Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*

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Sporulation in *Bacillus subtilis* is a model for how cells of one type generate other differentiated cell types. During sporulation two cellular compartments arise that differ from each other and from the progenitor cell. Differential gene expression between the two is governed by the successive appearance of four transcription factors whose activities are coordinated in crisscross fashion between the two cells.

HOW do cells of one type give rise to dissimilar types of cells? Much has been learned from the study of simple differentiating microbial systems, including the interconversion of **a** and σ mating cell types of the budding yeast *Saccharomyces cerevisiae*[1], the formation of swarmer and stalked cell types by the dimorphic bacterium *Caulobacter crescentus*[2] and the formation of heterocysts by the cyanobacterium *Anabaena*[3]. We now describe a particularly powerful microbial system for the study of differentiation—the formation of two distinct cell types by the spore-forming bacterium *B. subtilis*[4,5]. The expression of different genes in the two cell types is chiefly governed by four transcription factors. The action of these factors is restricted to one or the other cell and is coordinated by communication between the two cells.

In sporulation, an asymmetrically positioned septum partitions the developing cell (the sporangium) into two unequal compartments, the forespore and the mother-cell, each of which carries a chromosome from the last round of vegetative DNA replication (Fig. 1 *a*, *b*). The two compartments follow different programmes of gene expression: certain sets of genes are transcribed in the forespore and others in the mother-cell. After this initial stage, the septum migrates around the forespore (Fig. 1*c*), eventually engulfing it, pinching it off as a free protoplast (Fig. 1*d*) wholly enclosed within the mother-cell.

In the final stages of differentiation, a cortex of cell wall material is produced in the space between the mother-cell and the forespore membranes and coat polypeptides from within the mother cell are deposited around the forespore to form the tough protein shell encasing the mature spore (Fig. 1*e*, *f*). The forespore, a germline cell, becomes the spore from which may arise subsequent progeny. The mother-cell is discarded when development of the spore is complete.

The transition from one stage to the next is governed by six regulatory proteins[6,7] called sigma factors, which, by binding to RNA polymerase, determine which gene promoters are recognized[8]. These factors are the primary sigma factor of vegetative cells, σ^A, and five factors that become active during development called σ^H, σ^F, σ^E, σ^G and σ^K (in order of their appearance during sporulation). The σ^A and σ^H factors are active before the septum forms, and are not further considered. The other four, σ^F, σ^E, σ^G and σ^K, are specific to each cell type and direct gene transcription in one or the other compartment formed by septation.

Establishment of cell type

Gene expression characteristic of the forespore is governed by the action of σ^F within that compartment shortly after the septum forms[9]. Although this factor is produced in the undivided sporangium[10], and thus would be expected to be distributed to both compartments, the transcription of the genes under its control is delayed until after septation (P. Margolis and R.L., unpublished results) when the transcription it directs is confined to just one compartment[9]. This has been learned from experiments[9] in which the *E. coli lacZ* gene is fused to a gene under σ^F control; the location of β-galactosidase from the *lacZ* fusion is visualized by the use of antibodies against the enzyme and gold-conjugated secondary antibodies, followed by electron microscopy. Gold granules are almost exclusively located in the forespore (Fig. 2*a*).

Evidence that σ^F is produced before septation comes from the use of genetically mosaic sporangia in which the forespore chromosome is mutant for σ^F and the mother-cell chromosome is wild-type for σ^F (ref. 10). Such mosaic sporangia are obtained from cells mutant for σ^F by transforming them, at the start of sporulation, with wild-type DNA in conditions such that only one of the two chromosomes in the undivided sporangium is corrected[11]. After septation, mutant and wild-type chromosomes are then randomly distributed between the forespore and the mother cell. Sporangia in which the forespore carries the mutant chromosome can sporulate, but the resulting spores are of genotype *spo*$^-$ (that is, the progeny of the spores are mutant for σ^F and cannot themselves sporulate)[10]. But σ^F is necessary for transcription in the forespore, meaning that forespores with mutant σ^F must have acquired some quantity of σ^F produced

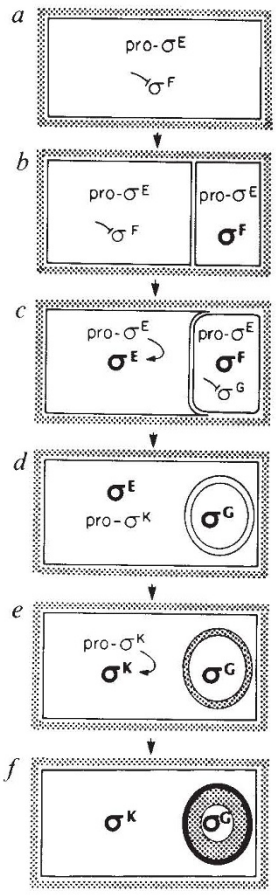

FIG. 1 The stages of morphogenesis and a model for the compartmentalized action of sporulation sigma factors. (See text for an explanation of the morphological stages.) The stippled areas indicate the cell wall around the sporangia and the cortex between the forespore and mother-cell membranes (*e*, *f*). The heavy line around the forespore in *f* indicates the coat. Each sigma factor is in an inactive (light lettering) or active (bold lettering) state. The σ^F and σ^G factors are held inactive by an inhibitory protein (see text) as indicated by the symbol —|; the σ^E and σ^K factors are held inactive in the form of proproteins as indicated. The precise time at which σ^F and σ^E are activated is not known but is arbitrarily indicated as occurring just before, and at the start of, septum migration, respectively. For simplicity the inactive form of σ^F is omitted from the mother cell in *c* and pro-σ^E is omitted from the forespore in *d*. Also omitted are σ^A and σ^H, which are active before septation and whose activities are not compartmentalized.

from the wild-type chromosome before septation. Thus σ^F must be so controlled that its activity, but not its synthesis, is restricted to the forespore (Fig. 1*a*, *b*).

An important clue to this unknown mechanism comes from studies of the operon *spoIIA*, which consists of three cistrons *spoIIAA*. *spoIIAB* and *spoIIAC*. and in which σ^F is itself encoded[12,13]. The product of the first cistron, SpoIIAA, is an inhibitor of that of the second[14], SpoIIAB, which is in turn an inhibitor of σ^F(refs 14,15) (encoded by the third cistron[16-18]). Although the mode of action of SpoIIAB is not known, it probably inhibits σ^F directly rather than by interfering with (or 'repressing') access to target promoters[9,14].

How is the expression of the genes controlled by σ^F achieved selectively only in the forespore? One hypothesis[9] is that SpoIIAA exists in inactive and active states, that it is inactive in the undivided cell and in the mother-cell after septation and that, in this state, it cannot prevent SpoIIAB from blocking the action of σ^F. Some feature of the post-septation sporangium must then drive SpoIIAA into the active state in the forespore only, inhibiting SpoIIAB and relieving σ^F from inhibition. Although the trigger for activation is not known, genetic experiments implicate the *spoIIE* operon[9], one of whose products is probably an integral membrane protein (P. Guzman, J. Westpheling and P Youngman, personal communication) and which governs proper formation of the septum[19] and may create the difference between the forespore and mother-cell compart ments to which SpoIIAA responds.

As gene expression in the forespore is determined by σ^F, so gene expression in the mother cell is determined by the expression factor σ^E, which is similarly produced in the undivided sporangium[10] and does not become active until after septation[20-23]. Thus immunoelectron microscopy[24] reveals that the transcription of a gene under the control of σ^E is chiefly confined to the mother-cell (Fig. 2*b*). Moreover, most if not all of the genes under the control of σ^E are required in, or have functions associated with, the mother-cell[25]: σ^E is evidently a regulator or post-septation gene expression specific to the mother-cell.

For σ^E, the inactive differs from the active form by an extension of 27 amino acids at the N terminus[20] (C. P. Moran Jr, personal communication). In other words, σ^E is first produced as an inactive proprotein, pro-σ^E, the activation of which is catalysed by the gene product SpoIIGA of the first cistron of the operon *spoIIG* (refs 21, 26), whose second cistron (*spoIIGB*) is the structural gene for pro-σ^E (refs 27, 28). Interestingly, processing of pro-σ^E also requires the action of σ^F (and of all of the gene products required for the activation of that factor)[21,22,29]. The inference is that an unknown gene under the control of σ^F is somehow required in the cleavage of pro-σ^E (see below).

The σ^E factor is not activated until after septation[20-23], suggesting the attractive hypothesis that processing of pro-σ^E is confined to the mother-cell[25] (Fig. 1*c*). It is also possible that pro-σ^E might be processed in both compartments, but that some other mechanism might limit the action of σ^E to the mother-cell. Reports of experiments involving the fractionation of the contents of the sporangium have indeed suggested that mature σ^E is equally present in the two compartments[30], but the experiments are technically difficult and the results may not be conclusive.

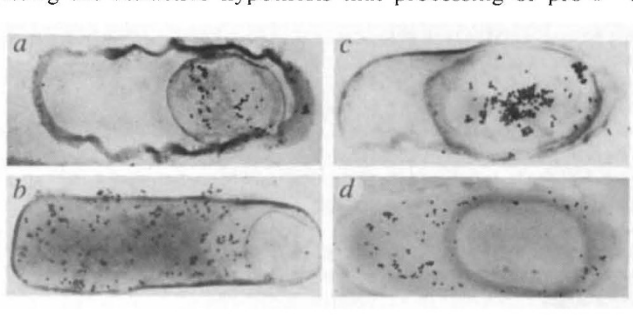

FIG. 2 Use of immunodecorated thin sections and electron microscopy to demonstrate compartmentalized gene expression. Thin sections of sporangia at intermediate (*a*, *b*) or late (*c*, *d*) stages of sporulation were stained with antibodies to β-galactosidase and with gold-conjugated secondary antibodies. Each sporangium contained *lacZ* fused to a gene under the control of σ^F (*a*), σ^E (*b*), σ^G (*c*) or σ^K (*d*). The experiment was done as described[9,24], except that the size of the gold granules was increased by silver enhancement using the IntenSE M kit (Janssen Biotech).

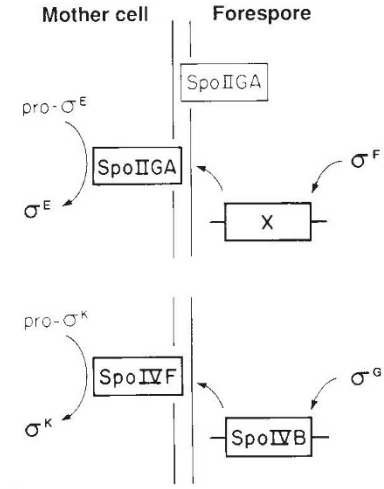

FIG. 3 Proposed signal transduction pathways governing the processing of pro-σ^E and pro-σ^K. The products of genes transcribed under the control of σ^F and σ^G in the forespore stimulate the processing of pro-σ^E and pro-σ^K, respectively, on the opposite side of the double membrane that separates the forespore from the mother cell (see text). For simplicity, pro-σ^E is omitted from the forespore in the upper panel.

If it is true that mature σ^E is generated only in the mother-cell, what might be the mechanism? It is unlikely that the putative processing enzyme SpoIIGA or its substrate pro-σ^E can be compartmentalized: the operon *spoIIG* is switched on before septation[10], so that both its products are likely to be present in both compartments afterwards. But the observation[21,22,29] that processing of pro-σ^E requires the action of the forespore transcription factor σ^F suggests this model of selective processing in the mother-cell: the product of an unidentified gene under the control of σ^F sends a signal across the membrane to the mother-cell, where it stimulates the processing of pro-σ^E (refs 9, 24; Fig. 3) and, because the stimulus is directional, processing takes place only in the compartment opposite that in which σ^F is active. This hypothesis is attractive because a similar directional signal controls the activity of a second transcription factor later in development.

Switch to late-acting sigmas

There are two phases in the compartmentalized expression of the sporulation genes, of which the early phase is governed by σ^F and σ^E. While the set of genes whose transcription is controlled by σ^F is still poorly characterized[14,15,31,32], that controlled by σ^E includes genes involved in the engulfment of the forespore and in the early steps of cortex and coat formation[33-37]. Gene expression in the late phase, on the other hand, which begins with engulfment (Fig. 1*d*) is controlled by the transcription factors σ^G and σ^K (refs 18, 38, 39), which are true compartment-specific regulatory proteins in that their synthesis and site of action is confined to one or the other cell type[24,38,40,41].

Specifically, σ^G governs the transcription of genes expressed in the forespore, which include genes encoding a family of small acid-soluble proteins which are abundant in the forespore[42]. Similarly, σ^K is the chief determinant of gene expression in the late-phase mother-cell, directing the transcription of genes encoding the most abundant proteins of the spore coat[37,43,44] and one or more genes involved in the formation of the cortex[45]. Immunodecoration demonstrates that gene expression directed by σ^G and σ^K is restricted to the forespore and the mother-cell respectively[24,41] (Fig. 2*c*, *d*).

The switch from early to late-phase expression requires not only mechanisms for turning on the synthesis of the late-phase σ-factors but also to remove the early-phase σ-factors from the RNA polymerase. The removal of the early-phase mother-cell factor σ^E may be explained by the observation that σ^E (but not pro-σ^E) is rapidly proteolysed when not bound to core RNA

polymerase (as, for example, after the initiation of a cycle of transcription)[46]; mother-cell depletion of σ^E could thus result from the inherent instability of the factor coupled with cessation of the synthesis of σ^E. As yet, it is not known whether the forespore factor σ^F is physically depleted or, alternatively, simply displaced from RNA polymerase by newly synthesized σ^G.

How is compartment-specific synthesis of the late-phase sigma factors achieved? In the forespore, σ^G is selectively synthesized because[14,15,32] its structural gene (*spoIIIG*) is controlled by σ^F (Fig. 1*c*). But, remarkably, σ^G is subject to a second kind of control acting at the level of its activity[38,47]: although the structural gene for σ^G is induced shortly after septation, transcription of the genes it controls is delayed until the forespore is fully engulfed (Fig. 1*d*). (The mechanism of this inhibition is not known, but genetic experiments[48] implicate SpoIIAB, which may therefore govern the activity of both forespore transcription factors.) But once the activity of σ^G has been unleashed, it maintains and amplifies its own synthesis by recognizing the promoter for its own structural gene.

By contrast, the mother-cell late-phase factor σ^K is controlled by three distinct regulatory mechanisms (Fig. 4), of which the first and most extravagant (but least important) involves a DNA rearrangement in the mother-cell chromosome to form its structural gene, called *sigK*[49,50]. What happens is that site-specific reciprocal recombination effects in-frame joining of partial genes encoding the amino- and carboxy-terminal portions of σ^K. The rearrangement entails the excision as a circle of an intervening DNA element (42 kilobases long) called *skin* (ref. 50) and is catalysed by the product of a recombinase gene (*spoIVCA*) which is itself located on the same intervening element[50,51] (Fig. 4). (The gene product SpoIVCA belongs to the Hin, Pin, Gin, TnpR family of site-specific recombinases[51].) The rearrangement also requires a small DNA-binding protein, SpoIIID, which may be part of the synaptic complex[50]. The σ^K gene rearrangement is confined to the mother-cell compartment because the transcription of both the *spoIVCA* recombinase gene (Y. Kobayashi, personal communication) and that (*spoIIID*) for the DNA-binding protein[52,53] (C.P.Moran, personal communication) is controlled by the early mother-cell factor σ^E.

Evidently the rearrangement need not be, and is not, reversible; both the mother-cell and its chromosome are discarded when the spore has matured. But the *skin* element is not normally excised either in the vegetative cell or in the forespore. It is interesting that the rearrangement as such is not essential for the proper compartmentalization of gene expression; the growth and development of cells in which the whole *skin* element has been deleted are not impaired although both mother-cell and forespore carry an intact copy of *sigK* from the onset of sporulation[50]. It is relevant that in *B. thuringiensis*, the gene corresponding to *sigK* is not interrupted by an intervening element[54].

In the second level of control, transcription of the intact *sigK* gene is directed by σ^E in conjunction with SpoIIID, which in this context is a positive regulatory protein[39,40] (Fig. 4).Once again, because σ^E and SpoIIID are specific to the mother-cell, this arrangement ensures that the transcription of *sigK* is also specific to the cell type carrying the reconstituted gene.

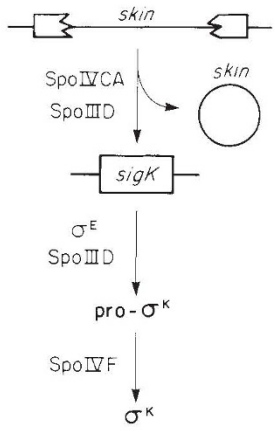

FIG. 4 Three levels of control of σ^K synthesis. The figure depicts the control of σ^K synthesis at the levels of DNA rearrangement, transcription, and proprotein processing (see text). The boxes with serrated edges at the top of the figure represent the two half-coding elements for σ^K in the chromosome before the DNA rearrangement.

The third level of control is the conversion of σ^K from an inactive precursor[39,55,56] (Fig. 1*e* and 4). As with σ^E, the primary product of *sigK* is a proprotein, in this case with an amino-terminal extension of 20 amino-acids[39]. The conversion of pro-σ^K to mature protein is governed by the *spoIVF* operon, which encodes either the processing enzyme or its regulator[56,57], and which is transcribed at an early stage in the mother-cell under control of σ^E (ref. 57).

Just as the transcription of the gene for σ^G is auto-catalytic, so is that of *sigK*, which is regulated positively by its product σ^K, again in conjunction with SpoIIID (ref. 39). Thus the compartment-specific synthesis of a transcription factor is again maintained and amplified by itself. But mature σ^K also directs the transcription of the regulatory gene *gerE*, encoding a small DNA-binding protein which acts in conjunction with σ^K to direct the transcription of a class of mother-cell genes expressed late in the existence of the mother-cell[37,43]. Thus the mother-cell hierarchy of gene regulation is more complex than that of the forespore, which involves just two known regulatory proteins, entailing the successive appearance of two sigma factors and two DNA binding proteins in the order σ^E, SpoIIID, σ^K and GerE (ref. 37).

Intercompartmental communication

The expression of genes in the forespore and mother-cell is not independent, but is coordinated by intercompartmental communication coupling the appearance of a sigma factor in one compartment to the activity of an earlier sigma factor in the other. Thus the appearance in the mother-cell of σ^E by the activation of the pro-protein depends on the activity of σ^F in the forespore. Likewise, the activity of σ^G in the forespore waits and evidently depends upon the engulfment of the forespore by the mother-cell, which is brought about by the action of genes under the control of σ^E, notably *spoIID* (whose product is required for pinching off the forespore as a free protoplast[5,19]) and the products of the *spoIIIA* operon (some of which are membrane-bound proteins: A.M.Guérout-Fleury and P.S., unpublished results) presumed to act from outside the forespore to relieve the inhibition of σ^G activity therein[47].

The coordination of gene expression in the two compartments is further illustrated by the coupling of the appearance of σ^K in the mother-cell to the action of σ^G in the forespore[45,55,56], which involves the following signal-transduction pathway: σ^G turns on the transcription of the forespore gene *spoIVB* whose product stimulates the activity in the mother-cell of the SpoIVF proteins, which in turn govern the processing of pro-σ^K (ref. 45; Fig. 3). The SpoIVF proteins are believed to be embedded in the outer membrane surrounding the forespore, and to be activated by the appearance in the forespore of SpoIVB (ref. 57).

Support for this view has been provided by the isolation of mutations in *B. subtilis* in which the dependence of active σ^K on the activity of σ^G has been removed; both gain-of-function mutations of *spoIVF* and a deletion of the amino acid sequence distinguishing pro-σ^K from the active protein have been obtained[56]. In both cases, transcription of genes under the control of σ^K begins an hour earlier than normally, and, as an apparent consequence, spore formation is impaired[56]. This demonstrates that the timing of late gene expression in the mother-cell hangs on the link between the activation of pro-σ^K and the activity of σ^G in the forespore.

An interesting evolutionary speculation now arises. The activation of σ^K by inter-compartmental signalling is reminiscent of the proposed coupling of σ^E activation in the mother-cell to forespore gene expression directed by σ^F (Fig. 3). Could it be that the mechanism linking pro-sigma processing in one cell to gene expression in the other directed by a different transcription

factor has been duplicated in the course of evolution? It is interesting that σ^G strongly resembles σ^F and σ^K, σ^E. Perhaps an ancestor of *B. subtilis* relied on σ^F and σ^E alone to govern the differentiation of mother-cell and forespore[58].

Crisscross regulation

The differential expression of genes in the forespore and mother-cell thus involves four σ-factors, each specific to a cell type. The first to act is σ^F, synthesized in the undivided sporangium but activated only after septation. That switches on transcription of a gene whose product may lead to the appearence of active σ^E in the mother-cell. Although σ^F also switches on transcription in the forespore of the structural gene for σ^G, gene expression directed by that factor does not begin until engulfment, which is driven by genes under control of σ^E in the mother-cell. The factor σ^E also brings about the transcription of the rearranged gene for σ^K, the last in the sequence, but the appearance of mature σ^Kdepends on the activation of pro-σ^K which is controlled by σ^G in the forespore. The compartmentalized action of all these factors ultimately derive from whatever restricts the activity of the first factor in the network, σ^F, to the forespore.

Thus the actions of four transcription factors are linked together in two different ways (Fig. 5). First, at the level of transcription, there are two parallel pathways in which σ^F directs the transcription in the forespore of the structural gene for σ^G and in which σ^E directs the transcription in the mother-cell of the gene for σ^K (together with the others involved in its production). Second, gene expression in the two cell types is linked in a crisscross fashion by mechanisms operating at the level of, and coordinating, the activities of the four factors: the selective activity of σ^F in the forespore signals the appearance of σ^E in the mother-cell, the activity of which regulates that of σ^G in the forespore, which in turn determines the appearance of σ^K in the mother-cell.

The parallel pathways ensure that the early factors σ^F and σ^E are replaced by the late-phase factors σ^G and σ^K respectively, while the crisscross pathway links all four factors in such a way that their sequential activity in the two cell-types is tightly coupled.

In the crisscross pathways, signals go from one cell to the other, transversing the double membrane separating the two cell types. Thus, the cells communicate by telling each other when to proceed to the next development stage. In each case signalling seems to be determined by specific morphological cues: asymmetric septation leads to activation of σ^F in the smaller compartment; some subtle feature of the sporulation septum created by the action of σ^F stimulates the processing of pro-σ^E; the engulfment of the forespore driven by the products of genes controlled by σ^E appears to activate σ^G; the free forespore protoplast and a signal transmitted across it by the action of σ^G stimulates the processing of pro-σ^K. None of the signalling processes is fully understood, but according to this point of view each sigma factor is tightly controlled by a series of checkpoints that tie its activation to the completion of a landmark event in the course of morphogenesis.

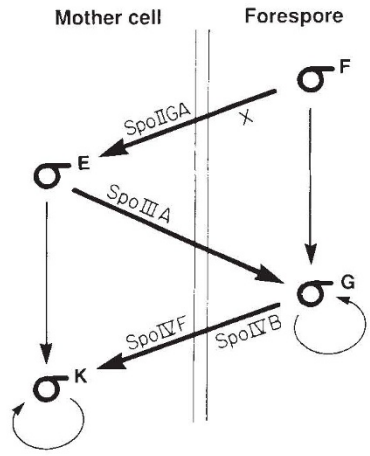

FIG. 5 Crisscross regulation of compartmentalized sigma factors. Summary of the proposed interrelationship of σ^F, σ^E, σ^G and σ^K in the post-septation sporangium (see text). The thin, vertical lines indicate intracellular pathways of transcriptional control; the circular arrows indicate autoregulation. The heavy diagonal lines indicate intercellular pathways acting at the level of sigma-factor activity.

The generation of differentiated cell types is a general feature of development, but the molecular mechanisms by which cell specialization is achieved differ widely among different organisms and among different cell types in the same organism. Nevertheless, the principles that have emerged from the study of differentiation in *B. subtilis*, namely the compartmentalization of transcription factors, the regulation of these factors by intercellular communication, and the linkage of the factors to morphological checkpoints, are likely to be common themes in developing systems of many kinds. □

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