The EMBO Journal vol.14 no.7 pp.1439–1445, 1995

Cell-type specificity during development in *Bacillus subtilis*: the molecular and morphological requirements for σ^E activation

Kamran Shazand[1], Niels Frandsen and Patrick Stragier[2]

Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France
[1]Present address: INSERM U33, Laboratoire Hormones, 94276 Le Kremlin-Bicêtre Cedex, France

[2]Corresponding author

Communicated by P.Stragier

Development in *Bacillus subtilis* involves the formation of two cell types with activation of the transcription factors σ^F in the forespore and σ^E in the mother cell. Activation of σ^E is due to the processing of the inactive precursor pro-σ^E, which requires the putative protease SpoIIGA and the presence of active σ^F. We have introduced missense mutations altering the promoter recognition properties of σ^F. These mutations abolish pro-σ^E processing, suggesting that σ^F is involved through its transcriptional activity and that the processing machinery responds to a signal generated by the product(s) of some unidentifed gene(s) transcribed in the forespore. The role of the septum in transducing this signal was investigated. Induction of σ^F during exponential growth in cells producing SpoIIGA and pro-σ^E led to a high level of processing and σ^E activity. Moreover, pro-σ^E was efficiently processed in a mutant strain blocked prior to septation and synthesizing σ^F in active form at the onset of sporulation. Therefore, the sporulation septum is not required for induction of pro-σ^E processing and pro-σ^E can be processed in the same cell in which σ^F is active. These results suggest that some unknown mechanism must exist to prevent σ^E from becoming active in the forespore.

Key words: asymmetric division/*Bacillus subtilis*/septum/sigma factor/sporulation

Introduction

Sporulation in *Bacillus subtilis* involves the presence of two different cells, the smaller forespore and the larger mother cell, which are generated by the synthesis of a septum in polar position ~1 h after the entry into stationary phase (Errington, 1993). This morphological event is followed by the activation of two transcription factors which induce differential gene expression in the two cells (Losick and Stragier, 1992). Genes controlled by σ^F appear to be exclusively transcribed in the forespore (Margolis *et al.*, 1991; Partridge *et al.*, 1991), whereas genes transcribed by σ^E are transcribed only in the mother cell (Driks and Losick, 1991). σ^F, the product of the *spoIIAC* gene, is synthesized prior to septation, but is maintained in inactive form by its interaction with the SpoIIAB protein (Duncan and Losick, 1993; Min *et al.*, 1993). Release of σ^F activity occurs only after septation, in the forespore, through the action of the SpoIIE and SpoIIAA proteins (Margolis *et al.*, 1991; Alper *et al.*, 1994; Diederich *et al.*, 1994). σ^E, the product of the *spoIIGB* gene, is synthesized before septation as an inactive larger precursor, pro-σ^E (Trempy *et al.*, 1985; LaBell *et al.*, 1987). Conversion to its active form can be induced, albeit inefficiently, when it is artificially synthesized during exponential growth concomitantly with the *spoIIGA* product, suggesting that SpoIIGA is the pro-σ^E processing enzyme (Stragier *et al.*, 1988). However, SpoIIGA is also synthesized in the pre-divisional cell, whereas σ^E becomes active only after septation, which indicates that some signal is required for activation of SpoIIGA (Stragier *et al.*, 1988). Presumably, processing takes place only in the mother cell, where σ^E is active (Losick and Stragier, 1992).

Many mutations have been described as preventing pro-σ^E processing (Stragier *et al.*, 1988; Jonas and Haldenwang, 1989; Beall and Lutkenhaus, 1991). With the exception of *spoIIGA*, they all interfere with the formation of active σ^F (Margolis *et al.*, 1991; Stragier *et al.*, 1994). Therefore, it has been postulated that activation of SpoIIGA requires the action of the product(s) of some gene(s) transcribed by σ^F in the forespore and, more specifically, that this signal acts vectorially across the sporulation septum and leads to pro-σ^E processing only in the mother cell (Margolis *et al.*, 1991; Losick and Stragier, 1992). However, some missense mutations in *spoIIAC* do not block pro-σ^E processing, although they abolish transcription of a σ^F-controlled gene (Errington *et al.*, 1990). This has led to an alternative model in which σ^F plays a 'structural' role in the processing machinery, without having to be proficient in directing transcription (Errington *et al.*, 1990; Illing and Errington, 1991a).

Whatever the mechanism by which σ^F activates pro-σ^E processing, a major issue is to understand how compartmentalization of σ^E activity is established. How does the presence of free σ^F in the forespore lead to σ^E being active only in the mother cell? Before it was known that the transcriptional activities of σ^F and σ^E are compartmentalized, it had been suggested that the sporulation septum plays a crucial role in the timing of pro-σ^E processing (Stragier *et al.*, 1988). Involvement of the sporulation septum seems even more necessary for transducing a signal from one cell to the other. The experiments reported in this paper were carried out in order to clarify the role of σ^F and the sporulation septum in pro-σ^E processing.

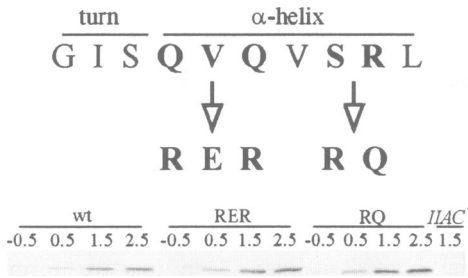

Fig. 1. Altered σ^F proteins. (Top) Residues 229–238 of σ^F are shown. Highlighted residues are supposed to interact with the −35 region of σ^F-recognized promoters. The changes introduced by localized mutagenesis are indicated by arrows. (Bottom) Western blot analysis of wild-type (wt) and mutated (RER and RQ) σ^F molecules in crude extracts of cells harvested every hour, starting 30 min before the onset of sporulation. The three strains are inactivated at the *spoIIAC* locus and at *spoIIIG* (to avoid possible cross-reaction of the anti-σ^F antibody with σ^G). They contain a copy of one of the *spoIIAC* alleles, under the control of the *spoIIA* promoter, integrated at *thrC*. The right-most lane contains an extract from cells without an intact copy of *spoIIAC*, harvested 1.5 h after the end of the exponential phase. The primary antibody is a polyclonal antibody directed against σ^F, kindly provided by L.Duncan.

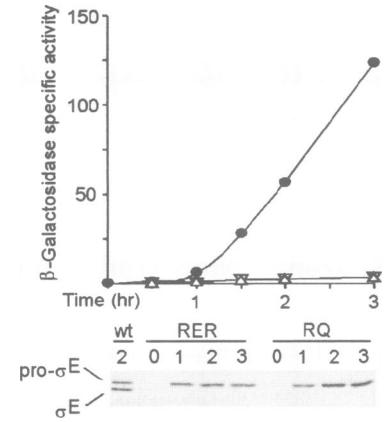

Fig. 2. Properties of the altered σ^F proteins. (Top) Expression of a translational *sspE*-2G−*lacZ* fusion integrated at the *amyE* locus in strains containing the RER (△) or the RQ (▽) *spoIIAC* alleles as compared with a wild-type strain (●). The mutated *spoIIAC* genes are under the control of the *spoIIA* promoter and integrated at the *thrC* locus. Time indicates hours after the onset of sporulation. (Bottom) Western blot analysis of pro-σ^E processing in the same set of strains. Cells were harvested every hour, starting at the onset of sporulation. The left-most lane contains an extract from wild-type cells harvested 2 h after the onset of sporulation. The primary antibody is a monoclonal antibody directed against σ^E, kindly provided by B.Haldenwang.

Results

Mutations inactivating σ^F transcriptional properties

To clarify the role of σ^F in σ^E activation we wanted to completely abolish its ability to act as a transcription factor, without altering its overall structure. Therefore, several residues of the DNA recognition helix of region 4.2 of σ^F were replaced by residues present at the homologous position in σ^A, the *Bacillus subtilis* major vegetative sigma factor (Lonetto *et al.*, 1992). Residues 232–234 (QVQ) were replaced by residues RER in one mutant, while residues 236–237 (SR) were replaced by residues RQ in another (Figure 1). We assumed that such residues would not interfere with the normal folding of the σ^F protein, since they are tolerated at that position in σ^A and its counterparts in many other bacteria (Lonetto *et al.*, 1992). We checked that they do not significantly modify the stability of σ^F by monitoring the presence of the altered σ^F proteins in crude extracts with a polyclonal antibody directed against wild-type σ^F (Figure 1). Strains inactivated for the wild-type *spoIIAC* gene and containing either mutated *spoIIAC* allele (under the control of the *spoIIA* promoter) were completely asporogenous and showed no σ^F transcriptional activity, as measured from expression of a *lacZ* fusion under the control of a σ^F-recognized promoter (Figure 2). Both strains were also completely deficient in pro-σ^E processing (Figure 2).

To obtain indirect evidence that the altered σ^F proteins were still normally folded and able to interact with other proteins, the mutated *spoIIAC* genes were introduced into a *spoIIAB spoIIAC* strain containing a *spoIIE*−*lacZ* fusion. A wild-type copy of *spoIIAC* leads to massive cell death at the onset of the stationary phase in the absence of the SpoIIAB product, presumably because of unregulated σ^F activity (Schmidt *et al.*, 1990; Coppolecchia *et al.*, 1991). Such a toxic effect was not observed with the mutated *spoIIAC* genes, suggesting that σ^F toxicity is due to over-expression of some σ^F-controlled gene(s) no longer transcribed by RNA polymerase associated with the altered forms of σ^F. The presence of the mutated *spoIIAC* genes had a negative effect on expression of the *spoIIE*−*lacZ* fusion, only 35–65% of the amount of β-galactosidase normally present 3 h after the onset of sporulation being found in these strains (unpublished data). We interpret this result as an indication that the altered σ^F molecules are able to compete for core RNA polymerase with σ^A, the sigma factor involved in expression of *spoIIE* (York *et al.*, 1992). Interestingly, the strain containing the QVQ→RER mutation does not produce disporic cells, which are usually observed in the absence of active σ^E (Lewis *et al.*, 1994). Therefore, this strain is apparently unable to build an asymmetric septum and is blocked at stage 0 (although this has not been checked by electron microscopy), an indication that the altered σ^F molecules interfere with transcription of the genes required for septum formation. Introduction of the same QVQ→RER mutation into a *spoIIAB*$^+$ *spoIIAC* background leads to the classic *spoIIAC* phenotype, with production of many disporic cells, suggesting that SpoIIAB is able to interact with these altered σ^F molecules (unpublished data).

Altogether, these results strongly suggest that the loss of transcriptional capacity of the mutated forms of σ^F is not accompanied by misfolding or instability of these proteins. Therefore, their inability to promote pro-σ^E processing is likely to be the consequence of the absence of the product(s) of some member(s) of the σ^F-controlled regulon.

Activation of pro-σ^E processing by σ^F during exponential growth

Artificially induced synthesis of both SpoIIGA and pro-σ^E during exponential growth leads to σ^E activity, as measured from expression of a *lacZ* fusion under the control of the σ^E-controlled *spoIID* promoter (Stragier *et al.*, 1988; Illing and Errington, 1991b). However, the

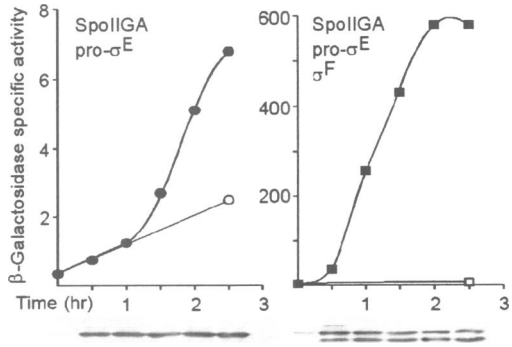

Fig. 3. Pro-σ^E processing during exponential growth. (Top) Expression of a transcriptional *spoIID–lacZ* fusion integrated at the *amyE* locus in strains harbouring a replicative plasmid with the *spoIIG* operon under the control of the *spac* promoter. Time indicates hours after the addition of IPTG in part of the cultures (●, ■), a control sample without IPTG being processed at the end of the experiment (○, □). The left panel corresponds to a control strain inactivated for *spoIIIG* (●, ○) (to avoid possible interference from σ^G molecules encoded by the σ^F-controlled *spoIIIG* gene); the right panel corresponds to a strain inactivated for *spoIIIG* and containing an extra copy of *spoIIAC* (encoding σ^F) under the control of the *spac* promoter and integrated at the *thrC* locus (■, □). Note the difference in scale. These experiments, as well as those presented in the other figures, have been done at least three times and the β-galactosidase levels were highly reproducible. The higher level of *spoIID–lacZ* induction observed in previous experiments in the presence of SpoIIGA and pro-σ^E (Stragier *et al.*, 1988) is apparently due to some difference between the JH642 and the 168 genetic backgrounds (unpublished data). (Bottom) Western blot analysis of pro-σ^E processing, after addition of IPTG, in the same set of strains and the same samples, using the same primary antibody as in Figure 2.

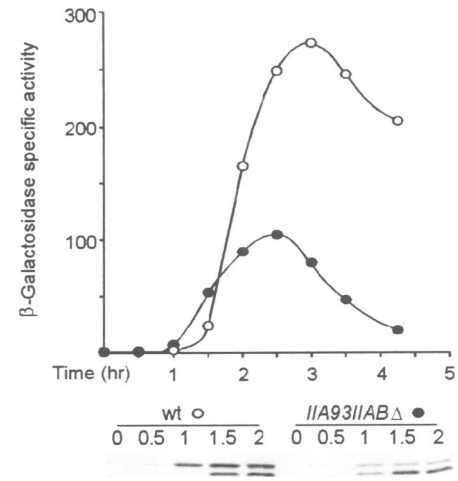

Fig. 4. Pro-σ^E processing in a stage 0 mutant. (Top) Expression of a transcriptional *spoIID–lacZ* fusion integrated at the *amyE* locus in a wild-type strain (○) and in a *spoIIA93 spoIIABΔ* mutant (●). Time indicates hours after the end of exponential growth. (Bottom) Western blot analysis of pro-σ^E processing in the same set of strains and in samples harvested at the hours indicated, using the same primary antibody as in Figure 2.

level of σ^E activity observed is very low and processing is not efficient enough to be visualized by Western blot analysis. This low level of processing was speculated to be mediated by the vegetative septa of the growing bacteria (Stragier *et al.*, 1988). We wondered if the unidentified product(s) of the σ^F-controlled regulon involved in activation of pro-σ^E processing during sporulation would also be able to enhance processing during exponential growth. To address this question, we constructed a strain containing an extra copy of the *spoIIAC* gene under the control of the isopropyl β-D-thiogalactoside (IPTG)-inducible *spac* promoter, as well as a replicative plasmid harbouring the *spoIIG* operon (encoding both SpoIIGA and pro-σ^E) under the control of *spac*. Addition of IPTG to an exponentially growing culture of that strain led to the immediate production of large amounts of processed σ^E (Figure 3) and to active transcription of a *spoIID–lacZ* fusion (Figure 3). Although induction of σ^F synthesis led to a dramatic decrease in the growth rate of the culture, it enhanced σ^E activity by about two orders of magnitude as compared with a strain producing only SpoIIGA and pro-σ^E (Figure 3). No activity was found in a strain producing only σ^F and pro-σ^E (data not shown). Therefore, pro-σ^E processing can be extremely efficient in non-sporulation conditions if SpoIIGA and the products of the σ^F-controlled genes are present.

Pro-σ^E processing in a stage 0 sporulation mutant

Mutations preventing asymmetric septation block pro-σ^E processing (Beall and Lutkenhaus, 1991, 1992; Levin and Losick, 1994) and it is believed that the sporulation septum is intimately involved in controlling SpoIIGA processing activity (Stragier *et al.*, 1988; Higgins and Piggot, 1992; Losick and Stragier, 1992). The high level of processing observed during exponential growth in the previous experiments indicated that it was possible to bypass the presence of the sporulation septum. However, it was still possible that the vegetative septa were playing an analogous role under these artificial conditions, as suggested previously (Stragier *et al.*, 1988). To clarify this issue, we took advantage of a strain recently isolated in our laboratory in which σ^F is synthesized in a constitutively active form at the onset of the stationary phase without killing the cell. Strain MO1300 is deleted for the *spoIIAB* gene and contains an additional mutation in the promoter of the *spoIIA* operon, *spoIIA93*, which reduces its transcription to 3–5% of the wild-type level (C.Karmazyn-Campelli, K.Shazand and P.Stragier, in preparation). Presumably, the lower amount of σ^F molecules accounts for the loss of toxicity of the *spoIIAB* deletion. Electron microscopy studies have shown that this strain is blocked at stage 0 (C.Karmazyn-Campelli, K.Shazand and P.Stragier, in preparation).

The *spoIIG* operon is expressed before asymmetric septation and its products, SpoIIGA and pro-σ^E, were expected to be present in strain MO1300. Therefore, when cells of this strain reach stationary phase in sporulation medium, they accumulate active σ^F as well as SpoIIGA and pro-σ^E, just as was engineered in vegetative cells in the experiments described above, but in the complete absence of septation. Western blot analysis showed that pro-σ^E is efficiently processed in strain MO1300, the mature form of σ^E being present 30 min earlier than in a wild-type strain (Figure 4). Moreover, introduction of a *spoIID–lacZ* fusion in strain MO1300 indicated that σ^E is active as soon as its processed form is detectable (Figure 4).

To rule out the possible involvement of some abortive septa or the existence of a small percentage of MO1300

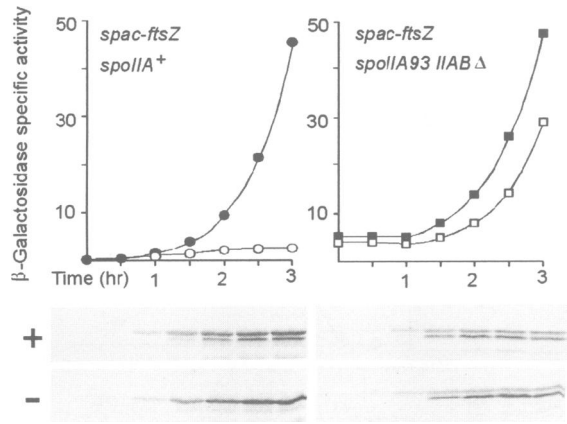

Fig. 5. Pro-σ^E processing in the absence of septation. (Top) Expression of a translational *sspE*-2G−*lacZ* fusion integrated at the *amyE* locus in strains in which the *ftsZ* gene is under the control of the *spac* promoter. Cultures were performed as described in Materials and methods and cells resuspended in the presence (●, ■) or absence of IPTG (○, □). The left panel corresponds to a control strain inactivated for *spoIIIG* (●, ○) (to measure only the σ^F-dependent expression of *sspE*-2G−*lacZ*); the right panel corresponds to a strain inactivated for *spoIIIG* and containing the *spoIIA93 spoIIABΔ* double mutation (■, □). Time indicates hours after the onset of sporulation. (Bottom) Western blot analysis of pro-σ^E processing in the same set of strains and the same samples, with (+) or without (−) addition of IPTG, using the same primary antibody as in Figure 2.

cells still being able to septate, we replaced the essential cell division gene *ftsZ* in that strain with a modified gene whose promoter has been substituted with the IPTG-inducible promoter *spac* (Beall and Lutkenhaus, 1991). Such cells can only grow in the presence of IPTG. If the culture is depleted of IPTG at an initial OD_{570} of 0.2–0.4 and grown in sporulation medium, the cells increase in mass for about three doublings before reaching stationary phase at the same optical density as in the presence of IPTG (Beall and Lutkenhaus, 1991). However, due to the lack of FtsZ molecules, they are unable to achieve asymmetric septation and pro-σ^E is not processed (Beall and Lutkenhaus, 1991). Depletion of FtsZ was carried out with the MO1300 derivative strain, as well as in a control *spoIIAB*$^+$ strain. As expected, the absence of FtsZ abolished σ^F activity in the *spoIIAB*$^+$ strain and prevented pro-σ^E processing (Figure 5). Conversely, depleting the cells of FtsZ did not affect σ^F activity in the MO1300 derivative and did not block pro-σ^E processing (Figure 5).

Discussion

σ^F transcriptional ability is required for pro-σ^E processing

It has been known for some time that disruption of *spoIIAC*, the gene encoding σ^F, leads to accumulation of σ^E in its inactive unprocessed form (Jonas and Haldenwang, 1989). However, some missense mutations in *spoIIAC* abolish σ^F transcriptional ability (as measured by expression of a *spoIIIG*−*lacZ* fusion) without preventing pro-σ^E processing (Errington *et al.*, 1990). This led to the proposal that σ^F could be required for pro-σ^E processing without being transcriptionally active (Errington *et al.*, 1990; Illing and Errington, 1991a). The *spoIIAC* missense mutations described in this paper have been designed to avoid any 'leakiness' (by changing several residues in the DNA recognition helix of region 4.2), without modifying the overall structure of the protein (by choosing residues already present at these positions in σ^A and its σ^{70} homologues). Our aim was not to create sigma factors with hybrid σ^F−σ^A recognition properties (since only part of region 4.2 was modified), but to generate a complete loss of promoter recognition and therefore of transcriptional capacity.

These altered σ^F proteins are normally accumulated in the cell and indirect evidence was obtained that they are able to interact with RNA polymerase (and inhibit expression of genes controlled by other sigma factors) and, in the case of the QVQ→RER mutant, with SpoIIAB, the anti-σ^F protein. Therefore, the parallel loss of expression of the σ^F-controlled *sspE*-2G−*lacZ* fusion and of pro-σ^E processing is a strong indication that transcription of some gene(s) controlled by sigma-F (and collectively designated by the *csfX* acronym) is required for processing to take place. We cannot exclude the possibility that the two clusters of residues altered in our mutants are both involved in the proposed 'structural' function of σ^F in activating pro-σ^E processing (Errington *et al.*, 1990). However, this possibility appears very unlikely since amino acids located at these positions in other sigma factors are known to be involved in DNA recognition (Lonetto *et al.*, 1992), which should create strong structural constraints and preclude additional interactions involving the same residues.

In order to explain how some missense mutations in *spoIIAC*, like *spoIIAC561*, have a dramatic effect on the expression of some *csf* genes (*spoIIIG*, as well as the *sspE*-2G−*lacZ* fusion, unpublished data) without preventing pro-σ^E processing, we have to assume that some genes, among which is *csfX*, are still expressed in these mutant backgrounds at a sufficient level for their products to carry out their function normally.

Pro-σ^E processing is not dependent on septum formation

It is now known that σ^F activity itself depends on formation of the sporulation septum (Margolis, 1993; Stragier *et al.*, 1994). This might be the basis of the septum requirement for pro-σ^E processing, but the sporulation septum could also play a direct role in σ^E activation. Simultaneous induction of SpoIIGA and pro-σ^E synthesis during exponential growth has been used to demonstrate that SpoIIGA is sufficient to obtain measurable σ^E activity and, therefore, presumably processing, although this could not be shown directly (Stragier *et al.*, 1988). This result was a strong indication that SpoIIGA is the actual pro-σ^E processing enzyme, but the low level of σ^E activity suggested that some component of the processing machinery was missing or that SpoIIGA was mostly in an inactive conformation under these experimental conditions (Stragier *et al.*, 1988). Now we report that the additional induction of σ^F, and thus of all the products of the σ^F-controlled regulon, enhances σ^E activity by about two orders of magnitude and leads to accumulation of large amounts of processed σ^E. This result is even more striking if one takes into account the fact that cells in which σ^F is induced are sick and rapidly stop growing and that the σ^F molecules are potential competitors of σ^E for core RNA polymerase.

What was supposed to be a very tightly controlled mechanism, intimately dependent on the specific conditions present in sporulating cells at the time of septum formation, can work extremely efficiently in exponentially growing cells.

These results cast doubts on the requirement for some specific properties of the sporulation septum in transducing the *csfX*-generated signal to the processing machinery, unless it is assumed that the vegetative septa are somehow modified as a consequence of σ^F activity. The availability of a stage 0 sporulation mutant in which σ^F is constitutively active without killing the cells has allowed us to rule out the participation of the sporulation septum in pro-σ^E processing. Although electron microscopy studies have consistently shown that this mutant is actually blocked before septation, we have introduced an additional *ftsZ* conditional mutation to avoid any misinterpretation of the results. Clearly, processing can take place in a stage 0 cell if σ^F is present in active form. It should be noted that in this stage 0 mutant the amount of σ^F molecules is much lower than in wild-type cells, which excludes the possibility that the observed processing could be an artificial consequence of over-producing the proteins encoded by the σ^F-controlled regulon.

How is σ^E activity restricted to the mother cell?

It is now widely accepted that σ^E and σ^F are mainly, if not exclusively, active in different cell types (Errington, 1993; Stragier *et al.*, 1994). Compartmentalization of σ^E activity has been proposed to result from pro-σ^E processing occurring exclusively in the mother cell, in response to the vectorial action of the product of a σ^F-controlled gene (Losick and Stragier, 1992). This model needs to be reassessed: if pro-σ^E processing can take place in the same cell in which σ^F-dependent transcription occurs, what are the mechanisms leading to σ^E and σ^F being active in two different cells?

It has recently been suggested, based on fluorescence microscopy studies, that the initial burst of activity of σ^F and σ^E could occur in both cells, followed by a shift to compartmentalized activity (Bylund *et al.*, 1994). This model easily accommodates the results reported in this paper, σ^F becoming active in both cells, presumably in response to a signal generated after asymmetric septation, the product(s) of some gene(s) controlled by σ^F activating SpoIIGA-mediated pro-σ^E processing in both cells and some unidentified mechanism leading to specific enhancement and maintenance of σ^F activity in the forespore and σ^E activity in the mother cell.

Alternatively, σ^F might become active only in the forespore. If this is actually the case, the results reported in this paper suggest that pro-σ^E processing occurs in the forespore, as has been proposed from fractionation studies (Carlson and Haldenwang, 1989; Kirchman *et al.*, 1993). Then some additional mechanism must prevent the processed σ^E molecules interacting with RNA polymerase and initiating the mother cell programme of gene expression in the forespore. Such an inhibition could be mediated by an anti-sigma factor, as has been shown for σ^F (Duncan and Losick, 1993; Min *et al.*, 1993) and σ^B (Benson and Haldenwang, 1993). This putative σ^E inhibitor could be the product of a gene controlled by σ^F. In that case, σ^F would simultaneously activate pro-σ^E processing and prevent σ^E from becoming active in the forespore. Since we have found conditions in which σ^F and σ^E are active in the same cell, some stoichiometry between σ^E and its inhibitor is probably necessary to completely shut off σ^E activity. It is also possible that the interaction between the two proteins is modulated by metabolic conditions that are optimal only in the forespore. Finally, although pro-σ^E processing can clearly take place in the same cell in which σ^F is active, that some special feature of the forespore prevents processing in that cell cannot be excluded.

If σ^F becomes active only in the forespore, the abundant presence of active σ^E in the mother cell implies that the signal generated by the CsfX protein(s) has to be transduced from the forespore to the mother cell. The SpoIIGA putative protease is likely to be a membrane bound protein (Stragier *et al.*, 1988; Peters and Haldenwang, 1991) and SpoIIGA molecules sitting on the mother cell side of the septum membrane could be activated by a short-range signal originating from the adjacent forespore membrane. Such a cell–cell signalling pathway would require the CsfX protein(s) to act outside the forespore cytoplasm. The sporulation septum would still play a major role in allowing local diffusion of the forespore signal, but restriction of σ^E activity to the mother cell would ultimately rely upon the existence of a forespore-specific mechanism of σ^E inhibition.

Materials and methods

Construction of spoIIAC mutations

Cloning techniques were as previously described (Sambrook *et al.*, 1989). The distal part of the *spoIIAC* gene was cloned in a phagemid and single-stranded DNA was prepared and hybridized with the following oligonucleotides (changes are underlined): 5′-CAAGCCTGGAAACC-CTTTCGCGAGAGATCCCGAGC-3′, (which converted residues 232–234 of σ^F from QVQ to RER and simultaneously introduced a *Nru*I site) and 5′-ATTTTCTTTTCAAGCTGTCGAACCTGCACCTGAG-3′ (which converted residues 236–237 of σ^F from SR to RQ and simultaneously introduced a *Taq*I site). Mutagenesis was carried out by using an oligonucleotide-directed *in vitro* mutagenesis kit (Amersham; RPN1523). After mutagenesis, plasmids were prepared from *Escherichia coli* TG1 and screened for the presence of the new restriction sites. Positive candidates were further analysed by DNA sequencing (Sanger *et al.*, 1977). Complete copies of the *spoIIAC* gene carrying the desired mutations were reconstituted by fusing the mutated *Ava*I–*Pst*I fragments (containing the last 28 codons of *spoIIAC*) to the *Cla*I–*Ava*I fragment containing the proximal part of the *spoIIAC* gene (Yudkin, 1987). The reconstituted *spoIIAC* genes were cloned downstream of the *Sca*I–*Hin*PI fragment containing the *spoIIA* promoter (Wu *et al.*, 1991), such that both *spoIIAA* and *spoIIAB* genes were inactivated and fused in-frame (Fort and Piggot, 1984) to ensure proper expression of *spoIIAC*. These constructs were cloned into a plasmid allowing marker exchange at the *thrC* locus of the *B.subtilis* chromosome (A.-M.Guérout-Fleury, N.Frandsen, K.Shazand and P.Stragier, in preparation). A similar construct with the wild-type copy of *spoIIAC* fully complemented a *spoIIAC* mutation.

lacZ fusions

Genetic techniques were as previously described (Stragier *et al.*, 1988). All *B.subtilis* strains were derivatives of strain JH642. All *lacZ* fusions were associated with a chloramphenicol resistance marker and introduced by marker exchange at the *amyE* locus of the *B.subtilis* chromosome (Shimotsu and Henner, 1986). The *sspE*-2G–*lacZ* translational fusion contains 80 bp of DNA upstream of the *sspE* transcription start as well as a mutation introducing a G residue at position −15, allowing this promoter to be efficiently recognized by σ^F (Sun *et al.*, 1991). The *spoIID*–*lacZ* transcriptional fusion has been described (Stragier *et al.*, 1988). The *spoIIE*–*lacZ* transcriptional fusion is a derivative of the pZΔ326-GV43 plasmid (Guzman *et al.*, 1988). β-Galactosidase activity

in sonicated extracts was measured as previously described (Stragier *et al.*, 1988) and is expressed as nmol 2-nitrophenyl-β-D-galactopyranoside hydrolyzed/min/mg protein.

Gene expression under the control of the spac promoter

The replicative plasmids containing the *spoIIG* operon or the *spoIIGB* gene under the control of the *spac* promoter have been described (Stragier *et al.*, 1988). The *ClaI–PstI* fragment containing the *spoIIAC* gene (Yudkin, 1987) was recovered as a *HindIII–SalI* fragment after a cloning step in a polylinker and then cloned downstream of the *spac* promoter in pDG148 (Stragier *et al.*, 1988). An *EcoRI–BamHI* fragment containing *spac–spoIIAC* and *lacI* was subsequently cloned in a plasmid allowing marker exchange at the *thrC* locus of the *B.subtilis* chromosome (A.-M.Guérout-Fleury, N.Frandsen, K.Shazand and P.Stragier, in preparation). Induced synthesis of SpoIIGA, pro-σ^E and σ^F was carried out by adding IPTG (1 mM final concentration) to cells growing in 2× YT medium and having reached an OD_{570} of 0.3. The fusion of the proximal part of *ftsZ* to the *spac* promoter was moved from plasmid pJSIZΔp (Beall and Lutkenhaus, 1991) to the pDG641 vector (Gonzy-Tréboul *et al.*, 1992), such that selection for erythromycin resistance (in the presence of 1 mM IPTG) led to integration of the plasmid by a single homologous event into the chromosomal *ftsZ* gene, which came under the control of the *spac* promoter. Cells containing the *spac–ftsZ* fusion were grown in sporulation medium (Schaeffer *et al.*, 1965) in the presence of 1 mM IPTG until they reached an OD_{570} of 1.0, when they were harvested at room temperature, washed and resuspended in sporulation medium at an OD_{570} of 0.35, with or without IPTG. Under these conditions both cultures reached stationary phase at around the same OD_{570}, ~2.5.

Western blot analysis

Clarified crude extracts prepared by ultrasonication were analysed by polyacrylamide gel electrophoresis (12.5% acrylamide) in the presence of sodium dodecyl sulfate. Proteins were detected by immunoblotting (Sambrook *et al.*, 1989), using the appropriate anti-sigma antibody and a secondary antibody conjugated to alkaline phosphatase (Sigma or Hyclone).

Acknowledgements

We thank Peter Setlow for providing the *sspE*-2G–*lacZ* fusion, Joe Lutkenhaus for the *spac–ftsZ* plasmid, Len Duncan for the anti-σ^F antibody, Bill Haldenwang for the anti-σ^E antibody and Rich Losick for stimulating discussions. K.S. was supported by the CNRS and by a grant from the Fondation Mérieux. N.F. was a fellow of the Danish Council of Research. This work was supported by grants from the CNRS (URA1139), INSERM (CRE930111) and the Human Frontier Science Program.

References

Alper,S., Duncan,L. and Losick,R. (1994) An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B.subtilis*. *Cell*, **77**, 195–205.

Beall,B. and Lutkenhaus,J. (1991) FtsZ in *Bacillus subtilis* is required for vegetative septation and for asymmetric septation during sporulation. *Genes Dev.*, **5**, 447–455.

Beall,B. and Lutkenhaus,J. (1992) Impaired cell division and sporulation of a *Bacillus subtilis* strain with the *ftsA* gene deleted. *J. Bacteriol.*, **174**, 2398–2403.

Benson,A.K. and Haldenwang,W.G. (1993) *Bacillus subtilis* σ^B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase. *Proc. Natl Acad. Sci. USA*, **90**, 2330–2334.

Bylund,J.E., Zhang,L., Haines,M.A., Higgins,M.L. and Piggot,P.J. (1994) Analysis by fluorescence microscopy of the development of compartment-specific gene expression during sporulation of *Bacillus subtilis*. *J. Bacteriol.*, **176**, 2898–2905.

Carlson,H.C. and Haldenwang,W.G. (1989) The σ^E subunit of *Bacillus subtilis* RNA polymerase is present in both forespore and mother cell compartments. *J. Bacteriol.*, **171**, 2216–2218.

Coppolecchia,R., DeGrazia,H. and Moran,C.P.,Jr (1991) Deletion of *spoIIAB* blocks endospore formation in *Bacillus subtilis* at an early stage. *J. Bacteriol.*, **173**, 6678–6685.

Diederich,B., Wilkinson,J.F., Magnin,T., Najafi,S.M.A., Errington,J. and Yudkin,M.D. (1994) Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor σ^F of *Bacillus subtilis*. *Genes Dev.*, **8**, 2653–2663.

Driks,A. and Losick,R. (1991) Compartmentalized expression of a gene under the control of sporulation transcription factor σ^E in *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA*, **88**, 9934–9938.

Duncan,L. and Losick,R. (1993) SpoIIAB is an anti-σ factor that binds to and inhibits transcription by regulatory protein σ^F from *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA*, **90**, 2325–2329.

Errington,J. (1993) Sporulation in *Bacillus subtilis*: regulation of gene expression and control of morphogenesis. *Microbiol. Rev.*, **57**, 1–33.

Errington,J., Foulger,D., Illing,N., Partridge,S.R. and Stevens,C.M. (1990) Regulation of differential gene expression during sporulation in *Bacillus subtilis*. In Zukowski,M.M., Ganesan,A.T. and Hoch,J.A. (eds), *Genetics and Biotechnology of Bacilli*. Academic Press, San Diego, CA, pp. 257–267.

Fort,P. and Piggot,P.J. (1984) Nucleotide sequence of the sporulation locus *spoIIA* in *Bacillus subtilis*. *J. Gen. Microbiol.*, **130**, 2147–2153.

Gonzy-Tréboul,G., Karmazyn-Campelli,C. and Stragier,P. (1992) Developmental regulation of transcription of the *Bacillus subtilis ftsAZ* operon. *J. Mol. Biol.*, **224**, 967–979.

Guzman,P., Westpheling,J. and Youngman,P. (1988) Characterization of the promoter region of the *Bacillus subtilis spoIIE* operon. *J. Bacteriol.*, **170**, 1598–1609.

Higgins,M.L. and Piggot,P.J. (1992) Septal membrane fusion—a pivotal event in bacterial spore formation? *Mol. Microbiol.*, **6**, 2565–2571.

Illing,N. and Errington,J. (1991a) Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of σ^E and σ^F in prespore engulfment. *J. Bacteriol.*, **173**, 3159–3169.

Illing,N. and Errington,J. (1991b) The *spoIIIA* operon of *Bacillus subtilis* defines a new temporal class of mother-cell-specific sporulation genes under the control of the σ^E form of RNA polymerase. *Mol. Microbiol.*, **5**, 1927–1940.

Jonas,R.M. and Haldenwang,W.G. (1989) Influence of *spo* mutations on σ^E synthesis in *Bacillus subtilis*. *J. Bacteriol.*, **171**, 5226–5228.

Kirchman,P.A., DeGrazia,H., Kellner,E.M. and Moran,C.P.,Jr (1993) Forespore-specific disappearance of the sigma-factor antagonist SpoIIAB: implications for its role in determination of cell fate in *Bacillus subtilis*. *Mol. Microbiol.*, **8**, 663–671.

LaBell,T.L., Trempy,J.E. and Haldenwang,W.G. (1987) Sporulation-specific σ factor σ^{29} of *Bacillus subtilis* is synthesized from a precursor protein, P^{31}. *Proc. Natl Acad. Sci. USA*, **84**, 1784–1788.

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.J., 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.

Lonetto,M., Gribskov,M. and Gross,C.A. (1992) The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.*, **174**, 3843–3849.

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

Margolis, P. (1993) Establishment of cell type during sporulation in *Bacillus subtilis*. PhD Thesis, Harvard University, Cambridge, MA.

Margolis,P., Driks,A. and Losick,R. (1991) Establishment of cell type by compartmentalized activation of a transcription factor. *Science*, **254**, 562–565.

Min,K.-T., Hilditch,C.M., Diederich,B., Errington,J. and Yudkin,M.D. (1993) σ^F, the first compartment specific transcription factor of *Bacillus subtilis*, is regulated by an anti-sigma factor which is also a protein kinase. *Cell*, **74**, 735–742.

Partridge,S.R., Foulger,D. and Errington,J. (1991) The role of σ^F in prespore-specific transcription in *Bacillus subtilis*. *Mol. Microbiol.*, **5**, 757–767.

Peters,H.K. and Haldenwang,W.G. (1991) Synthesis and fractionation properties of SpoIIGA, a protein essential for pro-σ^E processing in *Bacillus subtilis*. *J. Bacteriol.*, **173**, 7821–7827.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Schaeffer,P., Millet,J. and Aubert,J. (1965) Catabolite repression of bacterial sporulation. *Proc. Natl Acad. Sci. USA*, **54**, 704–711.

Schmidt,R., Margolis,P., Duncan,L., Coppolecchia,R., Moran,C.P.,Jr and

Losick,R. (1990) Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA*, **87**, 9221–9225.

Shimotsu,H. and Henner,D.J. (1986) Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene*, **43**, 85–94.

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.

Stragier,P., Margolis,P. and Losick,R. (1994) Establishment of compartment-specific gene expression during sporulation in *Bacillus subtilis*. In Piggot,P.J., Moran,C.P.,Jr and Youngman,P. (eds), *Regulation of Bacterial Differentiation*. American Society for Microbiology, Washington, DC, pp. 139–154.

Sun,D., Fajardo-Cavazos,P., Sussman,M.D., Tovar-Rojo,F., Cabrera-Martinez,R.M. and Setlow,P. (1991) Effect of chromosome location of *Bacillus subtilis* forespore genes on their *spo* gene dependence and transcription by $E\sigma^F$: identification of features of good $E\sigma^F$-dependent promoters. *J. Bacteriol.*, **173**, 7867–7874.

Trempy,J.E., Bonamy,C., Szulmajster,J. and Haldenwang,W.G. (1985) *Bacillus subtilis* sigma factor, σ^{29}, is the product of the sporulation-essential gene *spoIIG*. *Proc. Natl Acad. Sci. USA*, **82**, 4189–4192.

Wu,J.-J., Piggot,P.J., Tatti,K.M. and Moran,C.P.,Jr (1991) Transcription of the *Bacillus subtilis spoIIA* operon. *Gene*, **101**, 113–116.

York,K., Kenney,T.J., Satola,S., Moran,C.P.,Jr, Poth,H. and Youngman,P. (1992) Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. *J. Bacteriol.*, **174**, 2648–2658.

Yudkin,M.D. (1987) Structure and function in a *Bacillus subtilis* sporulation-specific sigma factor: molecular nature of mutations in *spoIIAC*. *J. Gen. Microbiol.*, **133**, 475–481.

Received on November 22, 1994; revised on January 11, 1995

Note added in proof

The *csfX* gene has now been identified [J.-A.Londoño-Vallejo and P.Stragier (1995), Cell–cell signaling pathway activating a developmental transcription factor in *Bacillus subtilis*. *Genes Dev.*, in press; and M.L.Karow, P.Glaser and P.J.Piggot (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*, in press].