Direct interaction between the cell division protein FtsZ and the cell differentiation protein SpoIIE

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SpoIIE is a bifunctional protein with two critical roles in the establishment of cell fate in Bacillus subtilis. First, SpoIIE is needed for the normal formation of the asymmetrically positioned septum that forms early in sporulation and separates the mother cell from the prespore compartment. Secondly, SpoIIE is essential for the activation of the first compartment-specific transcription factor σF in the prespore. After initiation of sporulation, SpoIIE localizes to the potential asymmetric cell division sites near one or both cell poles. Localization of SpoIIE was shown to be dependent on the essential cell division protein FtsZ. To understand how SpoIIE is targeted to the asymmetric septum we have now analysed its interaction with FtsZ in vitro. Using the yeast two-hybrid system and purified FtsZ, and full-length and truncated SpoIIE proteins, we demonstrate that the two proteins interact directly and that domain II and possibly domain I of SpoIIE are required for the interaction. Moreover, we show that SpoIIE interacts with itself and suggest that this self-interaction plays a role in assembly of SpoIIE into the division machinery.

Keywords: Bacillus subtilis/cell division/oligomerization/protein–protein interaction/sporulation

Introduction

A fundamental question in developmental biology is how cell division and cell fate are linked together. In organisms like the fly Drosophila melanogaster, the nematode Caenorhabditis elegans, the green alga Volvox carteri and the budding yeast Saccharomyces cerevisiae, the formation of an asymmetrically positioned division septum gives rise to two unequally sized progeny with different developmental potentials (reviewed by Horvitz and Herskowitz, 1992; Jan and Jan, 1998). In the Gram-positive bacterium Bacillus subtilis, asymmetric division is a key feature of spore formation. Early in sporulation, the developing cell divides asymmetrically into a smaller compartment, the prespore, which becomes the spore, and a larger compartment, the mother cell, which participates in the maturation of the spore and finally lyses to release it. The development of the two cells are dictated by the localized activation of sporulation-specific transcription factors. The first transcription factor, σF, which only becomes active in the prespore, governs the activation and expression of the remaining cell-specific transcription factors (as reviewed by Errington, 1996; Losick and Dworkin, 1999).

σF is made before asymmetric septation, together with two coordinately expressed regulatory proteins, SpoIIAA and SpoIIB (Gholamhoseinian and Piggot, 1989). At first, σF is held in an inactive complex by the anti-σ factor SpoIIB (Duncan and Losick, 1993; Min et al., 1993; Alper et al., 1994). SpoIIB is also a protein kinase, which phosphorylates the anti-anti-σ factor SpoIIAA on a specific serine residue, thereby maintaining it in an inactive state (Min et al., 1993; Diederich et al., 1994; Najafi et al., 1995; Duncan et al., 1996; Maginn et al., 1996). After asymmetric septation, σF remains inactive in the mother cell, but in the prespore it is released from SpoIIB (Margolis et al., 1991). This release is triggered by SpoIIE, a membrane-bound phosphatase, which dephosphorylates SpoIIAA-P and allows it to bind SpoIIB, thereby releasing σF (Duncan et al., 1995; Arigoni et al., 1996; Feucht et al., 1996). σF is then free to associate with core RNA polymerase and direct transcription. Thus, SpoIIE plays a key role in σF activation by regulating the phosphorylation state of SpoIIAA.

SpoIIE is an integral membrane protein and has a multi-domain structure (Figure 1) (Barak et al., 1996; Arigoni et al., 1999). It contains 10 membrane-spanning segments in its N-terminal domain (domain I), which targets the protein to the membrane. The large central domain (domain II) is poorly conserved and its function is unknown. The C-terminal domain (domain III) contains serine phosphatase activity and is structurally related to eukaryotic PP2C protein phosphatases (Adler et al., 1997). PP2C phosphatases are involved in regulating stress response pathways in both prokaryotes and eukaryotes (Das et al., 1996). SpoIIE is synthesized before asymmetric division and is localized initially in ring-like structures at one or both potential sites of asymmetric division near the cell poles (Arigoni et al., 1995). During asymmetric septation, most of the SpoIIE from the ring at the prespore is sequestered to the prespore side of the asymmetric septum, which could favour the activation of σF in the prespore (Wu et al., 1998). The prespore-distal SpoIIE ring (in the mother cell) then disappears (Arigoni et al., 1995; Pogliano et al., 1997).

In nearly all bacteria, cytokinesis begins with the polymerization of the essential GTPase FtsZ into a ring structure at the nascent division site (reviewed by Bramhill, 1997; Lutkenhaus and Addinall, 1997; Rothfield and Justice, 1997). The FtsZ ring provides a cytoskeletal scaffold that recruits other division proteins and directs the ingrowth of the septum (Bi and Lutkenhaus, 1991;
Addinall and Lutkenhaus, 1996a,b; Addinall et al., 1996, 1997; Ma et al., 1996; Hale and de Boer, 1997; Wang et al., 1997; Weiss et al., 1997; Yu et al., 1998). The crystal structure of FtsZ closely resembles that of eukaryotic α- and β-tubulin (Löwe and Amos, 1998; Nogales et al., 1998). Like tubulin, FtsZ undergoes GTP/GDP-dependent polymerization, forming protofilaments, sheets and mini-rings in vitro (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994, 1998; Erickson et al., 1996; Yu and Margolin, 1997). In B. subtilis, FtsZ localization shifts from a medial to a bipolar pattern after the onset of sporulation, forming a ring near each pole (Levin and Losick, 1996). Immunofluorescence microscopy has shown that localization of SpoIIE also requires FtsZ and is independent of other known division proteins like FtsA, FtsL, DivIC and DivIB (Levin et al., 1997; Feucht et al., 1999). Recently, it has been suggested that the phosphatase activity of SpoIIE is partially dependent on the FtsZ-dependent assembly of SpoIIE (King et al., 1999). In turn, in a spoIIE deletion mutant, polar FtsZ ring formation is impaired, suggesting that SpoIIE contributes to the switch from medial to polar septation (Khvorova et al., 1998). Additionally, it has been shown that SpoIIE plays a role in septal morphogenesis (Piggot, 1973; Illing and Errington, 1991). The precise role of SpoIIE in septation is still not clear, but the finding that the protein has two distinct and independent functions provides a fascinating example of co-ordination between morphogenesis and transcriptional regulation (Barak and Youngman, 1996; Feucht et al., 1996).

In this study, we have examined how SpoIIE is targeted to the asymmetric septum by analysing its interaction with FtsZ. Using purified FtsZ and full-length and truncated SpoIIE proteins, we demonstrate that the two proteins interact directly and that domain II and possibly domain I of SpoIIE are required for the interaction. Moreover, our data suggest that SpoIIE self-interaction plays an important role in the assembly of SpoIIE into the division machinery.

Results

**Purified SpoIIE interacts with SpoIIE and FtsZ from a B. subtilis cell extract**

To find interacting partners of SpoIIE that might be involved in regulation or targeting, we purified full-length SpoIIE and its cytoplasmic domain, SpoIIE (II–III) (see Figure 1), both fused to a strep-tag, by using streptavidin-coated magnetic beads. The beads carrying either full-length SpoIIE or SpoIIE (II–III) with SpoIIE and FtsZ from sporulating cell extracts. Cell extract was incubated with strep-tagged protein on magnetic beads and washed. Proteins from the beads were separated by SDS–PAGE and (A) silver stained or blotted (B) with anti-SpoIIE antibodies and (C) with anti-FtsZ antibodies. Lane 1, total cell extract; lane 2, cell extract incubated with purified strep-tagged SpoIIE (II–III); lane 3, cell extract incubated with purified strep-tagged FtsZ; lane 4, cell extract incubated with purified strep-tagged Mbl. In (A), the arrowhead for each lane points to the protein that is bound to the magnetic beads. Strep-tagged proteins are referred as to 5SpoIIE, 5SpoIIE (II–III) and 5Mbl. In (B) and (C), the positions of full-length SpoIIE, SpoIIE (II–III) and FtsZ are indicated by arrows. M, Biolabs broad range marker. The sizes of the proteins (in kilodaltons) are indicated on the left side of the figure.
were detected (Figure 2B and C, lane 2). FtsZ was also bound to the purified strep-tagged full-length SpoIIE (Figure 2C, lane 3). It was not possible to determine whether SpoIIE from the sporulating cell extract was also retained because the two proteins ran at the same position.

**Interaction of SpoIIE with itself as detected by the yeast two-hybrid system**

To confirm that SpoIIE interacts with itself, we exploited the yeast two-hybrid system. Residues 326–827 (domains II and III) were fused both to the activation and to the binding domain of the GAL4 transcription factor. If the fusion proteins interacted, expression of a lacZ reporter gene in the yeast host strain would be induced. With dithiothreitol (DTT) unexpectedly yielded two major binding domain and purified the overproduced protein.

To characterize further the interaction of SpoIIE with itself, we fused residues 568–827 (position 568), suggesting that this polypeptide is generated de novo and is not a degradation product.

The cytoplasmic domain of SpoIIE is composed of a large central domain (domain II) and the C-terminal phosphatase domain (domain III). To determine whether either of these domains alone was capable of supporting a protein–protein interaction, we fused residues 568–827 (domain III) or residues 326–567 (domain II) to the activation and binding domain of the GAL4 transcription factor. As shown in Table IA, none of the pairwise tests involving these fusion proteins gave a positive result.

These results demonstrate that the cytoplasmic part of SpoIIE interacts with itself, and this interaction may require both domain II and domain III.

**Domain II of SpoIIE is involved in oligomerization in vitro**

To characterize further the interaction of SpoIIE with itself, we investigated full-length and truncated SpoIIE proteins. Purification of the full-length SpoIIE was performed as described by Lucet et al. (1999). To obtain SpoIIE (II–III), we used a fusion to an Intein-chitin binding domain and purified the overproduced protein precursor on a chitin column. Self-cleavage of Intein with dithiothreitol (DTT) unexpectedly yielded two major products with an apparent $M_r$ of ~28 kDa. The upper and lower arrows indicate the position of SpoIIE (II–III) and SpoIIE (III), respectively. (B) Purified full-length SpoIIE (3.2 μM) and SpoIIE (III) (3 μM) were mixed and applied to a Superdex 200 gel filtration column equilibrated with buffer C. Elution was performed in the same buffer and fractions of 1 ml were collected and monitored by Western blotting with anti-SpoIIE antibodies. Full-length SpoIIE was eluted between 46 and 66 ml, corresponding to an apparent $M_r$ of 700 and 170 kDa. SpoIIE (III) was eluted between 84 and 91 ml, corresponding to an apparent $M_r$ of ~28 kDa. The positions of full-length SpoIIE and SpoIIE (III) are indicated by solid arrows. The additional immunoreactive bands (indicated by dotted lines) correspond to dimeric and higher molecular mass (possibly tetrameric) forms of SpoIIE.

**Table I. Interactions detected by the yeast two-hybrid system**

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<th>Binding domain fusion</th>
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<tbody>
<tr>
<td>SpoIIE (II–III)</td>
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<td>–</td>
</tr>
<tr>
<td>None</td>
<td>SpoIIE (II–III)</td>
<td>–</td>
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<tr>
<td>SpoIIE (II)</td>
<td>None</td>
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<tr>
<td>None</td>
<td>SpoIIE (II)</td>
<td>–</td>
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<td>SpoIIE (III)</td>
<td>None</td>
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<tr>
<td>None</td>
<td>SpoIIE (III)</td>
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<tr>
<td>SpoIIE (II–III)</td>
<td>SpoIIE (II–III)</td>
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<td>SpoIIE (II)</td>
<td>SpoIIE (II)</td>
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<td>SpoIIE (III)</td>
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<td>SpoIIE (II–III)</td>
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<td>SpoIIE (III)</td>
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<td>SpoIIE (II)</td>
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<tr>
<td>SpoIIE (II–III)</td>
<td>SpoIIE (II)</td>
<td>–</td>
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<tr>
<td>(A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>FtsZ</td>
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<td>–</td>
</tr>
<tr>
<td>SpoIIE (III)</td>
<td>FtsZ</td>
<td>–</td>
</tr>
</tbody>
</table>

*SpoIIE (II–III), SpoIIE (II) and SpoIIE (III) contain residues 326–827, 326–567 and 568–827, respectively.

**Fig. 3. Interaction of SpoIIE, SpoIIE (II–III) and SpoIIE (III).**

(A) SDS–PAGE of SpoIIE (II–III) and SpoIIE (III). SpoIIE (II–III) was eluted between 59 and 77 ml, corresponding to an apparent $M_r$ between 300 and 50 kDa. SpoIIE (III) was eluted between 84 and 90 ml, corresponding to an apparent $M_r$ of ~28 kDa. The upper and lower arrows indicate the position of SpoIIE (II–III) and SpoIIE (III), respectively. (B) Purified full-length SpoIIE (3.2 μM) and SpoIIE (III) (3 μM) were mixed and applied to a Superdex 200 gel filtration column equilibrated with buffer C. Elution was performed in the same buffer and fractions of 1 ml were collected and monitored by Western blotting with anti-SpoIIE antibodies. Full-length SpoIIE was eluted between 46 and 66 ml, corresponding to an apparent $M_r$ between 700 and 170 kDa. SpoIIE (III) was eluted between 84 and 91 ml, corresponding to an apparent $M_r$ of ~28 kDa. The positions of full-length SpoIIE and SpoIIE (III) are indicated by solid arrows. The additional immunoreactive bands (indicated by dotted lines) correspond to dimeric and higher molecular mass (possibly tetrameric) forms of SpoIIE.

**Interaction between FtsZ and SpoIIE**

Interaction between FtsZ and SpoIIE was also detected by the yeast two-hybrid system. Residues 326–827 (domains II and III) were fused both to the activation and to the binding domain of the GAL4 transcription factor. If the fusion proteins interacted, expression of a lacZ reporter gene in the yeast host strain would be induced. As shown in Table IA, when only one of the fusion proteins was produced in the yeast, no β-galactosidase activity was observed. By contrast, a positive interaction was detected when both fusion proteins were produced, suggesting that the soluble SpoIIE (II–III) interacts with itself. No interaction with SpoIIE (II–III) was detected when the full-length SpoIIE was fused to the binding domain of GAL4 (results not shown); we attribute this result to problems caused by the expression of the membrane domain (domain I) of SpoIIE in yeast.
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Fig. 4. Time course of dephosphorylation of SpoIIAA-P by full-length SpoIIE (A), SpoIIE (II–III) (●) and SpoIIE (III) (■). The assays were as described in Materials and methods.

anti-SpoIIE antibodies. As shown in Figure 3B, the two proteins eluted separately. In addition, we observed that purified full-length SpoIIE eluted from Superdex 200 with an apparent $M_r$ of 170–700 kDa, suggesting the presence of molecular species higher than a dimer in solutions with a high concentration of SpoIIE (Lucet et al., 1999). Some of the dimeric and higher molecular mass forms of SpoIIE are found to be SDS resistant and are easily detected on the Western blot, as has been found for other protein complexes (Patricelli et al., 1998). These results confirm that SpoIIE interacts with itself and suggest that oligomerization is one function of the previously uncharacterized domain II.

**Domains I and II do not affect the phosphatase activity of SpoIIE**

To analyse whether oligomerization of SpoIIE affects its phosphatase activity, we determined the rate of dephosphorylation of SpoIIAA-P by full-length SpoIIE, SpoIIE (II–III) and SpoIIE (III). As shown in Figure 4, no significant difference between the phosphatase activities was detected. The turnover number (moles of SpoIIAA-P dephosphorylated per mole of SpoIIE) was $7.6 \times 10^{-2}$ s$^{-1}$ for full-length SpoIIE, $8.6 \times 10^{-2}$ s$^{-1}$ for SpoIIE (II–III) and $8.5 \times 10^{-2}$ s$^{-1}$ for SpoIIE (III) at 30°C within the concentration range used (25–180 nM). These results suggest that in vitro the membrane-associated N-terminal domain and the central domain of SpoIIE do not regulate the enzymic activity of the phosphatase domain.

**SpoIIE and FtsZ form a stable complex**

Immunofluorescence microscopy has shown that localization of SpoIIE to the asymmetric division septum requires the FtsZ division protein but not the later assembling division proteins FtsA, DivIC, FtsL or DivIB (Levin et al., 1997; Feucht et al., 1999). Our data suggested that there is a direct interaction between FtsZ and SpoIIE (see Figure 2). To confirm such an interaction, we again employed the yeast two-hybrid system and fused FtsZ to the activation domain of GAL4 (FtsZ fused to the binding domain of GAL4 expressed the lacZ reporter gene and therefore could not be used in this test). The GAL4 fusion containing SpoIIE (II–III) interacted with FtsZ, but again no interaction was detected with the fusions containing isolated domains II or III (Table IB).

To investigate the interaction between SpoIIE and FtsZ in more detail, purified full-length SpoIIE and purified FtsZ were applied to a Superdex 200 gel filtration column equilibrated with buffer D. Elution was performed in the same buffer, and fractions of 1 ml were collected and monitored by Western blotting with anti-SpoIIE or anti-FtsZ antibodies. As shown in Figure 5A, purified full-length SpoIIE (3.2 μM) was applied to the column. SpoIIE was eluted between 47 and 64 ml. (B) Purified FtsZ (7 μM) was applied to the column. FtsZ was eluted between 59 and 84 ml. (C) Full-length SpoIIE (3.2 μM) pre-mixed with FtsZ (7 μM) was applied to the column. Note the shift of the FtsZ peak. (D) SpoIIE (III) (3.2 μM) pre-mixed with FtsZ (7 μM) was applied to the column. The elution patterns of SpoIIE (III) and FtsZ were unchanged. (E) Full-length SpoIIE (3.2 μM) pre-mixed with FtsZ (7 μM) without GTP was applied to the column. The elution patterns of full-length SpoIIE and FtsZ were unchanged. SpoIIE proteins and FtsZ are indicated by solid arrows. Dashed lines indicate dimeric and higher molecular mass (possibly tetrameric) forms of SpoIIE.
change in the elution pattern of FtsZ was found, and the two proteins eluted separately (Figure 5D). To check whether GTP (i.e. polymerization of FtsZ) is required for SpoIIE–FtsZ interaction, the experiment with full-length SpoIIE and FtsZ was repeated without GTP. As shown in Figure 5E, no significant change in the elution pattern of FtsZ was found, suggesting that SpoIIE interacts only with FtsZ in a polymeric or GTP-bound form.

We could not analyse SpoIIE (II–III) by gel filtration because of its tendency to interact tightly with the column material at low ionic strength. Higher ionic strength could not be employed because of its interference with the formation of SpoIIE–FtsZ complexes. We therefore incubated full-length SpoIIE, SpoIIE (II–III) or SpoIIE (III) with purified FtsZ, and subjected the mixtures to non-denaturing PAGE. The results of Levin et al. (1997; Feucht et al., 1999) demonstrated that SpoIIE interacts with itself. Moreover, purified full-length SpoIIE and SpoIIE (II–III) to a higher apparent molecular weight (Figures 5 and 6). Interestingly, SpoIIE binds only to FtsZ in the GTP-bound, presumably polymeric form (Figure 5E). In Escherichia coli, FtsZ is capable of interacting directly with several other division proteins, including FtsA, SulA and ZipA (Addinall et al., 1996; Huang et al., 1996; Hale and de Boer, 1997). In B. subtilis, only FtsA and now SpoIIE have been shown to interact directly with FtsZ. Wang et al. (1997) reported that FtsA interacts with the C-terminal domain of FtsZ, and it will therefore be useful to investigate whether SpoIIE binds to the same domain.

The interaction between SpoIIE and FtsZ requires domain II of SpoIIE. This was shown by two independent methods: the yeast two-hybrid system and gel filtration/non-denaturing PAGE. In the yeast two-hybrid system, FtsZ interacted with SpoIIE (II–III), but not with SpoIIE (II) or SpoIIE (III) (Table I). Moreover, full-length SpoIIE (Figures 5 and 6) and SpoIIE (II–III) (Figure 6), but not SpoIIE (III) (Figures 5 and 6), shifted FtsZ to a higher apparent molecular weight. These results show that at least part of domain II is required for interaction with FtsZ (and for oligomerization; see below). At the moment, we favour the notion that this domain of SpoIIE is responsible for the protein–protein interactions and that domain III, which functions independently as a phosphatase, is required indirectly, e.g. to stabilize domain II or control its folding.

It is also possible that domain I contributes to the interaction with FtsZ in vivo. Insertion of SpoIIE into the membrane might impose a topology that favours interaction with FtsZ as it assembles at the membrane. King et al. (1999) reported that replacement of domain I of SpoIIE with the first two membrane-spanning domains of E. coli MalF protein localizes the fusion protein to the cytoplasmic membrane. It may be that the presence of the membrane-spanning segments of MalF overrides localization signals in domain II of SpoIIE, or else that domain I of SpoIIE also plays a role in the proper localization of SpoIIE. The latter suggestion is supported by the finding that a SpoIIE mutant protein with a deletion of all membrane segments was dispersed throughout the cytoplasm (Arigoni et al., 1999). Possibly the SpoIIE membrane anchor helps to bring SpoIIE to where FtsZ ring formation is taking place.

Until now not much was known about the function of the poorly conserved domain II (Arigoni et al., 1999). The present study reveals that it is involved not only in binding FtsZ, but also in self-oligomerization. The yeast two-hybrid system showed that SpoIIE (II–III) interacts with itself. Moreover, purified full-length SpoIIE and SpoIIE (II–III) behaved like large oligomeric species on gel filtration chromatography, whereas SpoIIE (III) behaved as a monomer. Preliminary sedimentation equilibrium analysis of purified SpoIIE (II–III) suggests that the sample has, like full-length SpoIIE, a heterogeneous
Materials and methods

SpoIIE protein has two distinct and independent functions: (i) a morphogenetic activity needed for the formation of the asymmetric septum; and (ii) a phosphatase activity needed for release of the critical first cell-specific sigma factor σF in the prespore. It seems that domain I and domain II are involved in the first function by sequestering the protein to the membrane and targeting it to FtsZ rings. The finding of a direct interaction with FtsZ provides new insight into the function of SpoIIE in asymmetric septum formation. One reasonable hypothesis is that interaction of the membrane-bound SpoIIE protein with FtsZ facilitates recruitment of FtsZ to the membrane. This recruitment, together with the oligomerization of SpoIIE, could strongly promote formation of the FtsZ ring. In fact, Khvorova et al. (1998) have shown that in a spoIIE deletion strain, FtsZ ring formation is delayed and the proportion of bacteria with FtsZ rings is substantially reduced. Moreover, Wu et al. (1998) have reported that SpoIIE tends to localize sequentially to the potential division sites, and that the prespore septum forms at the pole where SpoIIE appears first.

The second function of SpoIIE seems to depend only on domain III. There was no significant difference between the phosphatase activities of full-length SpoIIE, SpoIIE (II–III) and SpoIIE (III) (Figure 4), showing that at least domain I and domain II are involved in the first function by sequestering the protein to the membrane and targeting it to FtsZ rings. The finding of a direct interaction with FtsZ provides new insight into the function of SpoIIE in asymmetric septum formation. One reasonable hypothesis is that interaction of the membrane-bound SpoIIE protein with FtsZ facilitates recruitment of FtsZ to the membrane. This recruitment, together with the oligomerization of SpoIIE, could strongly promote formation of the FtsZ ring. In fact, Khvorova et al. (1998) have shown that in a spoIIE deletion strain, FtsZ ring formation is delayed and the proportion of bacteria with FtsZ rings is substantially reduced. Moreover, Wu et al. (1998) have reported that SpoIIE tends to localize sequentially to the potential division sites, and that the prespore septum forms at the pole where SpoIIE appears first.

The second function of SpoIIE seems to depend only on domain III. There was no significant difference between the phosphatase activities of full-length SpoIIE, SpoIIE (II–III) and SpoIIE (III) (Figure 4), showing that at least in vitro domains I and II do not regulate the phosphatase activity of SpoIIE. This result might also imply that SpoIIE is needed for its role in septum formation, and not for its activity in regulating the release of σF in the prespore. On the other hand, it has been shown that expression of a SpoIIE mutant protein without membrane-spanning segments leads to a severe reduction in sporulation frequency (Arigoni et al., 1999), demonstrating the importance of co-ordination of the two functions.

Our data are summarized in the schematic model for SpoIIE–FtsZ interaction shown in Figure 7. FtsZ localizes to potential division sites and recruits SpoIIE by interaction with domain II and possibly also domain I. The local concentration of SpoIIE is thereby increased, which leads to the oligomerization of SpoIIE. In turn, SpoIIE provides a membrane anchor for the FtsZ polymer. When the septum has formed, most of the SpoIIE is sequestered into the membrane on the prespore side by an unknown mechanism. SpoIIE remains localized in the asymmetric septum, whereas FtsZ depolymerizes and becomes dispersed throughout the cytoplasm, suggesting either that SpoIIE remains associated with the septum because of its membrane domain and its oligomerization state or that it has an affinity for some feature of the septum other than

Materials and methods

Strains and plasmids

The strains and plasmids used in this study are described in Table II. All plasmids were constructed in E.coli strain DH5α. DNA manipulations and E.coli transformations were carried out using standard methods (Sambrook et al., 1989). The sequence of all cloned PCR products was confirmed by DNA sequencing. Bacillus subtilis chromosomal DNA was prepared by a scaled-down method based on the one described by Errington (1984).

Growth media

Nutrient agar (Oxoid) was used as a solid medium for growing B.subtilis. Sporulation was induced by growth in a hydrolysed casein medium, followed by resuspension in a starvation medium (Sterlini and Mandelstam, 1969; Partridge and Errington, 1993). Times (hours) after resuspension of cells in the starvation medium were denoted t0, t1, t2 and so on. Media used for growing E.coli were 2xTY (Sambrook et al., 1989) and nutrient agar (Oxoid) supplemented with ampicillin (100 μg/ml).

Yeast two-hybrid analysis

Plasmid pSG1904 was constructed by cloning a 1506 bp PCR amplified spoIIE fragment from SG38 chromosomal DNA. The oligonucleotides used for PCR, 5'-ATGCCCATGGAAGCCGAAAGTGGCGGAGAT-3' and 5'-GCGGATCCCATATATCCATCTGCGAAG-3', introduced SfiI and BamHI sites for insertion into vector plasmid pAS2-1,
### Table II. Strains and plasmids

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Plasmids

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<td>pACT2</td>
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<td>A. Marston (unpublished)</td>
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*Numbers in parentheses after spoIE refer to the first and last nucleotides of the insert from the spoIE coding sequence.

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**Construction of strep-tagged SpoIIE fusions and purification of the recombinant proteins**

To join the 5’ end of spoIE in-frame with a strep-tag, oligonucleotides 5’-GGAATTCATATGCGGATCCTTGGTTGGCGCTTTTGGGCGGCAGTT-GCGGATCTGACTGGAAAGCCGAACAGCACTC-3’ and 5’-TCA-TTAAGTCATATGCTTACTGATTTATCAAGTGCTTGTGAC-3’, generating a strep-tag (bold) at the 5’ end and a NdeI site at each end of the PCR product, were used to amplify a 1280 bp spoIE fragment from pRB1011. The PCR product was digested with NdeI and subcloned into NdeI-digested and gel purified pRB1011, thereby replacing the 5’ end of spoIE and yielding plasmid pIL1. To generate an N-terminal strep-tag fusion to only the cytoplasmic domain of SpoIIE, a 1521 bp spoIE fragment was amplified by PCR from SG38 chromosomal DNA. The oligonucleotides were 5’-CATGGTCACTGCTGGGTTCCTTGGGCGTCACCCAGTT-CCGGCACTTGCTGTTGGTTGTCTCATTACCAGGAAAGTGTC-3’ and 5’-GGGATCCATATGCTTACTGATTTATCAAGTGCTTGTGAC-3’, introducing a strep-tag (bold) and NcoI and BamHI sites for insertion into NcoI–BamHI-digested pET-21d to give plasmid pSG1909. Plasmids pIL1 and pSG1909 were transformed into E. coli C41 (DE3) and the proteins were overexpressed for 4 h at 30°C after induction with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). The cells from 1 l culture were resuspended in 25 ml of 50 mM Tris–HCl pH 8 containing 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, 0.5 mg/ml lysozyme, 0.5 mg/ml DNase, 0.5 mg/ml RNase (buffer A) and disrupted by sonication (three times for 10 s). The membrane fractions were recovered by centrifugation at 29 000 g for 1 h and washed three times with the same buffer. The washed membranes were resuspended in 20 ml of buffer A, supplemented with 350 mM NaCl and 5% Triton X-100 for the full-length SpoIIE, or with 1 M NaCl and 10% Triton X-100 for the cytoplasmic domain, and stored at 4°C for at least 3 h. After centrifugation at 29 000 g for 30 min, the supernatants were incubated overnight at 4°C with 200 μl of streptavidin-coated magnetic beads (DYNAL) washed in the same buffer. The magnetic beads were then washed with 10 ml of the same buffer to remove unspecific binding. The binding of the strep-tagged protein to the magnetic beads was checked by SDS–PAGE.

**Interaction between strep-tagged SpoIIE proteins and FtsZ from sporulating B. subtilis cell extract**

The strep-tagged SpoIIE fusion proteins [full-length SpoIIE and SpoIIE (II–III)] bound to magnetic beads were incubated overnight at 4°C with cell extract taken 1.5 h after the initiation of sporulation. The cells were broken in 50 mM Tris–HCl pH 7 containing 250 mM KCl, 0.1 mM DTT, 1 mM MnCl2, 2 mM MgCl2, 10% glycerol, 1% Triton X-100, 1 mM GTP. After extensive washes, aliquots were loaded onto SDS–PAGE gels, silver stained and Western blotted using anti-SpoIIE antibodies or anti-FtsZ antibodies.

**Construction and purification of C-terminal SpoIIE protein by a self-cleavage affinity tag**

To generate a C-terminal fusion of the cytoplasmic domain of SpoIIE to Intein, a 1521 bp spoIE fragment was amplified by PCR from SG38 chromosomal DNA. The oligonucleotides used, 5’-CATGGTCACTGCTGGGTTCCTTGGGCGTCACCCAGTT-CCGGCACTTGCTGTTGGTTGTCTCATTACCAGGAAAGTGTC-3’ and 5’-CCACAGTCTCGTCTCCATTACCATGGAAAGTGTC-3’, were introduced at the 5’ end of each plasmid in aNcoI site, cutting pRB1011. The resulting plasmid pSG1911 was transformed into E. coli C41 (DE3) and the fusion protein was overproduced overnight at 16°C with induction with 0.3 mM IPTG. The cells from 1 l culture were resuspended in 20 ml of 20 mM Tris–HCl pH 8 containing 250 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100 (buffer B), and disrupted by sonication on ice (three times for 10 s). After centrifugation at 29 000 g for 30 min, the clarified extract was loaded onto a 6 ml chitin column equilibrated in buffer B. The column was washed with 200 ml of the same buffer supplemented with 250 mM NaCl at a flow rate of 1 ml/min. The column was flushed quickly with

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18 ml of 50 mM DTT freshly diluted in buffer B supplemented with 250 mM NaCl and left overnight at 4°C. The protein of interest was eluted using buffer B without DTT, and 1 ml fractions were collected and analysed by SDS–PAGE. SpoIE (H–III) and SpoIE (III) were purified further on a Superdex 200 gel filtration column equilibrated in 100 mM Tris–HCl pH 8 containing 500 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10% glycerol (buffer C). Fractions of 1 ml were collected and analysed by SDS–PAGE.

**Assay for dephosphorylation of SpoIIAA-P**

Non-radioactive phosphatase assays were performed using the Ser/Thr phosphatase assay system (Promega) as described by the manufacturer. Dephosphorylation was carried out at 30°C in 100 μl volume containing 20 μM SpoIIAA-P. The reaction was started by the addition of full-length SpoIE, SpoIE (II–III) or SpoIE (III) (25–180 nM).

**Gel filtration chromatography assay**

*Bacillus subtilis* FtsZ was purified according to the method published previously (Wang and Luktenhaus, 1993). Purified full-length SpoIE, SpoIE (III) and FtsZ were dialysed overnight at 4°C in 50 mM Tris–HCl pH 7 containing 250 mM KCl, 0.1 mM DTT, 1 mM MnCl₂, 2 mM MgCl₂, 10% glycerol, 0.5% Triton X-100, 1 mM GTP (buffer D), and applied separately or after mixing to a FPLC Sephacryl S200 gel filtration column previously equilibrated in the same buffer. Elution was performed with the same buffer at 0.5 ml/min and fractions of 1 ml were collected. Each fraction (20 μl) was tested by Western blotting with either anti-SpoIE antibodies or anti-FtsZ antibodies. Staining of proteins using Coomassie blue was used to calculate the column volume using blue dextran (70 kDa), γ-galactosidase (44 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.3 kDa) from the molecular sieve coefficient (gel filtration calibration kit, Bio-Rad). Gel filtration data are presented in a second determinant with extensive sequence similarity to the morphogene.

**Gel shift experiment on non-denaturing PAGE**

Purified SpoIE proteins and purified FtsZ were incubated together at 4°C in buffer D. Samples were added to sample buffer and subjected to non-denaturing 8% PAGE overnight. GTP was added to the running buffer at 1 mM. Proteins were detected by immunoblotting.

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**References**


Interaction between FtsZ and SpoIIIE


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-σ factor that is also a protein kinase. Cell., 74, 735–742.


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