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Septal localization of the SpoIIIE chromosome partitioning protein in *Bacillus subtilis*

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The 787 amino acid SpoIIIE protein of *Bacillus subtilis* is required for chromosome partitioning during sporulation. This process differs from vegetative chromosome partitioning in that it occurs after formation of the septum, apparently by transfer of the chromosome through the nascent septum in a manner reminiscent of plasmid conjugation. Here we show that SpoIIIE is associated with the cell membrane, with its soluble C-terminal domain located inside the cell. Immunofluorescence microscopy using affinity-purified anti-SpoIIIE antibodies shows that SpoIIIE is targeted near the centre of the asymmetric septum, in support of a direct role for SpoIIIE in transport of DNA through the septum. We also report on the isolation of a mutation affecting the N-terminal hydrophobic domain of SpoIIIE that interferes with targeting to the septum and blocks DNA transfer. This mutation also causes de-localization of the activity of the normally prespore-specific sigma factor, σ^F, consistent with the notion that SpoIIIE can form a seal between the chromosomal DNA and the leading edge of the division septum.

Keywords: *Bacillus subtilis*/chromosome partitioning/SpoIIIE protein/sporulation

Introduction

Under starvation conditions, *Bacillus subtilis* undergoes a differentiation process that culminates with the release of a dormant endospore (Piggot and Coote, 1976; Errington, 1993). The process begins with an asymmetric cell division in which a septum is formed near one pole of the developing cell, dividing it into a small prespore (which later becomes a mature spore) and a larger mother cell (which later lyses to release the spore). Formation of the asymmetric septum results in enclosure of ~30% of one chromosome in the small prespore compartment; the rest of the chromosome is then translocated into the prespore compartment by a process requiring SpoIIIE (Wu and Errington, 1994; Wu *et al.*, 1995). Therefore, the prespores of *spoIIIE* mutant cells contain only 30% of a chromosome, with the other 70% remaining in the mother cell, together with the whole of the mother cell chromosome. It has been shown that the SpoIIIE-dependent chromosome translocation is postseptational, processive and hence analogous to conjugative DNA transfer (Wu *et al.*, 1995). This analogy is reinforced by striking sequence similarity between SpoIIIE and the Tra proteins from several conjugative plasmids of *Streptomyces* spp. (Wu *et al.*, 1995). However, the nature of the mechanism whereby SpoIIIE directs translocation of the prespore chromosome was unclear. In principle, it could act by pulling or condensing the DNA from inside the prespore, by pushing the DNA from the mother cell, or by acting as a DNA pump within the asymmetric septum.

Formation of the asymmetric septum is followed by activation of two sporulation-specific transcription factors, σ^F in the prespore and σ^E in the mother cell, which then specify different programmes of gene expression in the two compartments (Losick and Stragier, 1992; reviewed by Errington, 1996). Although all of the *spoIIIE* mutants examined so far are impaired in prespore chromosome translocation, class I *spoIIIE* mutants correctly localize σ^F activity, while class II mutants show σ^F activity in both the prespore and the mother cell compartments (Wu and Errington, 1994). Apparently the SpoIIIE protein is also required for the correct localization of the σ^F activity, at least in cells in which prespore chromosome translocation has not been completed. Class II mutants also differ from class I in producing undetectable amounts of SpoIIIE antigen (Wu and Errington, 1994). Therefore, we postulated that the SpoIIIE protein has a second function, which is required for correct localization of σ^F activity (at least under conditions in which the prespore chromosome occludes the septum), and that it probably exerts this function by making a seal between the septum and the DNA (Wu and Errington, 1994). Class I mutants retain this sealing ability by virtue of the partially functional protein that they make. This is lost in class II mutants presumably because of the lack or instability of the SpoIIIE protein. Such considerations provided circumstantial evidence in favour of SpoIIIE operating in the asymmetric septum.

Because of its apparent roles in both chromosome translocation and the compartmentalization of σ^F activity, we have investigated the localization of the SpoIIIE protein. We show that SpoIIIE is associated with the membrane, with its C-terminal putative DNA transfer domain located inside the cell, and that the protein becomes localized to the centre of the division septum during sporulation. We also show that the putative membrane-binding domain is required for the targeting of SpoIIIE to the asymmetric septum and for the localization of the σ^F activity. The results are consistent with the idea that during sporulation, the SpoIIIE protein becomes localized to the leading edge of the closing asymmetric septum and that it forms a seal between the DNA and the septum, through which the DNA is driven.

Results

SpoIIIE is membrane-associated

The predicted amino acid sequence of the SpoIIIE protein contains several stretches of predominantly hydrophobic

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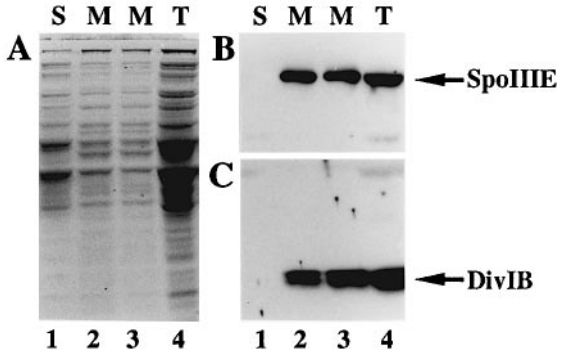

Fig. 1. Membrane association of the SpoIIIE protein. Wild type strain SG38 was induced to sporulate by the resuspension method. Cells were harvested 2 h after the initiation of sporulation, protoplasted, and fractionated. The cytoplasmic fraction (S; lane 1), membrane fraction (M; lanes 2 and 3), and whole protoplast lysates (T; lane 4) were analysed for (**A**) total protein (Coomassie Blue-stained gel), and for (**B**) SpoIIIE and (**C**) DivIB proteins by Western immunoblotting using anti-SpoIIIE and anti-DivIB antibodies, respectively.

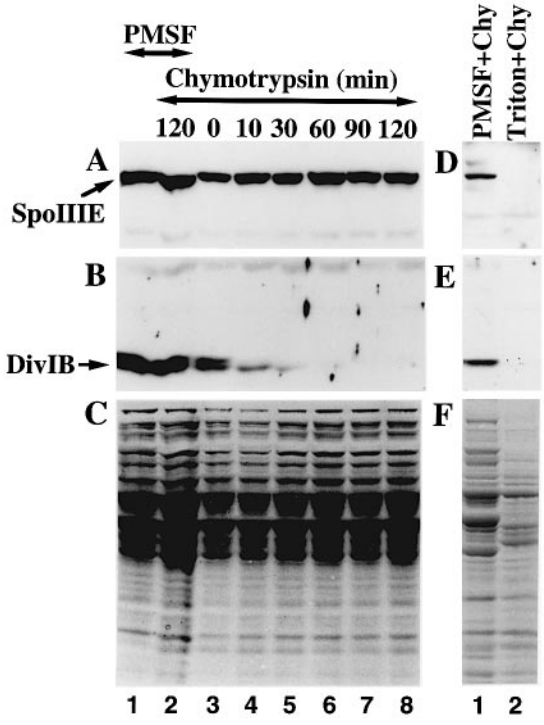

Fig. 2. Resistance of SpoIIIE in protoplasts to chymotrypsin. Wild type strain SG38 was induced to sporulate and the cells were harvested and protoplasted as described in the legend to Figure 1. Washed protoplasts were then treated with chymotrypsin for various times (0–120 min). The same samples were analysed for (**A**) SpoIIIE and (**B**) DivIB proteins by Western immunoblotting, and for (**C**) total protein by Coomassie Blue staining. Lane 1, protoplasts treated with PMSF only for 120 min; lane 2, PMSF was added to the protoplasts prior to the addition of chymotrypsin; lanes 3–8, protoplasts were treated with chymotrypsin for 0, 10, 30, 60, 90 and 120 min respectively then PMSF was added to inactivated the chymotrypsin. To demonstrate that the SpoIIIE protein was susceptible to degradation by the protease (**D–F**), protoplasts were treated with chymotrypsin in the presence of either PMSF (lane 1), which had no effect on protoplast integrity, or triton (lane 2), which caused complete lysis. D, E and F were treated as A, B and C, respectively.

and non-polar residues near its N-terminus, suggesting that this part of the protein is integrated into the cell membrane (Butler and Mandelstam, 1987; Foulger and Errington, 1989). To test this, sporulating cells of the wild type strain SG38 were harvested 2–2.5 h after induction of sporulation, protoplasted, lysed and then fractionated. Samples of the particulate (membrane) and soluble (cytoplasm) fractions, and of the total protoplast lysate, were analysed by SDS–PAGE and Western immunoblotting using an antiserum raised against the C-terminal region of SpoIIIE (Figure 1B). Duplicate samples were also analysed by immunoblotting for the presence of a known membrane-binding protein, DivIB (Harry *et al.*, 1993), as a positive control (Figure 1C), and for total protein by Coomassie Blue staining (Figure 1A). In a series of experiments, both SpoIIIE and the DivIB control protein were detected only in the membrane fraction (M in Figure 1B and C), confirming the association of SpoIIIE with the membrane. Similar results were obtained with extracts of vegetative cells (results not shown). In control experiments, the soluble cytoplasmic proteins, SpoIIAA (from SG38; Min *et al.*, 1993; Alper *et al.*, 1994) and β-galactosidase (from SG38::pPS1395, which contains a σ^F-dependent *gpr′-′lacZ* fusion and thus produces β-galactosidase during sporulation; Sussman and Setlow, 1991), were detected, as expected, almost exclusively in the cytoplasmic fraction (data not shown). In some blots, a cross reactive protein of unknown identity, which was found only in the soluble fraction, served as a further control (see also below).

Cytoplasmic location of the C-terminal domain of SpoIIIE

To understand the role of SpoIIIE in chromosome translocation it was important to establish whether the hydrophilic C-terminal domain of SpoIIIE is located inside or outside the cytoplasmic membrane. We therefore tested whether the protein was accessible to protease on the surface of protoplasts, as described by Harry *et al.* (1993). Washed protoplasts of sporulating wild type cells (strain SG38) were treated with chymotrypsin for different periods of time (up to 2 h). Samples were then analysed for the presence of SpoIIIE, DivIB (a known surface exposed protein; Harry *et al.*, 1993) and total protein. There was no obvious change in the total protein profile (Figure 2C), indicating that the internal cytoplasmic compartments of the protoplasts were not accessible to the chymotrypsin. The DivIB protein, known to be located outside the cell (Harry *et al.*, 1993), was degraded rapidly, as expected (Figure 2B). In contrast, the amount of SpoIIIE protein remained unchanged (Figure 2A), indicating that the C-terminal domain was protected presumably by being located inside the protoplast. The apparent stability of SpoIIIE was not due to intrinsic resistance to the protease, because when the protoplasts were disrupted, either by the addition of Triton (Figure 2D–F), or by osmotic lysis (data not shown) most of the cellular protein and all of the detectable SpoIIIE were degraded. Similar results were obtained with protoplasts from vegetatively growing cells, and in experiments using a different protease, thermolysin (results not shown).

These results strongly suggest that the C-terminal hydrophilic domain of SpoIIIE lies within the cytoplasm during growth and sporulation. Although one of the loops predicted to be located outside the cell (see below) contains

two cleavage sites for chymotrypsin (cleavage at the peptide bonds in which the carbonyl group is contributed by aromatic amino acid residues), it was possible that the chymotrypsin treatment would yield some SpoIIIE bands of smaller sizes, but these were not detected. Presumably, these potential cleavage sites are not surface exposed.

Subcellular localization of SpoIIIE in the division septum

To determine the subcellular location of the SpoIIIE protein we employed immunofluorescence microscopy (Harry *et al.*, 1995; Pogliano *et al.*, 1995) using affinity purified anti-SpoIIIE antibodies (Figure 3). To relate the position of the SpoIIIE fluorescence to the cell, the samples were also stained with DAPI to visualize the DNA (note that the DAPI images are shown in red, rather than in the natural blue colour). The specificity of the antiserum was confirmed by the absence of a significant signal in cells of a *spoIIIE* null mutant, 647 (Figure 3A–C). We then examined wild type cells (strain SG38) early in sporulation (80 min), a time when some of the cells should be undergoing prespore DNA transfer (Hauser and Errington, 1995). Because DNA transfer is a relatively rapid process and the population of cells enters sporulation asynchronously, only a few cells in any given sample will actually be undergoing DNA translocation. The few cells detected in this state did indeed show discrete fluorescent foci. Usually it was a single discrete focus, lying in or close to the constriction in the prespore nucleoid, which corresponds to the location of the septum through which the DNA is thought to be driven (Wu *et al.*, 1995) (Figure 3D–F). Surprisingly, later in sporulation, when most of the cells would be expected to have completed DNA transfer and to have proceeded to the later stages of sporulation (120–135 min), each of the prespore nucleoids still had a single strong SpoIIIE focus associated with them (Figure 3G–I). This suggested that the SpoIIIE protein does not disperse after the completion of DNA transfer and remains associated with the prespore nucleoid, or the membranes surrounding the prespore. These foci always lay close to the edge of the prespore nucleoid, but their precise position was variable; sometimes lying between the prespore and mother cell nucleoid but often further away from the mother cell and even at the distal pole of the cell. These observations suggested that the protein is initially localized to a specific site at the middle of the asymmetric septum when it forms but that its position can drift during subsequent development.

We then examined cells of a class I *spoIIIE* mutant (36.3). This mutant produces normal amounts of full-length SpoIIIE protein but DNA translocation does not occur (Wu and Errington, 1994). SpoIIIE foci were present in all of the sporulating cells of this mutant, and, as seen in wild type cells early in sporulation, were always located precisely between the bulk of the prespore and mother cell nucleoids, where the spore division septum forms (Figure 3J–L). Thus it appears that the mutant SpoIIIE protein assembles into the membrane correctly but that it remains in the position of the septum after engulfment. Perhaps the SpoIIIE focus is fixed in position by association with the chromosomal DNA protruding out of the prespore into the mother cell.

spoIIG mutants form prespore-like cells at both poles of the sporangium, leaving a large central compartment devoid of DNA (Setlow *et al.*, 1991; Lewis *et al.*, 1994; arrows in Figure 3N), and prespore development is blocked prior to engulfment. Examination of these cells revealed SpoIIIE foci at both ends of each DNA-free central compartment (Figure 3M–O) where DNA transfer through the septa would have occurred. As in the *spoIIIE* mutant, the foci did not move out around the periphery of the prespore nucleoid, suggesting that the movement in wild type cells depends on partial or complete prespore engulfment.

In non-septate or non-sporulating cells (as judged by the DAPI appearance), weak, randomly distributed SpoIIIE foci were sometimes evident (Figure 3D and J, for example), but in a few cases (~6% of cells undergoing medial division) it was also localized near the centre of the septum in both the mutant and the wild type (see arrowheads in Figure 3H and I). Since the SpoIIIE protein has been found to be required for recovery of vegetatively growing cells from interruption of DNA replication (Sharpe and Errington, 1995), the presence of SpoIIIE localized in some cells undergoing medial division may reflect its involvement in post-septational chromosome partitioning.

Mutations affecting the putative membrane-binding domain of SpoIIIE prevent compartmentalization of σ^F activity and the targeting of SpoIIIE to the division septum

We have previously postulated that SpoIIIE has a second function in sealing off the prespore and the mother cell compartments during translocation of the prespore chromosome (Wu and Errington, 1994). The above results, indicating that SpoIIIE is located near the middle of the spore septum, during and immediately after chromosme translocation, are consistent with the idea that the protein can seal off the two compartments and allow correct localization of σ^F activity when chromosome translocation fails. If so, it should be possible to isolate mutants in which targeting of the SpoIIIE protein to the asymmetric septum was eliminated; such mutants should have a class II phenotype due to the loss of the sealing function but, unlike previously described class II mutants, make normal amounts of SpoIIIE protein.

We described previously the isolation of 23 new *spoIIIE* mutations by chemical mutagenesis of the plasmid pSG255, which carries the N-terminal half of the *spoIIIE* gene (Wu *et al.*, 1995). All but one of the new mutations gave a class II phenotype. When the strains containing these new class II mutations were screened for the presence of SpoIIIE protein, only one produced detectable amounts of protein (Figure 4B). The mutation, designated *spoIIIE912*, was introduced into the wild type strain CU267 by congression and the resulting mutant named 912. Further characterization of the mutant confirmed that it had a class II phenotype. The typical defect in prespore chromosome partitioning is shown in Figure 3Q. The reduction in prespore DNA content contrasts with that of the wild type sporulating cells in Figure 3H. The characteristic overexpression of a σ^F-dependent gene (rather than the block in expression characteristic of class I mutants; Wu and Errington, 1994) is shown in Figure 4A. Finally, expression of the σ^F-dependent *gpr′-′lacZ*

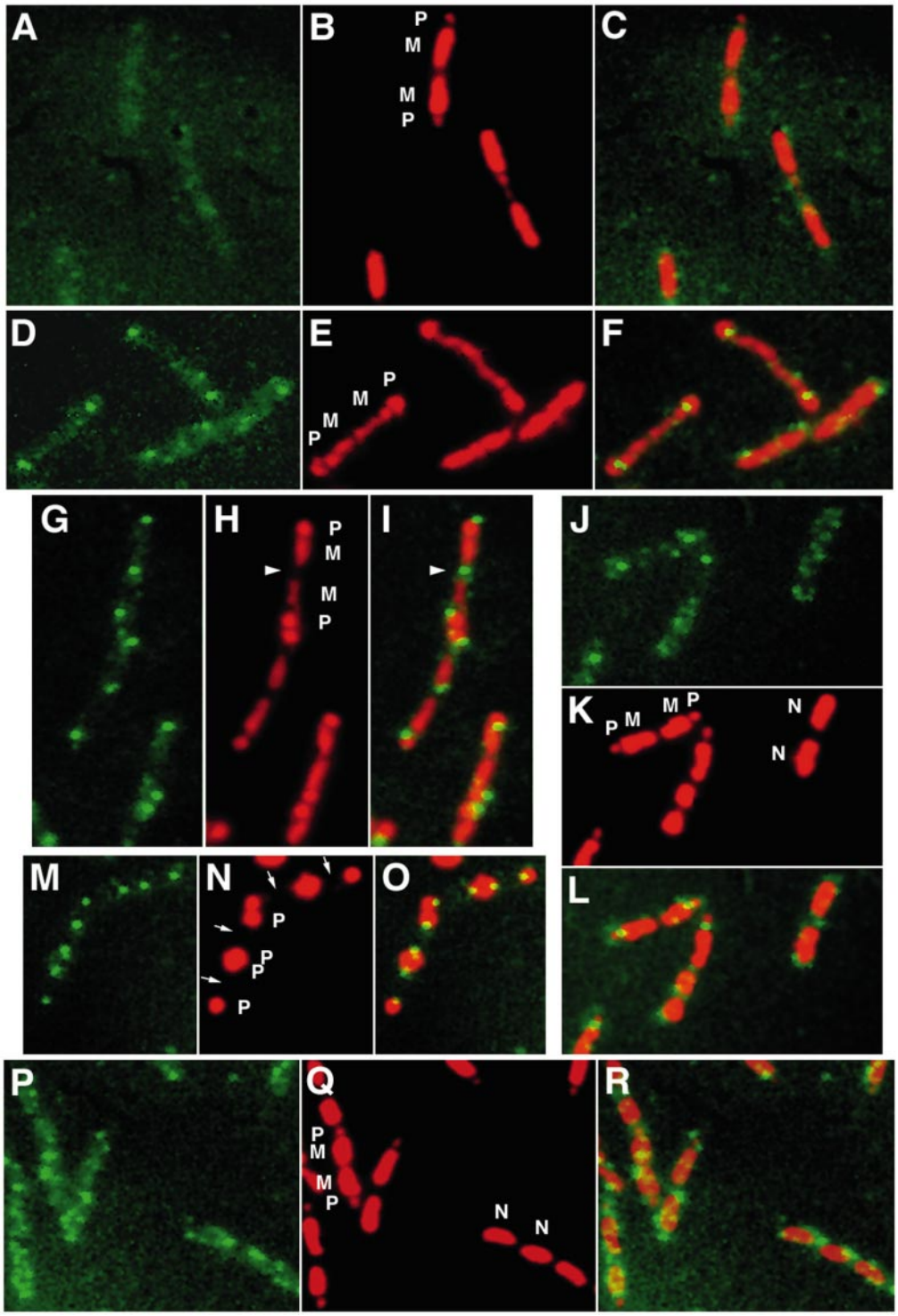

Fig. 3. Subcellular localization of SpoIIIE in a *spoIIIE* null mutant, (647, **A**–**C**); the wild type strain (SG38, **D**–**I**); a *spoIIIE* class I mutant (36.3, **J**–**L**); a *spoIIG* mutant (901, **M**–**O**), and in *spoIIIE* mutant 912 (**P**–**R**). Cells were induced to sporulate by the resuspension method and harvested between 120 and 135 min after the initiation of sporulation (except for the samples in D–F which were taken after 80 min), then fixed and processed for immunofluorescence microscopy. (A, D, G, J, M and P) Images of cells viewed with a fluorescein filter showing the distribution of SpoIIIE. (B, E, H, K, N and Q) Images of the same fields obtained with a DAPI filter, showing the distribution of nucleoids. (C, F, I, L, O and R) Overlays of panels A and B, D and E, G and H, J and K, M and O, and P and Q, respectively, showing the location of SpoIIIE protein in relation to the nucleoids and the mother cell compartment. Arrowheads in panels H and I show that the SpoIIIE protein is also located at the septum in some cells undergoing medial division. Arrows in panel N point to the central 'mother cell' compartment that is devoid of DNA. Examples of the different forms of nucleoid visible are labelled as follows: P, prespore nucleoid; M, mother cell nucleoid; N nucleoid of preseptation or non-sporulating cell.

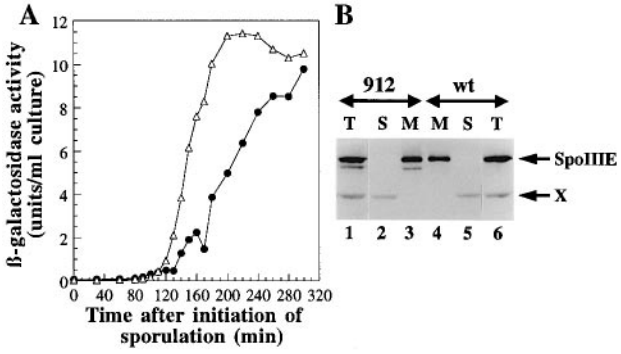

Fig. 4. Effect of the *spoIIIE912* mutation on the expression of σ^F-dependent *gpr′-′lacZ* fusion at its natural chromosomal location (**A**) and association of the mutant SpoIIIE protein with membranes (**B**). The mutant 912 containing plasmid pPS1395 (*gpr′-′lacZ*) integrated at the *gpr* locus (△) and isogenic wild type strain SG38 (●) were induced to sporulate and assayed at intervals for β-galactosidase activity (A). Cells from the sporulating cultures were also harvested 2 h after the initiation of sporulation, fractionated, and analysed for SpoIIIE protein by Western immunoblotting as described in the legend to Figure 1. Lanes 1–3 are mutant strain 912; lanes 4–6 are wild type strain SG38; lanes 1 and 6 are total protoplast lysate (T); lanes 2 and 5 are cytoplasmic fractions (S); lanes 3 and 4 are membrane fractions (M). X indicates the position of an unknown cross reactive protein, visible in some blots, that behaves as a soluble protein.

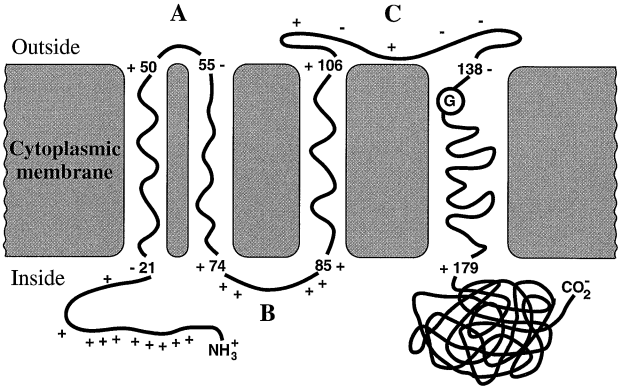

Fig. 5. Topological model of SpoIIIE protein. The model is based on a hydrophobicity plot using the algorithm of Kyte and Doolittle (1982) (window size 19), and the 'positive inside rule' of von Heijne (1986). Positively (K and R) and negatively (D and E) charged residues are indicated. Proposed surface loops are labelled A–C. The circled glycine residue indicates the position of the substitution resulting from the *spoIIIE912* mutation.

fusion at its natural chromosomal location (excluded from the prespore in *spoIIIE* mutants) was detected in the mother cell compartment (data not shown), indicating that σ^F activation was aberrantly localized, as is typical of class II mutants (Wu and Errington, 1994).

The mutation *spoIIIE912* was cloned, sequenced and found to cause substitution of a glycine in the last hydrophobic segment of SpoIIIE (see below) with glutamic acid. Cell fractionation studies showed that this mutant form of SpoIIIE was still associated with the membrane (Figure 4B). In the blot shown, the unknown cross reactive protein mentioned above (labelled X), found only in the soluble fraction, is particularly evident. Strikingly, however, though the mutant protein showed some tendency to form weak foci; these were scattered round the cell rather than localizing between the prespore and mother cell nucleoids, indicating that the protein is affected in targeting (Figure 3P–R). Similar results were obtained with a newly constructed mutant (904) containing a deletion of most of the putative membrane-binding domain (residues 21–159) (data not shown). These results strongly suggest that the putative N-terminal membrane-binding domain of SpoIIIE is responsible for the targeting to the septum and that this targeting is necessary to allow SpoIIIE to help seal off the prespore and mother cell compartments when chromosome partitioning is underway.

Discussion

Topological organization and subcellular localization of SpoIIIE

The extremely hydrophobic nature of the predicted sequence of the N-terminal region of SpoIIIE suggested that it was likely to act as a membrane anchor (Foulger and Errington, 1989). We confirmed that the protein was membrane associated in fractionation experiments, such as the one shown in Figure 1. We then showed that the C-terminal hydrophilic domain of SpoIIIE lies inside the cytoplasm on the basis of its resistance to protease degradation, in contrast to the behaviour of a known extracellular protein DivIB (Harry *et al.*, 1993). Location of the C-terminal region inside the cell accords with the notion that the protein interacts directly with chromosomal DNA during translocation of the DNA through the septum (see below).

The finding that the C-terminal region of the protein seems to lie inside the cytoplasm has implications for the likely membrane topology of the N-terminal domain of the protein (Figure 5). Hydrophobicity plots (Kyte and Doolittle, 1982; window size 19) revealed three likely transmembrane segments (residues 21–74, 84–105 and 133–180). Of these, the first and last segments are long enough to span the membrane twice. As shown in Figure 5, having the first long segment cross the membrane twice produces a structure that accords well with the 'positive-inside rule' of von Heijne (1986). Thus, the hypothetical extracellular loops labelled A and C in Figure 5 would both have little or no net charge, whereas the intracellular N-terminus of the protein and loop B would be strongly positive. If this part of the model is correct, the final long hydrophobic segment (41 residues, with no charges) must cross the membrane only once, to keep the C-terminal domain of the protein inside the cell (in accordance with the data from Figure 2). It is tempting to speculate that the structure of this long hydrophobic segment may play a role in targeting the protein to the septum, or activating it therein (see below). Clearly, more work is needed to confirm this predicted topology, and we cannot exclude other possibilities, such as the complete embedment of the hydrophobic region in the lipid bilayer with no surface exposure.

The recent application of immunofluorescence methods to bacterial systems (Maddock and Shapiro, 1993; Arigoni *et al.*, 1995; Harry *et al.*, 1995; Pogliano *et al.*, 1995) allowed us to examine the subcellular localization of SpoIIIE protein. Affinity purified anti-SpoIIIE antibodies specifically detected SpoIIIE protein, as was demonstrated by the absence of any significant signal in cells of a *spoIIIE* null mutant (Figure 3A–C). In wild type cells

early in sporulation, and in cells of *spoIIIE* missense mutants, which make normal amounts of a defective SpoIIIE protein and are blocked in DNA transfer, SpoIIIE protein was detected as a discrete single focus located between the main bulk of the prespore and mother cell chromosomes (Figure 3). This strongly supports the notion that SpoIIIE is localized in the nascent septum during DNA transfer. Later in wild type development, after the completion of engulfment, the SpoIIIE foci were still associated with the prespore, but their position was apparently able to drift. The drift in position is probably due to the process of engulfment because it was blocked in cells of a *spoIIG* mutant. We suggest that the SpoIIIE focus remains associated with the pair of membranes that surround the prespore after DNA translocation is complete and that the variable position is due to rotation of the prespore inside the enveloping mother cell. The movement did not occur in *spoIIIE* mutant cells either, even though these cells can complete engulfment (Wu and Errington, 1994). In these mutants we suggest that either the postulated free rotation of the prespore is blocked because the prespore is tethered to the mother cell by the prespore DNA, or because the SpoIIIE protein within the prespore membrane is held in position by the DNA.

We never detected SpoIIIE protein in any state other than as a single, near spherical focus, or occasionally two touching foci. In contrast, other proteins associated with septation, such as FtsZ, which forms a ring at the leading edge of the septum (Levin and Losick, 1995), and SpoIIE which marks the sites of incipient asymmetric septation (Arigoni *et al.*, 1995), form elongated zones of fluorescence spanning the width of the cell. We also have observed predominantly elongated regions of fluorescence for proteins associated with septation, using the same microscopic system as was used to detect SpoIIIE (D.H. Edwards and J.Errington, unpublished results). We suggest that SpoIIIE protein may therefore differ from other division proteins studied so far in becoming associated with the septum only during the late stages of septal closure, or when the septum closes around the DNA. However, since the protein is not very abundant, it is possible that it is associated with the septum throughout division but that we can only visualize it when the localized protein concentration peaks late in septal closure.

Implications for SpoIIIE function in DNA transport through the septum

The finding that SpoIIIE protein is targeted to the septum clearly has important implications for its role in prespore chromosome partitioning. In particular, it excludes possible models in which SpoIIIE acts inside the prespore to directly or indirectly draw in the DNA, for instance, by increasing DNA supercoiling. In Figure 6, we suggest a direct role for SpoIIIE in DNA transport through the septum, based on its localization, its similarity to the Tra proteins of several conjugative plasmids from Gram-positive organisms (Wu *et al.*, 1995), and other considerations discussed below. In the model, SpoIIIE is anchored to the leading edge of the spore septum via its N-terminal hydrophobic domain. The C-terminus of the protein lies in the cytoplasm where it can interact with DNA bisecting the septum and drive the DNA through the septal annulus. The presence of a P-loop (ATP binding) motif (Saraste *et al.*, 1990), the critical residues of which are highly conserved in proteins related to SpoIIIE (Wu *et al.*, 1995), suggests that the protein uses ATP hydrolysis to drive DNA translocation.

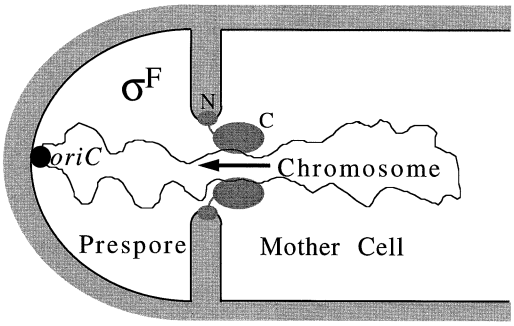

Fig. 6. Model for SpoIIIE function during sporulation. The outline of a cell just completing polar septation is shown in cross section, with an exaggerated annulus occupied by the extended prespore chromosome. The SpoIIIE protein is anchored in the membrane at the leading edge of the septum through its putative N-terminal membrane-binding domain. The hydrophilic C-terminal domains of the protein are located inside the cell where they can interact directly with the chromosomal DNA to effect DNA translocation. Although only two protein subunits are shown, in three dimensions the annulus may be lined by more SpoIIIE subunits to provide a continuous ring, which could effectively seal off the prespore and mother cell compartments from each other. We postulate that *spoIIIE* class I mutants can form the pore and thus seal off the two compartments, allowing σ^F to be correctly compartmentalized, even though DNA translocation fails. The directionality of chromosome translocation could be imposed by a tight association of the *oriC* region of the chromosome and the pole of the cell, dependent at least in part on *soj-spo0J* function (see Sharpe and Errington, 1996).

The directionality of the DNA transfer process mediated by SpoIIIE is an interesting problem. During sporulation, the bulk of the prespore chromosome is translocated into the small compartment (Wu *et al.*, 1995). In contrast, in minicells (produced by vegetative cells of a *divIVB* mutant) that are morphologically similar to prespores, the DNA appears to be transported out of the small cell (Sharpe and Errington, 1995). We recently showed that the *soj-spo0J* locus may provide a chromosomal anchor that imposes directionality on DNA transfer during sporulation (Sharpe and Errington, 1996), as indicated in Figure 6.

Figure 6 also indicates how another feature of the *spoIIIE* mutant phenotype may arise. We reported previously that *spoIIIE* null mutants (designated class II mutants) were not only defective in prespore chromosome translocation but they also allowed aberrant, delocalized activation of the normally prespore-specific sigma factor σ^F (Wu and Errington, 1994). In contrast, missense mutations (class I mutations), affecting the C-terminal putative DNA transfer domain, allowed normal compartmentalization of σ^F. If several SpoIIIE molecules were to form a complete ring at the leading edge of the septum, the protein would effectively form a seal between the DNA in the centre of the annulus and the membrane at the leading edge of the septum. Such an assembly would be compatible with our detection of SpoIIIE foci, given the probable limits to the sensitivity of immunofluorescence. Class I mutants would form the ring of SpoIIIE subunits, allowing functional separation of the prespore and mother cell compartments, even under conditions in which chromosome transfer fails and septal closure is blocked

Table I. Bacterial strains, plasmids and phages

Strain	Relevant genotype[a]	Construction, source or reference[b]
Bacillus subtilis		
CU267	*ilvB2 leuB16 trpC2*	S.Zahler[c]
SG38	*trpC2 amyE*	Errington and Mandelstam (1986)
36.3	*trpC2 spoIIIE36*	Piggot (1973)
647	*trpC2* Ω*(spoIIIE::aphA-3)647*	Wu and Errington (1994)
901	*trpC2* Ω*(spoIIGA::aphA-3)901*	Wu and Errington (1994)
904	*trpC2 spoIIIE904*	pSG813 (*Nco*I) + SG38 DNA→CU267 (ILV, C^s)
912	*trpC2 spoIIIE912*	920 DNA→CU267 (ILV, C^s)
920	*trpC2 spoIIIE::*pSG255 *spoIIIE912 cat*	NTG-mutagenized pSG255→SG38 (C)
Escherichia coli		
DH5α	F^- *endA1 hsdR17*(r_k^- m_k^+) *supE44* λ^-*thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ*(lacZYA-argF)U169 Ø80dlac*Δ*(lacZ)M15*	Gibco BRL
Plasmids		
pSG255	*bla cat spoIIIE* (598–2264)	Wu *et al.* (1995)
pSG812	*bla cat spoIIIE903* (696–1134, 1545–1959)	pSG818 [*Sst*I], [*Sal*I] digest, religated
pSG813	*bla cat spoIIIE904* (696–1134, 1552–1959)	pSG812 *Sal*I, [Nuclease S1] digest, religated
pSG817	*bla cat spoIIIE* (696–2264)	pSG255 [*Eco*RI], [*Cla*I] digest, religated
pSG818	*bla cat spoIIIE* (696–1959)	pSG817 [*Bcl*I], [*Pst*I] digest, religated
pSG829	*bla cat spoIIIE912* (598–2264)	[*Xba*I], *Eco*RV digest and religation of chromosomal DNA from 912::pSG255 (Spo^-)
Phage		
ϕ105J115	*spoIIIE*$^+$ transducing phage	East and Errington (1989)

[a]Numbers in parentheses after *spoIIIE* refer to the first and last nucleotides of the insert, numbered according to Butler and Mandelstam (1987) (see also Foulger and Errington, 1989).
[b]For newly constructed strains, the arrow indicates that the DNA was transformed into the recipient strain. The letters in parentheses indicate the selection imposed: ILV: Ilv^+ and Leu^+; C, chloramphenicol resistance; C^s, chloramphenicol sensitivity. For the plasmids constructed in this work, the DNA and the enzymes used to digest it are given. [ ] indicates that the overhang generated by a enzyme has been end-filled.
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by DNA. In class II mutants, with no SpoIIIE protein present, no such seal would be formed allowing diffusion of some factor required for σ^F activation, or σ^F itself, to diffuse between the two compartments. SpoIIIE might even have a more direct role in compartmentalization of σ^F activation, perhaps by interaction with the SpoIIE protein, which is directly involved in the regulation of σ^F activity (Duncan *et al.*, 1995; Arigoni *et al.*, 1996; Feucht *et al.*, 1996) and was recently shown to localize to the spore septum (Arigoni *et al.*, 1995).

Whatever the precise role of SpoIIIE in compartmentalization of σ^F activity, if the protein needs to be present in the septum to fulfil this function, missense *spoIIIE* mutants affected in targeting to the septum should exhibit a class II phenotype. Therefore, we screened a collection of new class II mutants by Western blotting and found one mutant (912) that produced normal amounts of SpoIIIE antigen. The mutant protein showed little tendency to localize in septa (Figure 3P–R). The mutation *spoIIIE912* resulted in insertion of a charged residue into the final long hydrophobic segment of the N-terminal domain of SpoIIIE. The finding that a mutant impaired in targeting to the septum exhibited a class II phenotype lends strong support to the concept that SpoIIIE can help to seal off the prespore and mother cell compartments when septal closure is occluded by DNA. The *spoIIIE912* mutation also strongly implicates the N-terminal domain of SpoIIIE in targeting to the septum. One possible effect of the glycine to glutamate substitution resulting from this mutation would be to shorten the final long hydrophobic segment of the N-terminal domain of the protein. Perhaps the preferred configurations of the long hydrophobic segments of SpoIIIE are tailored to the unusual membrane curvature that is likely to characterize the leading edge of the septal annulus.

Irrespective of the likely function of the N-terminal domain in targeting to the septum, it is likely that SpoIIIE recruitment to the septum is enhanced by the presence of occluding DNA. We showed previously that SpoIIIE can operate in vegetative cells if DNA is trapped by the central vegetative septum (Sharpe and Errington, 1995). Our detection of SpoIIIE in a small proportion of cells undergoing medial division (Figure 3), would be in accordance with such a vegetative function for SpoIIIE. Its mode of action might then be similar to that suggested for sporulating cells in Figure 6, but without the polar attachment to impose a specific directionality.

Materials and methods

Bacterial strains and media

The *B.subtilis* and *Escherichia coli* strains used in this study are described in Table I, together with plasmids and phages. Solid media used for growing *B.subtilis* were nutrient agar (Oxoid) and lactose glutamate minimal agar (LGMA). 5 μg/ml chloramphenicol, 5 μg/ml kanamycin, 1 μg/ml erythromycin and 25 μg/ml lincomycin, or 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), were added as required. Media used for growing *E.coli* were 2xTY (Sambrook *et al.*, 1989) and nutrient agar (Oxoid) supplemented with 100 μg/ml ampicillin, as required.

General methods

Bacillus subtilis cells were made competent for transformation with DNA by the method of Anagnostopoulos and Spizizen (1961) as modified by Jenkinson (1983). The *spo* mutants were made isogenic by transforming *B.subtilis* strain CU267, selecting for Ilv^+ Leu^+ transformants and screening for Spo^- congressants on LGMA plates. The congressants were checked by complementation with a recombinant *spoIIIE*$^+$ transducing phage ϕ105J115. *Bacillus subtilis* chromosomal DNA was prepared by a scaled down method based on that of Errington (1984).

DNA manipulations and *E.coli* transformations were done by standard methods (Sambrook *et al.*, 1989).

Cloning and sequencing of the spoIIIE912 mutation

The mutation *spoIIIE912* in strain 912 was transferred to the integration plasmid pSG255 and sequenced as described by Wu *et al.* (1995).

Induction of sporulation

Bacillus subtilis cells grown in hydrolysed casein growth media at 37°C were induced to sporulate by the resuspension method of Sterlini and Mandelstam (1969), as modified by Partridge and Errington (1993). Times (h) after resuspension of cells in the starvation medium were denoted t_0, t_1, etc.

Assay for β-galactosidase activity

β-galactosidase activity was measured by the method of Errington and Mandelstam (1986). One unit of β-galactosidase catalyses the production of 1 nmol 4-methylumbelliferone per min under the standard conditions.

Protoplasting, fractionation and chymotrypsin treatment

Cultures were harvested 2 h after induction of sporulation, or, for vegetative cultures grown in Oxoid Penassay Broth, at an OD_{600} of 1.0. The cell pellet was resuspended in approximately one half volume (relative to the original culture) of 1× SMM (0.5 M sucrose, 20 mM maleic acid, 20 mM $MgCl_2$ pH 6.5) containing 2 mg/ml lysozyme as described by Errington (1990). The mixture was incubated at 37°C with gentle shaking for 1–1.5 h until at least 95% of the cells were protoplasted with no detectable lysis (examined by microscopy). Alternatively, cells were protoplasted with 4 mg/ml lysozyme in 1× SMM and incubated at 20°C for 25 min, giving similar results. The protoplasts were then pelleted and resuspended in the same volume of 1× SMM.

Cell fractionation from protoplasts was done essentially as described by Harry *et al.* (1993). A 1.5 ml vol of protoplasts were pelleted and resuspended in 0.25 ml of TM buffer (20 mM Tris–HCl pH 8.0; 5 mM $MgCl_2$) containing 2 mM PMSF and 5 μg/ml DNase I. The sample was incubated at room temperature for 20 min, checked by microscopy to ensure that most of the protoplasts had lysed, then microfuged for 15 min at 4°C. The supernatant (cytoplasmic fraction) was added to an equal volume of 2× SDS–PAGE loading buffer and the pellet (membrane fraction) was resuspended in 0.25 ml of 1× SDS–PAGE loading buffer containing 2 mM PMSF.

To test the susceptibility of SpoIIIE in protoplasts to protease, 6 ml of protoplasts were mixed with chymotrypsin to a final concentration of 50 or 62.5 μg/ml. Aliquots of 0.8 ml were removed after 0, 10, 30, 60, 90 and 120 min incubation at room temperature and transferred to Eppendorf tubes containing 40 μl of PMSF (100 mM, freshly prepared). As a control, 0.8 ml of protoplasts were mixed directly with 40 μl of PMSF (100 mM), without chymotrypsin treatment, and left at room temperature for 2 h. Another control sample was also prepared by adding 40 μl of PMSF (100 mM) to 0.8 ml of protoplasts prior to the addition of the chymotrypsin (to a final concentration of 50 μg/ml) and incubated at room temperature for 2 h. All of the samples were then centrifuged and the pellets resuspended in 120 μl of 1× SDS–PAGE loading buffer containing 2 mM PMSF. To test the sensitivity of SpoIIIE to the protease, 0.8 ml protoplasts were pelleted and resuspended in 60 μl 1× SMM containing chymotrypsin (62.5 μg/ml), to which 0.6 μl Triton-100 was added. After incubation at 20°C for 90 min, the sample was mixed with 60 μl 2× SDS–PAGE loading buffer containing 4 mM PMSF.

SDS–PAGE and Western immunoblot analysis

Proteins were separated by SDS–PAGE and analysed by Western immunoblotting using anti-SpoIIIE and anti-DivIB antibodies as described by Wu and Errington (1994). Samples were heated at 65°C for 5 min prior to loading. The rabbit anti-DivIB antibodies were kindly provided by Prof. R.G.Wake, and were used at a dilution of 1/5000.

Immuno-affinity purification of the anti-SpoIIIE antibodies

The GST–SpoIIIE fusion protein (Wu and Errington, 1994; R.Allmansberger, unpublished) was extracted from an SDS–polyacrylamide gel, then ~30 μg of the protein was immobilized onto a 20 μl Affi-Gel-10 column as described by the supplier (Bio-Rad). These affinity columns were then used for purifying the anti-SpoIIIE antiserum by the following method (L.Harry, O.Resnekov and R.Losick, personal communication). The columns were washed with 20 vol of 10 mM Tris–HCl (pH 7.5), then 20 vol of elution buffer (4.5 M $MgCl_2$, 0.1% BSA), and finally with 20 vol of 10 mM Tris–HCl (pH 7.5). Antiserum (50 μl) was then passed through the column five times to allow antibody binding. The column was washed with 10 mM Tris–HCl (pH 7.5), and then 10 mM Tris–HCl (pH 7.5), 500 mM NaCl until no detectable amount of protein was washed out. The bound antibodies were then eluted with 50 μl of elution buffer and stored at –20°C.

Immunofluorescence microscopy

Cells of sporulating cultures were fixed and processed for immunofluorescence microscopy as described by Harry *et al.* (1995), using affinity purified rabbit polyclonal anti-SpoIIIE antibody (at a dilution of 3/100) and anti-rabbit IgG (whole molecule) FITC-conjugated antibody (Sigma). The DNA-specific dye, 4,6-diamidino-2-phenylindole (DAPI; Sigma) was used at a concentration of 0.2 μg/ml to visualize and discriminate between the nucleoids of non-sporulating or preseptation cells, prespores and mother cells. Cells were viewed by epifluorescence microscopy and images were captured and analysed as described by Lewis and Errington (1996).

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