduction in this system, what are the roles of the other *spo0* genes that have been identified (Table 2)? The *spo0H* gene codes for a sigma factor that is necessary for high-level transcription of the *spo0F*, *spo0A*, and *kinA* genes (39); therefore, its role in this process is indirect in that an Spo0H mutant lacks σ^H and therefore cannot produce sufficient levels of the phosphorelay components to allow sporulation to occur. In addition, σ^H is implicated in the transcription of the *spoIIA* operon and therefore has transcriptional roles in genes that are subsequently activated by Spo0A~P

Table 2 Genes involved in initiation of sporulation

Locus	Protein product (mol wt)	Function
spo0A	29,691	Response regulator, transcription repressor, and activator
spo0F	14,286	Response regulator
spo0B	22,542	Phosphoprotein phosphotransferase
spo0H	25,447	Sigma factor
spo0K	5-gene operon	Oligopeptide permease
spo0E	9,791	Negative regulator
obg	47,668	G protein, essential for growth
kinA	69,170	Cytoplasmic transmitter kinase
kinB	47,774	Membrane transmitter kinase
abrB	10,773	Multifunctional repressor or preventer
hpr	23,718	Multifunctional repressor or preventer

(64). Several other *spo0* loci were identified in the genetic studies. These loci have been cloned and characterized, but their function in at least two cases remains obscure.

The *spo0K* mutation has been found to define the oligopeptide permease system that is homologous to the *opp* operon of *S. typhimurium* (32, 40). The original *spo0K* mutant consisted of two mutations, an *opp* mutation that blocked the function of the oligopeptide permease, and a second mutation that causes strains bearing the *opp* mutation to acquire an *spo0* mutant phenotype (32). The mutation leading to sporulation deficiency, however, is clearly in the *opp* operon. The function of the oligopeptide permease is to transport small peptides, up to five amino acids, from the outside. The permease consists of five proteins: OppA, which codes for an external specificity determinant protein that recognizes peptides; OppB and OppC, which are integral membrane-spanning proteins making up the core of the permease; and OppD and OppF, which are ATP-binding proteins that are located on the cytoplasmic side of the membrane and provide the energy for peptide transport. Curiously, although OppD and OppF are both required for peptide transport in *S. typhimurium,* only OppD is required in *B. subtilis* for both peptide transport and for sporulation. Mutants in the *oppF* gene can transport at least some peptides but are deficient in competence (40). The transport of some peptides across the membrane is now believed to be the step in competence requiring the *opp* system (16), and therefore different peptides apparently utilize different ATP-binding domains for their transport. Thus, both *oppD* and *oppF* mutants are competence defective, whereas only *oppD* mutants are defective in sporulation.

What peptide is transported that is required for sporulation? This is the major underlying question that remains to be answered. It has been postulated that extracellular differentiation factors are produced that may help in the recognition of cell density, and these might be peptides in nature and transported by the oligopeptide permease system (15). Thus, lack of the *opp* system prevents such peptides from being internalized and, therefore, some aspect of sporulation is defective. The postulated role of the *opp* system in *S. typhimurium* is the recycling of cell-wall peptides that are cut from the peptidoglycan cross-linkers to allow cell-wall growth. The turnover of cell-wall peptides may play an important role in the signaling of growth and sporulation, and the inability to transport these peptides may lower their internal concentration, which could be a signal for growth rather than sporulation (32).

The *spo0E* gene codes for a small protein of 9791 Daltons, and the original mutations in this gene were found to be nonsense mutations in that portion of the gene coding for the carboxyl end of the protein (33). Investigators concluded that the *spo0E* gene product is required for the onset of sporulation

and must have a positive role in this process. Subsequent studies showed that this conclusion was in error; deletions of the *spo0E* gene, rather than give rise to a sporulation-defective phenotype, in fact resulted in sporulation proficiency (35). Thus, the phenotype of the original mutations in the *spo0* gene is apparently caused by production of truncated peptides brought about by the nonsense mutations in the gene. Furthermore, overproduction of this gene product on a multicopy plasmid results in sporulation deficiency, which again suggests that this protein can interfere with sporulation, either when it is overproduced or through a modification by carboxyl truncation. Deletions of the *spo0E* gene give rise to secondary mutations in strains bearing them. These mutations are in other components of the phosphorelay, suggesting that deletion of the *spo0E* gene causes inappropriate timing of the expression of phosphorelay compensated for by secondary mutations in the pathway. Thus, the *spo0E* gene product must play some negative role in the pathway's control. An *spo0E* deletion has no effect on the relative transcription of the *kinA*, *spo0F*, *spo0B*, or *spo0A* genes, so Spo0E may control the flow of phosphate through this pathway (35). Consistent with this hypothesis is the fact that deletions of the *spo0E* gene suppress many of the missense mutations in the *spo0F* gene, which can be interpreted to mean that the pathway is much more efficient at using phosphate from low-level defective or unstable Spo0F proteins (J. A. Hoch, unpublished data). If indeed the *spo0E* gene product is a negative regulator of the pathway, then it is of some interest to determine how this works and what the target of its action is.

A third *spo0* gene, *spo0J*, has been described and recently cloned and sequenced (27, 29). The *spo0J* locus consists of two genes, resides very near the origin of replication of the chromosome, and the gene products have homology to the *korB-incC* genes of certain plasmids. These genes are somehow involved in the regulation of plasmid partition, which may indicate that the *spo0J* genes have a role in segregation of the chromosome into the two compartments that ultimately become mother cell and forespore. The forespore compartment results from an asymmetric septum produced for the initial segregation of the chromosome. This septum should certainly have special partition functions that differentiate it from a cell-division septum. Perhaps the segregation of the chromosomes is different in division and the initial sporulation event and this segregation or partition is mediated by the *spo0J* gene products. However, the exact function of *korB-incC* proteins in something as easy to study as plasmid replication and segregation is still quite murky—the functions of the *spo0J* genes may be very complex. Because *spo0J* mutations prevent subsequent spore gene expression, the chromosome segregation may have a direct effect on the control of subsequent genes in sporulation. That is, the physical act of segregating the chromosome into a

forespore compartment may directly affect transcription of subsequent genes in the sporulation process.

The Transition State and Its Regulators

The transition state is roughly defined as that period of time between the end of exponential growth and the onset of stage II of sporulation. In cultures in the laboratory, this transition state lasts 1–2 h and is characterized by the initial synthesis of several proteins characteristic of early stationary phase. These proteins include enzymes such as amylase, subtilisin, and neutral proteases. Other phenomena such as the production of antibiotics also characterize stage II. In nature, the transition state may exist as an extended partially quiescent state, where nutrients are insufficient for sustained growth and division, but the insufficiency is not enough to kick off the sporulation process. Thus the cell produces extracellular carbohydrate and protein-degrading enzymes to scavenge the environment for sources of carbon and nitrogen. Antibiotics are produced to protect the ecological niche in which the organism has found itself.

In nature cells presumably spend most of their time in this type of state, only rarely having the opportunity for exponential growth when unexpected nutrient abundance occurs. The transition state is for the most part controlled by the same regulators that control sporulation. The *spo0* mutants in general never produce any of the structures characteristic of spores, and they are inhibited in the production of many of the products characteristic of the transition state, such as proteases and antibiotics. Thus, a regulatory commonality is associated with early stationary phase or transition state and sporulation. Many of these features common to both are controlled by a group of proteins termed transition-state regulators. The best characterized of these are the products of the *abrB* gene, the *hpr* gene, and the *sin* gene, although other regulators of this sort have been described. Although several important and complete reviews of the functions of transition-state regulators have recently appeared, it is worthwhile here to place their function in context with the overall control of sporulation (44, 50, 52).

The AbrB transition-state regulator is one of the most well-studied regulators of this type. The *abrB* gene codes for a protein of 10,500 Daltons that assembles into a hexameric structure for its interaction with DNA (36). AbrB prevents gene expression during exponential growth by binding to promoters of genes that are usually activated during stationary phase or the early part of sporulation (54, 68). In this regard, functioning of AbrB is a bellwether for nutritional excess and low population density where the cell has an excess of nutrients and has no desire whatsoever to enter the sporulation process. Under these conditions AbrB is maximally active, and its role is to

prevent the activation of numerous genes that are associated with stationary phase or are not required under these conditions. AbrB mainly serves as a preventer of the expression of such genes and may not be the primary regulator of their expression; i.e. it prevents the expression of these genes during exponential growth even if such genes are regulated by their normal regulators. The competitive advantage that AbrB provides the cell is to minimize the expression of extraneous gene products that would interfere with the maximal rate of growth of the cell under nutrient-excess conditions. It plays a role formally but not mechanistically similar to the Cap protein of *E. coli* that prevents the expression of many unessential genes under conditions of catabolite excess. AbrB, however, does not appear to be the *B. subtilis* equivalent of Cap, but rather functions to indicate the level of Spo0A~P in the cell. Insomuch as Spo0A~P levels are controlled by catabolites and mechanisms similar to catabolite repression, AbrB is superficially similar in function to Cap.

The regulation of genes by AbrB and its mechanism of binding to sensitive promoters has been extensively studied. AbrB seems to work in a concentration-dependent manner, and no small molecule effectors have been found that either inhibit or enhance its activity toward any promoters (54). The concentration of AbrB in the cell is controlled by two factors: autoregulation by AbrB itself of its own promoter and repression of AbrB transcription by Spo0A~P (49). Thus the concentration of AbrB, and therefore its ability to prevent transcription, is controlled directly by the level of Spo0A~P in the cell. Spo0A~P binds to a region just downstream of the two promoters of *abrB* and prevents its transcription. Thus, the product of the phosphorelay, Spo0A~P, is directly responsible for controlling many genes by regulating the concentration of the transition-state regulator AbrB within the cell. Many of the genes and processes that AbrB controls are hallmarks of the stationary phase, including subtilisin and neutral protease production, motility, and competence.

A second transition-state regulator is encoded by the *hpr* gene. This gene was first identified from studies of mutants that overproduce proteases and was subsequently shown to be identical to the gene for mutations called *scoC*, for altered sporulation control, and *cat*, for catabolite resistant sporulation. Cloning and sequencing of the gene and its mutations has shown that the phenotypes result from the loss of the *hpr* gene product, and therefore the Hpr protein functions as a negative regulator of the protease genes, as well as other genes involved in catabolite repression and sporulation control (34). Overproducing the *hpr* gene product using a multicopy plasmid results in a sporulation-defective phenotype, suggesting strongly that at least some as yet unidentified genes are required for sporulation and negatively controlled by the Hpr transition-state regulator. Hpr binds to promoters for the subtilisin

and neutral protease genes and most likely asserts its effects on these promoters by inhibiting transcription of their genes (21). No effector molecules are known that enhance or inhibit this DNA binding, and therefore it is not certain what environmental or metabolic stimulus controls the function of the Hpr regulator. Transcription of the *hpr* gene has been shown to be constitutive in an *spo0A* mutant and normal in an *spo0A-abrB* double mutant, suggesting that the overproduction of AbrB in an *spo0A* mutant serves to directly activate the transcription of the *hpr* gene. However, extensive experiments to confirm these observations have not been undertaken, and therefore AbrB itself is not proven to be a positive activator of this gene. If AbrB is an activator of *hpr*, then the synthesis of Hpr would be indirectly tied to the level of Spo0A~P in the cell through the level of AbrB.

One of the most interesting transition-state regulators is the product of the *sinR* gene, Sin. Sin was originally identified as the product of the gene that inhibits sporulation when present on a multicopy plasmid (12, 13). Overproduction of Sin by this means not only inhibits sporulation, but protease production and purified Sin protein can bind specifically to promoters including the subtilisin promoter (14, 21). Sin also must have some repressive effects on one or more components of the phosphorelay, because the production of Spo0A-controlled genes is curtailed by overproduction of the Sin protein. Sin appears to be constitutively expressed during growth and not subject to transcriptional controls. However, the function of the Sin protein is controlled by the product of the *sinI* gene, Isin (2). Isin functions as an inhibitor of the activity of Sin, and presumably the transcriptional regulation of genes mediated by Sin can be overcome through interaction with the Isin protein. The *sinI* gene, on the other hand, is transcriptionally controlled and its promoter is a target for binding of the Hpr protein, the AbrB protein, and Spo0A~P (21; M. A. Strauch, personal communication). Thus, not only Spo0A but also the two transition-state regulators that may be controlled by Spo0A serve to regulate transcription of *sinI*. Both Hpr and AbrB most likely act in a negative fashion to prevent production of Isin under conditions when Spo0A~P concentration is at a low level. Sin can repress sporulation genes under these conditions. When Spo0A~P accumulates through the action of the phosphorelay, AbrB and Hpr production fall and Isin can be transcribed. The fact that Spo0A~P binds to this promoter suggests that Spo0A~P positively activates the *sinI* gene to complete the formation of Isin and thereby inactivate the function of any remaining Sin.

If the above scenario is true, then the activities of all three transition-state regulators, AbrB, Hpr, and Sin, are basically controlled by the level of Spo0A~P in the cell. Whether any metabolic or environmental effectors play a role in the control of any of these transition-state regulators is still open to question. It seems unlikely that environmental input would only be affected

through the kinases phosphorylating Spo0F. Unfortunately, the process of finding an effector molecule for regulatory proteins of this type is not straightforward. These could be the major regulators functioning to control the activity of the phosphorelay by preventing the flow of phosphate through the phosphorelay and lowering the production of Spo0A~P. They could do this by preventing the transcription of one or more components of the phosphorelay, or they could promote the transcription of inhibitors of the components of the phosphorelay. How any of these proteins act to prevent sporulation is not clear except that they all seem to be negative regulators of transcription.

Control of Phosphate Flux in the Phosphorelay

An article of faith in sporulation is that the environment somehow controls the onset of stationary phase and sporulation. Unfortunately, the actual effector molecules from the environment that accomplish this task are completely obscure. Environmental stimuli presumably activate sensor kinases of two-component regulatory systems to autophosphorylate and to transfer phosphate to their response-regulator proteins. Two kinases are probably responsible for all of the phosphate input into the phosphorelay, and little has been discovered of the nature of the small molecules or environmental messengers that might control their activities. However, kinase regulation may not be the only point of control of phosphate flux in the phosphorelay, and its complexity has been postulated as a means by which the cell may exert control at many levels (4).

KinA, which supplies the bulk of the phosphate to the phosphorelay, at least under laboratory conditions, appears to be a soluble enzyme with no apparent homology to any other enzymes or proteins except for the conserved homology to the kinases. Thus, the primary amino acid sequence of KinA has not helped to identify potential effectors for this kinase. Recently *cis*-unsaturated fatty acids were found to be inhibitory to KinA activity on Spo0F (51). The most inhibitory fatty acids have at least one unsaturated double bond in the *cis* configuration and a chain length of 16–20 carbon atoms. The homologous *trans*-isomers are not inhibitory, nor are saturated straight or branched-chain fatty acids. KinA may not function like a classic sensor kinase with a positive effector, but rather its activity could be simply inhibited during exponential growth by the pool of *cis*-unsaturated fatty acids. Such fatty acids, although rare in the *B. subtilis* cell, may act as specific signals, linking the initiation of sporulation to the status of membrane biosynthesis and septation, or to some other membrane-associated activity, where the free fatty acid acts as a signal. This statement reflects our ignorance of the role of fatty acids in metabolic signaling.

Through extensive kinetic analyses, C. E. Grimshaw and colleagues (personal communication) have shown that KinA is greatly stimulated by simply the presence of its substrate, Spo0F. This raises the possibility that KinA is basically inactive in its own autophosphorylation reaction in the absence of Spo0F, and any residual activity may be inhibited by the concentration of *cis*-unsaturated fatty acids in the cell. When *spo0F* transcription is induced at the end of exponential growth by a series of complicated controlling factors, the expression of Spo0F might be sufficient to activate the kinase to convert Spo0F to Spo0F~P. No other positive effector need be required. Although this is not the classical view of how such kinases are activated, it would fit the kinetic data, which show KinA to be on the order of 3% as active in the absence of Spo0F as in its presence, and Spo0F appears to induce an isomerization reaction in the enzyme to increase its activity. Thus, any environmental input into KinA activity could be indirect through the control of the transcription of *spo0F*.

KinB is a protein with six potential membrane-spanning regions, suggesting that the enzyme is an integral membrane protein with the kinase portion of the molecule in the cytoplasm of the cell (61). The *kinB* gene is present in an operon along with a gene, *kapB*, that codes for KapB, a protein of 14,600 Daltons that appears from its deduced amino acid sequence to be a moderately charged soluble protein. Inactivation of either *kinB* or *kapB* in the presence of the *kinA* mutation reduces the residual level of sporulation of the double mutant to almost zero. This result indicates that KapB is required for the activity of KinB, although one cannot rule out the possibility that KinB is not synthesized in the absence of KapB. The most likely scenario is that KapB is required to facilitate the activity of KinB, either by acting as an effector ligand–binding domain, or by facilitating the interaction of KinB with Spo0F. KapB itself appears to have no homology to response regulators and no homology to any other known protein. The reason for the membrane location of KinB is a mystery.

The *spo0E* gene product may be involved in negative control of the level of Spo0F~P, because deleting the *spo0E* gene results in suppression of poorly active mutant Spo0F proteins (J. A. Hoch, unpublished data). This sparing effect on Spo0F could be accomplished in several ways, perhaps by preventing the degradation of Spo0F~P either through an inherent phosphatase activity, or by activating the phosphatase activity of either KinA or KinB. For example, KinA has a potent phosphatase activity for Spo0F~P. Spo0E may be responding to some effector molecule, since removal of the carboxyl-terminal portion of the protein results in a sporulation-defective phenotype and presumably a constitutively active protein. What effector molecule this carboxyl portion of the protein might be interacting with is open to question.

The most likely role of Spo0E is to control the flow of phosphate through the phosphorelay in a negative fashion in response to the level of some other environmental or metabolic factor.

The *spo0B* gene resides in the same transcription unit as the gene for an essential GTP-binding protein, Obg (60). Although the juxtaposition and common control of these two genes suggests that one plays a role in the other's activity, the evidence is entirely circumstantial. Sporulation can only initiate during a certain window in a cell cycle, and the Obg protein may convey some cell-cycle control to the activity of the Spo0B phosphotransferase. Obg is essential for growth, and temperature-sensitive mutants of this protein have been isolated that do not appear to properly septate at the restricted temperature (J. Kok & J. A. Hoch, unpublished data). Whether Obg has a direct effect on septation remains to be seen, but Obg is the only candidate at present for a protein with an essential function in the cell that may convey information about the position of the cell within the cell cycle.

Transcriptional Regulation of the Phosphorelay

Regulation of the cellular concentration of the components required for the production of Spo0A~P initiates control of the flow of phosphate through the phosphorelay. This mechanism of control is readily apparent during conditions of vigorous vegetative growth in nutrient-excess conditions in which the amounts of two of the major components, Spo0F and Spo0A, are strongly controlled by transcriptional mechanisms. During the early portion of the transition state, repression of these genes is released and strong induction of both occurs (66). σ^H plays a crucial role in the induction of both *spo0A* and *spo0F* during this state, as well as being involved in the transcription of the *kinA* gene (39). Spo0A~P has some role in regulating the level of σ^H because this gene is repressed by the transition-state regulator, AbrB (63), which is subject to direct control by Spo0A~P (49). Because the σ^H required for induction is controlled by Spo0A~P, some mechanism must insure that a low level of phosphorelay components that are not under σ^H control exists in the cell. This is accomplished by differentially controlled tandem promoters for both the *spo0A* gene and the *spo0F* gene (5, 24, 58, 65).

A vegetative promoter utilizing σ^A is located upstream of the *spo0A* and *spo0F* genes and transcribes both genes at a very low level during growth in conditions of nutrient excess (Figure 4). Both of these vegetative promoters appear to be repressed as the culture moves from vegetative growth into the transition state. For the *spo0A* gene, Spo0A~P binds directly downstream of the vegetative promoter and inhibits transcription from it (55). In the *spo0F* gene, an Spo0A~P binding region occurs within the vegetative promoter, presumably again shutting off this promoter (56). The binding of Spo0A~P to both of these regions is also thought to activate transcription from the

downstream σ^H promoter of both genes. The *spo0A* promoter has an additional Spo0A~P binding site just downstream of the presumed repression site of the vegetative promoter, whereas in the *spo0F* gene, Spo0A~P binding at the site of the vegetative promoter may accomplish both tasks—repressing the vegetative promoter and activating the σ^H promoter. An Spo0A~P (repression) binding site for both σ^H promoters also covers the −35 region of the *spo0A* promoter, and another is just downstream of the start site of transcription in the *spo0F* gene. Both of these sites are thought to modulate the expression of these genes in a fashion similar to autoregulation; once the Spo0A~P concentration reaches a level sufficient to accomplish its functions in the cell, Spo0A~P represses any further synthesis of both Spo0A and Spo0F (56). At this time Spo0A~P may also act as a repressor of the *kinA* gene because an Spo0A-binding site is located just downstream of this gene's start site (K. Trach, M. Strauch & J. A. Hoch, unpublished data).

This autoregulation scheme, in which the product of the phosphorelay Spo0A~P acts as a positive activator of genes not only coding for itself but also for *spo0F* required for the production of Spo0A~P, is basically an autocatalytic positive feedback loop (Figure 4). Such an arrangement might

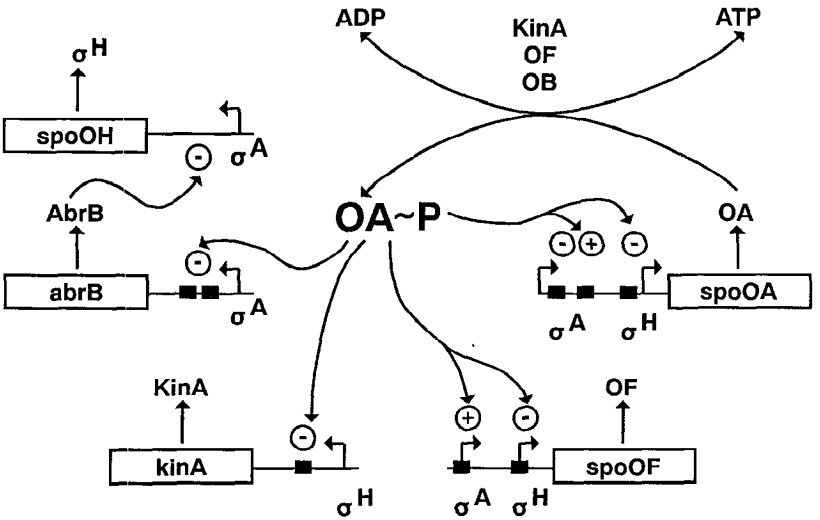

Figure 4 Autoregulation of production of Spo0A~P by Spo0A~P. The complex interactions between Spo0A~P and promoters it controls are shown. Low levels of Spo0A~P are required to repress the *abrB* promoter, which results in the relief of repression of the production of σ^H. σ^H is required for high-level expression of *kinA*, *spo0F*, and *spo0A*. Spo0A~P is a positive activator of σ^H promoters for the *spo0A* and *spo0F* genes. Spo0A~P may be a repressor for the *kinA* gene, and for *spo0F* and *spo0A* genes when Spo0A~P concentration reaches a sufficient level.

be advantageous if the cell finds itself in conditions where it needs to rapidly produce Spo0A~P in order to respond to a sudden shift from nutrient-excess to nutrient-poor conditions. In theory, the entire sporulation-induction scheme could be regulated by simply controlling the kinases that are responsible for the initial input of information into the phosphorelay. When these are off, the transcription of the genes for *spo0A* and *spo0F* is shut down except for the low-level transcription from the vegetative promoters for these genes. When the kinases become activated, a small amount of Spo0A~P is produced, which causes a rapid rise in the production of Spo0F and Spo0A through the positive feedback loop. The suggestion of such a control mechanism, although pleasing and simple, reflects our ignorance of the influence of other factors on the transcription of these genes.

Glucose strongly affects transcription of *spo0A* (65). The presence of glucose in the media prevents switching from the vegetative to the σ^H promoter of the *spo0A* gene (5). This could occur by a variety of mechanisms. For example, catabolite repression could be mediated through a DNA-binding protein with some of the properties of the Cap protein of *E. coli* that serves to prevent the transcription of the *spo0A* σ^H promoter. This hypothesis seems to be a favorite of many, especially because Chambliss and colleagues have shown that promoters controlled by catabolites including *spo0A* have a specific sequence in common, suggesting that a specific protein binds to this sequence to effect the catabolite repression of such genes (17, 62). Until such a protein is found and shown to directly bind to this promoter, however, an equally likely hypothesis is that catabolite repression is mediated through inhibition of the production of Spo0A~P, either by the inhibition of the enzymatic activity of one of the components of the pathway, by the prevention of transcription of some other gene such as the σ^H gene, or by the deactivation of the kinases by preventing effector-molecule formation.

The transition-state regulator Hpr is known to prevent sporulation when overproduced, and mutants that are deficient in this protein have catabolite-resistant sporulation. But Hpr cannot be the hypothetical catabolite-resistance protein identified by Weikert & Chambliss (62) because Hpr has no effect on the amylase gene that is presumably controlled by their regulator, and the DNA sequence of the Hpr-binding site does not correlate to the common site mentioned above (21). One of the roles of Sin, when overproduced, is to prevent the transcription of some genes controlled by Spo0A~P. Sin might transcriptionally control *spo0A* or *spo0F* genes, or both, or regulate other components of the phosphorelay scheme required for production of Spo0A~P. Finally, the possibility exists that other regulators that have not yet been identified could be controlling transcription of the genes for the phosphorelay. Clearly, the function of the transition-state regulators in this sporulation scheme is to prevent sporulation, rather than to promote it. These may be the

proteins subject to the mythical nutrient regulation that we have so far been unable to explain. In the absence of transition-state regulators, presumably the production of Spo0A~P and the induction of sporulation would depend solely on activation of the kinases.

Transcriptional Properties of Spo0A~P

The Spo0A transcription regulator is clearly required for the initiation of sporulation and also plays a major role in the control of its own synthesis. This protein works both as a transcription repressor and as a transcription activator by binding at 0A boxes (5-TGNCGAA-3) in promoters (49). Several studies have shown that only the phosphorylated form of this enzyme is active in transcription (57). Site-directed mutations in the aspartic acid pocket required for phosphorylation of Spo0A give rise to a protein that is inactive in vivo, either as a repressor or an activator (55). Studies of the DNA-binding ability of Spo0A and Spo0A~P have shown that the phosphorylated form of the protein is 20–50 times more efficient at binding to its sites on the *abrB* promoter than the unphosphorylated form (49). The unphosphorylated form binds to promoters with the same specificity as the phosphorylated form, and the same is true for proteins with mutations in the aspartic acid pocket that cannot be phosphorylated. Phosphorylation may affect either the affinity of the protein for its target or the dissociation constant of the DNA-Spo0A~P complex.

The *abrB* gene appears to be extremely sensitive to the concentration of Spo0A~P in the cell. This conclusion comes from several observations, the major one being that repression of the *abrB* gene is controlled directly by Spo0A~P and occurs toward the middle of exponential growth when the Spo0A~P concentration in the cell must be quite low. A mutation in the *spo0A* gene renders the production of AbrB constitutive, subject only to its own autoregulatory controls (53). In a mutant lacking both KinA and KinB, which cannot produce enough Spo0A~P to induce a positively controlled gene such as *spoIIA*, the regulation of *abrB* is normal (61). Thus, the small amount of Spo0A~P that can be formed by crosstalk with other kinases on *spo0F* is sufficient to repress *abrB* transcription. This sensitivity may be because the *abrB* promoter contains two Spo0A~P binding sites (0A boxes) separated by one helical turn of the DNA. One possibility is that this arrangement of 0A boxes builds a high-affinity site for Spo0A~P; another is that two molecules of Spo0A~P can bind to this site and stabilize through protein-protein interactions, as well as protein-DNA interactions.

Spo0A~P is responsible for activating the transcription of three operons, *spoIIA*, *spoIIE*, and *spoIIG*, required for the transition from stage II to stage III of sporulation (42, 57, 67). It accomplishes this by activating transcription of these operons from either a σ^A promoter (in the case of *spoIIE* and *spoIIG*)

or a σ^H promoter (in the case of *spoIIA*). The difference between these promoters and the *abrB* promoter, for example, is believed to be the lower concentration of Spo0A~P required for the activation of *abrB*. In addition, mutations in *spo0A*, such as *spo0A9V*, which modifies one of the very carboxyl-terminal amino acids of the protein, result in a transcription factor that can repress *abrB* efficiently, but cannot activate the transcription of *spoIIA* (37). Thus, positive activation of genes requires a certain conformation of the very carboxyl-terminal amino acids that is not required when Spo0A~P acts as a repressor. The carboxyl terminal portion of *spo0A* may need to make physical contact with the transcription complex in the case of activated promoters. The promoters for the three stage II genes have several partial 0A boxes, and in vitro studies have shown that Spo0A binds over a large portion of these promoters, extending past the –100 nucleotide of two of them (42, 67). Thus, the structure of these promoters may be such that several Spo0A~P molecules must bind in concert in order to provide the structure required for transcription activation.

The studies of Moran and colleagues on the *spoIIG* promoter and the *spoIIE* promoter have shown that these promoters are not high-affinity binding sites for the transcription complex and probably require the presence of Spo0A~P to allow the transcription complex to recognize the promoters (42, 67). Whether this occurs because of Spo0A~P–transcription complex contacts, or whether *spo0A* binding to these promoters causes a more favorable confirmation of the DNA, or both, has not been determined. The key to understanding the transcription of these stage II genes is the perception that a certain concentration barrier of Spo0A~P must be breached before these promoters turn on. No other regulatory factors have been implicated in the control of these genes, although it is certainly possible they exist.

How It Works

One can consider a bacterial culture in the laboratory as being in one of three general states: nutrient-excess exponential growth, nutrient and population density–limited transition state, and stationary phase/sporulation. Bacteria compete in nature by growing rapidly, and therefore under any conditions where nutrients are available, the first priority of a cell is to grow and divide. Under these conditions, the phosphorelay is transcribed at a maintenance level and little, if any, Spo0A~P is present in the cell. This leads to high-level production of the AbrB protein, which keeps stationary-phase genes from being transcribed and possibly interfering with the cell's growth and division. Utilization of the available nutrients leads to a more nutrient-limited situation and conversion from an exponentially dividing culture to one in which growth and division occur at a much slower rate as the cells enter the transition state. This transition-state period can be quite short in cultures in the laboratory,

but may occur for extended periods in nature where limitation of nutrients may be common. The conversion to the transition state occurs with the release of transcriptional controls on the genes for the phosphorelay and the presumed accumulation of low levels of Spo0A~P. This quantity of the activated transcription factor is sufficient to cause repression of the *abrB* gene and, therefore, to release from repression those stationary-phase gene functions that are required for life in the transition state. Presumably, accumulation of Spo0A~P is not sufficient to kick off transcription of the stage II genes. Stage II gene transcription must result when nutrients become so limited that the cell cannot maintain itself in the transition state.

Several enzymes are produced during the transition state. These include amylase and other enzymes that degrade complex carbohydrates, and enzymes such as subtilisin and neutral protease that serve to produce amino acids for carbon and energy as well as nitrogen sources. During this stage, the cell is hunting through the environment for all of the possible available carbon sources it can find to continue to grow and divide, albeit, at a much reduced rate. Secondary pathways of metabolism may now be induced to take advantage of all available carbon sources that might be around. The cell produces antibiotics during this period, presumably to fend off the invasion of other bacteria or fungi that could take advantage of the substrates generated by these extracellular enzymes and to protect its space in its ecological niche. *B. subtilis* has a large armament of offensive weapons in the form of antibiotic pathways for the ongoing warfare occurring in complex habitats. *B. subtilis* and many other bacteria have developed a semiquiescent transition state called *competence,* in which the cells develop the ability to take up extraneous DNA, but that is controlled in a complex fashion by several regulators (6, 7). Thus, the transition state is a time of intensive transcription and regulation of a wide variety of genes and operons that exist solely to maintain the cell in exceedingly varied environmental states. Transition-state regulators, such as Hpr and Sin, must come into their own during these states and prevent sporulation from occurring as long as sufficient nutrients are available for the cell in the transition state. They accomplish this task by keeping Spo0A~P below the threshold level at which initiation of the transcription of stage II genes occurs and the subsequent cascade of events that ultimately leads to a spore.

It is axiomatic that sporulation is controlled by nutrient availability, but none of the research to date has been able to show what nutrients actually cause repression of sporulation and how they act. Similarly, nutrients and/or other events must be responsible for the activation of the phosphorelay, yet we have no clear picture of which nutrients these are or how they activate the kinases. We know glucose has an effect, but it is unclear what derivative form of glucose actually exerts the control, and through what proteins it works

and how it works. Finally, transition-state regulators clearly exist to prevent sporulation, and they must operate by sensing the fact that sufficient nutrients exist such that sporulation should not occur, yet we have no real clue as to what nutritional compounds are controlling the activity of Hpr, Sin, or the other less-well-studied proteins that may prevent sporulation. Advances in molecular biology have allowed us to define the genes and isolate the proteins involved in this regulation but have been of limited value in determining how the proteins function and what interacts with them.

The end of the transition state is signaled by the activation of the stage II genes, *spoIIA*, *spoIIG*, and *spoIIE*, by Spo0A~P. At this time, the nutritional value of the environment has reached a point at which it is no longer advantageous to continue the battle, and the cell initiates sporulation and goes inert until conditions improve. The production of proteins like amylase and subtilisin shuts down, and the remaining energy available to the cell is directed toward the production of the morphological structures that ultimately characterize the quiescent spore. This is a time when form determines function (47), chromosomes condense (43), sigmas cascade (48), and compartments talk to one another (25). These exciting studies, too complex to review here, have brought forth interesting and provocative theories of gene regulation and some of them might even be right.

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