A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*

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Spore formation in *Bacillus subtilis* begins with an asymmetric cell division that superficially resembles the division of vegetative cells. Mutations in the *spoIIIE* gene of *B. subtilis* partially block partitioning of one chromosome into the smaller (prespore) compartment of the sporulating cell. Point mutations that specifically block prespore chromosome partitioning affect a carboxy-terminal domain of SpoIIIE that shows significant sequence similarity to the DNA transfer (Tra) proteins of several conjugative plasmids of *Streptomyces*. In wild-type sporulating cells, the prespore chromosome passes through an intermediate stage resembling the state in which *spoIIIE* mutant cells are blocked. The prespore chromosome is then transferred progressively through the newly formed spore septum. We propose that translocation of the prespore chromosome occurs by a mechanism that is functionally related to the conjugative transfer of plasmid DNA.

[Key Words: *spoIIIE* gene; cell division; Tra proteins; septation; nucleoid]

Received March 6, 1995; revised version accepted April 11, 1995.

Successful cell division requires an accurate mechanism for partitioning the products of a round of DNA replication to the daughter cells created by formation of a division septum. It is generally agreed, at least for *Escherichia coli*, that DNA partitioning is an active process that usually occurs before the septum begins to be formed (Donachie 1993). Despite many years of work, the mechanism of chromosome partitioning remains obscure. Although it has been suggested that newly synthesized sister chromosomes move apart abruptly, inferring the existence of a mitotic apparatus (Hiraga et al. 1989; Hiraga 1992), the most recent results indicate that partitioning occurs progressively during cell growth (van Helvoort and Woldringh 1994).

Cell division in vegetative *Bacillus subtilis* closely resembles that of *E. coli* in morphological terms, and several gene products needed for division seem to be conserved in both organisms (Beall et al. 1988; Yanouri et al. 1993). *B. subtilis* also undergoes a modified cell division, during the process of sporulation. Again, this process seems related to vegetative division morphologically (Hitchins and Slepecky 1969), and the formation of the septum requires gene products in common with vegetative division (Beall and Lutkenhaus 1991, 1992; Levin and Losick 1994). However, during sporulation the division septum is placed close to one pole of the parent cell and one of the newly formed chromosomes must un-

dergo an extreme movement to achieve its near polar position (Ryter 1965).

We showed recently that DNA partitioning during sporulation specifically requires the product of the *spoIIIE* gene (Wu and Errington 1994). In *spoIIIE* mutants the spore septum forms in its normal asymmetric location, but only a minor proportion (~30%) of the prespore chromosome is localized in the small compartment. The remainder is located incorrectly in the larger compartment, along with the whole of the mother cell chromosome. The SpoIIIE phenotype, with the prespore nucleoid bisected by the septum, could arise by failure of the chromosome to move to the pole of the cell before septation or by failure of the chromosome to be translocated through the septum after it has formed around the nucleoid.

Soon after the formation of the spore septum, the σ^F transcription factor becomes active specifically in the prespore compartment (Margolis et al. 1991; Partridge et al. 1991). The classic *spoIIIE36* mutation was once thought to block σ^F activation, but it now appears that the effects on gene expression are an indirect consequence of the impairment in DNA partitioning: In the mutant, σ^F becomes active specifically in the prespore, as usual, but most σ^F -dependent genes fail to enter this compartment. Despite the fact that the prespore chromosome has been trapped (or bisected) by the septum, the two compartments seem to be physiologically separated in this mutant. Surprisingly, however, a *spoIIIE* null mutation also results in inappropriate release of σ^F in both the prespore and the mother cell, indicating that

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spoIIIE is also required for correct localization of σ^F activity.

To improve our understanding of SpoIIIE function, we have now characterized the phenotypic effects of a collection of *spoIIIE* mutants. The class I mutations, which are defective specifically in prespore DNA partitioning (i.e., they do not affect σ^F localization), all lie in the carboxy-terminal coding region of the gene and are mainly missense mutations. The carboxy-terminal domain of SpoIIIE shows striking sequence similarity to the transfer (Tra) proteins of various conjugative plasmids of *Streptomyces*. By analogy to conjugation, SpoIIIE could work by driving the major part of the prespore chromosome through the nascent spore septum. In accordance with this idea, we have shown that in wild-type sporulating cells, the prespore chromosome enters the prespore compartment after septation, in a progressive manner. The results indicate that prespore chromosome partitioning proceeds by a mechanism that is quite different from the one that normally operates in vegetative cells and instead may be functionally related to conjugative DNA transfer.

Results

spoIIIE mutations that specifically block prespore DNA partitioning all affect a carboxy-terminal domain of the protein product

We reported previously that the classically isolated *spoIIIE36* mutation specifically blocks partitioning of the prespore DNA, with little immediate effect on gene expression in either of the cells. The early cell-specific transcription factors σ^F and σ^E are activated correctly, in their appropriate compartments, but some prespore-specific genes, such as *gpr*, do not become active because they fail to enter the prespore compartment (Wu and Errington 1994). An in vitro-constructed null mutation, *spoIIIE647*, caused a similar defect in prespore DNA partitioning, but it also affected σ^F activation, which occurred inappropriately in the mother cell, as well as in the prespore (Wu and Errington 1994). Our laboratory strain collection contained nine other alleles of *spoIIIE*, as judged by complementation with the *spoIIIE*⁺ transducing phage ϕ 105J115 (East and Errington 1989). The mutations were all transformed into an isogenic background, and their effects on DNA partitioning during sporulation were examined. All blocked prespore DNA partitioning in a manner indistinguishable from the effects of the *spoIIIE36* and *spoIIIE647* mutations described previously (results not shown; Wu and Errington 1994). Transformation of each mutant with a *spoIIIG*'-'*lacZ* fusion (plasmid pSG139) allowed the effects on σ^F localization to be assessed, because this fusion lies in a region of the chromosome that fails to enter the prespore compartment in *spoIIIE* mutants. Mutations *spoIIIE47*, *spoIIIE82*, *spoIIIE601*, *spoIIIE602*, *spoIIIE603*, and *spoIIIE629* (designated class I) behaved like *spoIIIE36*, in conferring a Lac⁻ phenotype, indicating that σ^F activity was localized correctly in the prespore. The remaining

three mutations, *spoIIIE604*, *spoIIIE644*, and *spoIIIE649*, like *spoIIIE647*, produced blue colonies and were designated class II mutations. A full description of the phenotypic effects of the various mutations will be described elsewhere.

The DNA sequences of the class I mutant alleles should give information on the region of SpoIIIE involved specifically in prespore chromosome partitioning. Integration plasmids pSG255 and pSG801 covering the amino- and carboxy-terminal coding portions of *spoIIIE* were first used to determine in which half of the gene each of the mutations lay. Then, plasmids carrying the mutations were excised from the chromosome and recovered by transformation of *E. coli*. The *spoIIIE913* mutant allele (see below) was recovered directly from the chromosome by PCR amplification.

As shown in Table 1, the previously isolated class I *spoIIIE* mutations (allele numbers <900) all lay in the carboxy-terminal coding region of *spoIIIE* (Fig. 1, below). Most were point mutations that would produce single amino acid substitutions. Mutation *spoIIIE36* consisted of a cluster of closely linked missense changes. Mutation *spoIIIE82* was a nonsense mutation, but it lay close to the carboxy-terminal coding end of the gene. In contrast, the class II mutations were all nonsense or frameshift mutations that would severely truncate the protein product.

Table 1. Sequences of *spoIIIE* mutations

Mutation	DNA sequence alteration ^a	Effect on SpoIIIE protein ^b
Class I		
<i>spoIIIE36</i>	G2352A, C2390G, G2391C	V427M, N439K, V440L
<i>spoIIIE47</i>	G3055A	G661D
<i>spoIIIE82</i>	C3162T	Q697*
<i>spoIIIE602</i>	G3028A	G652D
<i>spoIIIE629</i>	G2305A	G411E
<i>spoIIIE911</i>	C2254T	A394V
<i>spoIIIE913</i>	G2199A	G376R
Class II		
<i>spoIIIE604</i>	C1377T	Q102*
<i>spoIIIE644</i>	Δ (G2601)	510, frameshift, + 8 aa
<i>spoIIIE647</i>	Ω (1184–3039::aphA-3)	37, insertion, + 11 aa
<i>spoIIIE649</i>	(ATCA)2268(GG)	397, frameshift, + 14 aa

^aNumbering according to the sequence described by Butler and Mandelstam (1987), incorporating the correction by Foulger and Errington (1989). The wild-type and mutant bases are given, respectively, before and after the number indicating the position of the mutation in the sequence. (Ω) Insertion; (Δ) deletion.

^bNumbering according to Foulger and Errington (1989). For amino acid substitutions, the letters before and after the number giving the position represent, respectively, the residues in the wild-type and the mutant proteins. For frameshift and insertional mutations, the number indicates the last unchanged residue in the protein. The number of additional amino acids (aa) added after the site of the mutation are given. (*) Stop codon.

Absence of class I mutations in the amino-terminal coding region of *spoIIIE*

It was striking that the class I mutations were all located in the carboxy-terminal coding half of *spoIIIE* (Fig. 1). To test whether similar mutations could be isolated in the amino-terminal half of the gene, plasmid pSG255, containing this region, was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in *E. coli* and the purified mutagenized plasmid DNA was transformed into strain 687, which carries a *lacZ* fusion to the σ^F -dependent *spoIIIG* gene and thus forms blue colonies on plates containing X-gal. From ~5000 chloramphenicol-resistant transformants [which would contain Campbell-integrated copies of the plasmid], two mutants showing a white colony color phenotype on plates containing X-gal were isolated. Transducing phage $\phi 105J115$, carrying the wild-type *spoIIIE* gene, was used to check that the new mutations lay in *spoIIIE*; lysogeny with the phage resulted in the restoration of the blue colony color phenotype. In a parallel experiment, the mutagenized plasmid DNA was also transformed into wild-type strain SG38 and *Spo*⁻ mutants were isolated. From ~8000 transformants, 23 mutants were isolated. A *spoIIIG'*-*lacZ* fusion was introduced into each of the mutants by transformation with chromosomal DNA from strain 687, so that the effects of the mutations on σ^F -dependent transcription could be determined. Only one of the mutants (containing a mutation designated *spoIIIE913*) showed a block in *spoIIIG'*-*lacZ* expression, as expected for a class I mutation.

The three class I mutations derived by mutagenesis of pSG255 were cloned and sequenced. The two mutations derived by direct transformation of strain 687 had identical lesions, so only one, designated *spoIIIE911*, will be considered further. Both *spoIIIE911* and *spoIIIE913* were missense mutations lying close to the downstream end of the *spoIIIE* insert in pSG255 (Table 1). The locations of these mutations close to the cluster of previously isolated class I mutations suggest that the domain defined

by the class I mutations does not extend far into the insert in plasmid pSG255 and/or that only the amino-terminal part of *SpoIIIE* is important for the localization of σ^F activity.

The carboxy-terminal domain of *SpoIIIE* resembles the *Tra* proteins from several conjugative plasmids of *Streptomyces*

The mutations specifically blocking prespore chromosome partitioning all lay in the C-terminal coding half of *spoIIIE*. As one way of elucidating the function of this domain, we searched for sequence similarities in protein sequence data bases. Strong sequence similarities were reported previously between *SpoIIIE* and chromosomal genes from diverse bacteria, *Coxiella burnetii* (Oswald and Thiele 1993) and *Campylobacter jejuni* (Miller et al. 1994). Unfortunately, the functions of these genes are not yet known. Among the sequences showing lower but still highly significant similarity scores to *SpoIIIE* were four proteins from plasmids of another Gram-positive bacterium, *Streptomyces*. As shown in Figure 2, the proteins could be aligned with *SpoIIIE* over a carboxy-terminal region of ~500 amino acids. The sequences were particularly closely related over a central region of ~200 amino acids. This region coincided with the most highly conserved part of *SpoIIIE* as compared with the two chromosomal homologs (Fig. 2). The central region included the A and B motifs (Walker et al. 1982) that are highly conserved in proteins that bind ATP (Saraste et al. 1990). However, these short motifs represented only a small proportion of the highly conserved region, so it is unlikely that the sequence similarities simply reflect a common NTP-binding function.

The plasmids encoding the proteins related to *SpoIIIE* all mediate conjugation. The mechanism of conjugation in *Streptomyces* differs from that of the more familiar F system of *E. coli* in that it requires fewer plasmid-encoded products. In fact, in three of the four cases, the

Figure 1. Locations of *spoIIIE* mutations and consequences for the SpoIIIE protein. (Top) The SpoIIIE protein, with the scale indicated in numbers of amino acids. A hydrophobic region and the regions of similarity between SpoIIIE and the Tra proteins of several plasmids from *Streptomyces* (see Fig. 2) are shaded. In the latter case, the intensity of shading indicates the degree of similarity. (A,B) The positions of the ATP-binding motifs (Walker et al. 1982; Saraste et al. 1990) in this highly conserved region. (Bottom) The *spoIIIE* gene (sequence from Butler and Mandelstam 1987; Foulger and Errington 1989; scale in kb), with the locations of the *spoIIIE* mutations shown below.

Figure 2. Sequence similarities between SpoIIIE and proteins encoded by conjugative plasmids. Asterisks (*) over the SpoIIIE sequence denote residues that are identical in two chromosomally encoded genes that are closely related to SpoIIIE (see text). MACAW was used to identify blocks of significant sequence similarity between SpoIIIE and the Tra proteins. The final alignment was optimized manually. Residues that are identical in both SpoIIIE and one or more of the Tra proteins are highlighted. The protein sequences aligned with SpoIIIE [787 amino acids; Butler and Mandelstam 1987; Foulger and Errington 1989; protein information resource (PIR), SO9411] were Spi (303 amino acids; PIR, JC1485) from pSA1.1 of *Streptomyces cyaneus* (Tomura et al. 1993); TraSA [336 amino acids; European Molecular Biology Laboratory (EMBL), Z19593] from pSAM2 of *Streptomyces ambofaciens* (Hagège et al. 1993); TraB (652 amino acids; GenBank, D14281) from pSN22 of *Streptomyces nigrificiens* (Kataoka et al. 1991); and TraA (621 amino acids; PIR, P22409) from pJ101 of *Streptomyces lividans* (Kendall and Cohen 1988).

gene related to *spoIIIE* has been shown to carry out the single function needed for DNA transfer (Kendall and Cohen 1988; Kataoka et al. 1991; Hagège et al. 1993). Figure 1 shows that the region of sequence similarity to the Tra proteins coincides with the region of SpoIIIE in which the class I mutations all lie.

Prespore chromosome partitioning occurs postseptation in wild-type sporulating cells

Our previous observations of *spoIIIE* mutants indicated a role for SpoIIIE in chromosome partitioning but not whether the protein acted before or after septation (Wu and Errington 1994). The sequence similarity to the Tra proteins suggested that SpoIIIE might drive prespore chromosome transfer through the nascent spore septum in a conjugation-like manner. A prediction of this model was that in wild-type cells formation of the spore septum should occur before the prespore compartment becomes filled with DNA. The timing of prespore DNA partitioning has not been directly compared with that of asymmetric septation. To do this, we used a newly developed ethanol fixation procedure to visualize the septa (see Materials and methods) and used fluorescence microscopy and a DNA-specific dye (Setlow et al. 1991) to determine the DNA contents of newly formed prespore compartments. Figure 3A illustrates the detection of central and asymmetric septa in wild-type sporulating cells. In accordance with our new ideas on the function of SpoIIIE protein, most cells with clear asymmetric septa had relatively small and rather variable amounts of DNA in the prespore compartment. Quantitation of the amounts of

segregated DNA in such cells confirmed that few contained fully segregated chromosomes: Most contained ~30%–70% of a chromosome equivalent (Fig. 3A,C). To exclude the possibility that the asymmetric septa were optical artifacts generated in some way by partitioning of the prespore chromosomes, we examined vegetative cells of a minicell-producing mutant of *B. subtilis* (SG10). The minicells made by these mutants are prespore-like in size and shape, and they too originate by near polar division. As shown in Figure 3E, the polar septa made by these organisms were readily detectable by the microscopic methods used to observe prespore septa, despite the fact that they were devoid of DNA (Fig. 3F). Polar septa were not detected in vegetative cells of a nonminicell mutant (results not shown).

The demonstration that many sporulating cells with visible spore septa contained only partially segregated chromosomes strongly suggested that septation precedes chromosome partitioning in sporulating cells. The fact that there were few, if any, cells with <30% of the chromosome compartmentalized in the prespore accords with our previous suggestion (Wu and Errington 1994) that the first step in partitioning involves chromosome attachment to the cell pole, leading to the entrapment of ~30% of the chromosome in the newly formed small compartment. The near absence of cells with apparent prespore DNA contents of >70% was either the result of the systematic underestimation of DNA content, which occurs with increasing levels of DNA condensation (Setlow et al. 1991; P.M. Hauser and J. Errington, unpubl.), or the disappearance of the septa, resulting from the further development of the prespore.

Figure 3. Septation and DNA partitioning in sporulating cells. (A) Wild-type strain SG38. (B) *spoIIG* mutant strain 901. (Note that under these conditions, *B. subtilis* cells typically grow in chains.) The septa of sporulating cells taken 75 or 90 min after induction of sporulation were visualized by ethanol fixation and phase-contrast microscopy. Fluorescence microscopy of the same cells stained with DAPI allowed the DNA contents of the prespore compartments to be measured. The relative DNA content of each small prespore compartment (P) was determined as a percentage of that of the whole cell (T), by measuring the fluorescence intensity as described previously (Wu and Errington 1994). The numbers obtained were multiplied by a factor of 2 so that they express the amount of DNA segregated as a fraction of one chromosome equivalent, there being a total of two chromosomes per sporulating organism (Setlow et al. 1991). (C,D) Frequency histograms plotted using data similar to that illustrated in A and B, respectively. (E,F) Phase-contrast and fluorescence micrographs, respectively, showing the detection of anucleate minicells by the *divIVB* mutant of *B. subtilis* (strain SG10). Microscopic magnification, 2500 $\times$.

As a further test of the relative timing of septation and prespore chromosome partitioning, we repeated the experiment in a *spoIIG* mutant strain, in which prespore chromosome partitioning occurs sequentially at both poles of the sporulating organism (Lewis et al. 1994), and septa remain visible because engulfment is blocked. Again, a substantial proportion of the prespores demarcated by asymmetric septa exhibited relatively low DNA contents (Fig. 3B). Moreover, cells that had clear septa at both poles (examples are starred in Fig. 3B) often contained a considerable amount of DNA in the large central compartment: This was usually associated with a correspondingly smaller amount of DNA in the adjacent prespore compartment, indicating that partitioning was incomplete (Fig. 3B). The apparent DNA contents of these cells were often close to that of a whole chromosome, for reasons mentioned above.

These results strongly supported the view that the main phase of prespore chromosome partitioning occurs after asymmetric septum formation is well under way or near to completion.

SpoIIIE can drive DNA translocation through a previously formed spore septum

Although comparison of the kinetics of septum formation, as revealed by electron microscopy (Partridge and

Errington 1993) or ethanol fixation (Hauser and Errington 1995), suggested that the latter showed only septa that were complete or near to completion, it was important to exclude the possibility that chromosome partitioning was occurring when the septal annulus was still quite large. We reported previously that in the presence of a *spoIIIE* mutation septation appears to proceed to completion but the prespore chromosome fails to segregate properly and segments of the chromosome lie on either side of the septum (Wu and Errington 1994). Assuming that the DNA is not severed by the closing septum, there must be a discontinuity in the septum. To look for such a discontinuity, or pore, in the septum, previously we used electron microscopy to examine cells of a *spoIIG spoIIIE36* double mutant, in which septa form at both poles of the sporangium and further development of the septum is blocked. The proportion of cells with a visibly discontinuous septum was very small (<5%) and equivalent to that of the *spoIIIE*⁺ control (Wu and Errington 1994). A few cells with incomplete septa were expected, being caught in the process of forming the septum. Because the sections that we examined were about one-tenth of a cell diameter thick, we estimate that if a "pore" remains in the septa of *spoIIIE* mutant cells, it represents only a small fraction of a cell diameter. We reasoned that if these mutant cells were trapped at an intermediate stage of partitioning,

Table 2. Rescue of prespore chromosome partitioning in a *spoIIIE* mutant by infection with $\phi 105J115$ (*spoIIIE*⁺)

Infecting phage	Frequency of rescue of prespore chromosome partitioning ^a		
	$t_{3.5}$ ^b	$t_{4.5}$	$t_{5.5}$
None	<0.2 (79)	<0.2 (77)	<0.2 (84)
$\phi 105J106$		<0.2 (76)	<0.2 (80)
$\phi 105J115$		9 (78)	16 (80)

^aStrain 902 (*spoIIIE36 spoIIIGB901*) was induced to sporulate. After 3.5 hr ($t_{3.5}$), the culture was divided into three portions, which were infected with the appropriate phage or left uninfected. Most of the cells of this mutant had formed septa at both poles at this time, but as shown previously (Wu and Errington 1994), the majority of their DNA was located in the central compartment, rather than in the polar prespore compartments. At intervals, samples were examined by phase-contrast and fluorescence microscopy. Cells in which partitioning was at least partially rescued (prespore nucleoid brighter than that of the central compartment; a conservative estimation), were counted and presented as a percentage of the total cells viewed. The total frequency of sporulation (percentage of cells with at least one spore septum; either rescued or not rescued) is given in parentheses.

^bTime after induction of sporulation; phage was added at $t_{3.5}$.

because of the absence of functional SpoIIIE protein, it might be possible to drive partitioning of the trapped chromosomes by providing them with the wild-type protein. We tested this idea by inducing sporulation in a culture of the double mutant strain; then after 3.5 hr (as in the experiments reported previously), we infected one aliquot of the culture with a recombinant transducing phage, $\phi 105J115$, carrying the wild-type *spoIIIE* gene (*spoIIIE* should still be expressed at this time; Foulger and Errington 1989). A second aliquot of the culture was infected with the vector phage $\phi 105J106$, with no *spoIIIE* gene, at a similar multiplicity of infection (m.o.i. ~5.0). $\phi 105$ is known to be able to infect sporulating cells but does not undergo a lytic cycle (Osborne and Sonenshein 1976). Samples of both infected cultures and an uninfected aliquot were sampled at intervals and examined by phase-contrast and fluorescence microscopy (summarized in Table 2).

The $t_{3.5}$ time point was chosen because most cells that are capable of initiating sporulation have done so by this stage. At the time of infection, the majority of cells thus showed a typical "disporic" phenotype, with septa at both poles, but in keeping with the effects of the *spoIIIE36* mutation, the majority of the DNA was located in the large central compartment, with only a minor component located in each of the prespores (Fig. 4A,B). Infection with the control phage, $\phi 105J106$, had little effect on the appearance of the cells (Fig. 4C,D). However, 1 hr after infection with $\phi 105J115$ (*spoIIIE*⁺), the culture contained cells in which prespore chromosome partitioning appeared to have been partially completed (Table 2). After 2 hr, numerous cells of this type were evident (Fig. 4E,F), together with some cells in

which partitioning was complete at both poles. That the latter cells contained fully segregated chromosomes was confirmed by measurement of their fluorescence relative to that of the nucleoids of a standard preparation of cells (data not shown). The cells in which chromosome partitioning was rescued were too numerous to be explained by the action of wild-type SpoIIIE prior to septation in a subpopulation of cells that were late in initiating sporulation: The number of sporulating cells showed only a slight increase over the 2-hr incubation period in all three cultures (Table 2).

These results strongly suggest that prespore chromosome partitioning can occur after the presumptive aperture in the spore septum has closed to the point where it cannot be detected by conventional electron microscopic methods.

Prespore chromosome partitioning is dependent on asymmetric septation

If partitioning of the prespore chromosome occurs through the nascent septum, it seemed possible that formation of the septum might be required for chromosome

Figure 4. Rescue of prespore chromosome partitioning post-septation in a *spoIIIE spoIIIGB* double mutant. Strain 902 (*spoIIIE36 spoIIIGB901*) was induced to sporulate. After 3.5 hr ($t_{3.5}$) the culture was divided into three portions, which were infected with the appropriate phage or left uninfected. Phase-contrast (A,C,E) and fluorescence (B,D,F) micrographs of representative fields of cells are shown. (A,B) Control culture (uninfected) at the time of infection ($t_{3.5}$). Asterisks indicate disporic cells (i.e., which have a septum at both poles of the cell). (C,D) Two hours after infection with $\phi 105J106$ ($t_{5.5}$). (E,F) Composite images 2 hr after infection with $\phi 105J115$ ($t_{5.5}$). Microscopic magnification, 2000 $\times$.

partitioning. To test this we made use of a strain (SU105) carrying a mutation in the *divIB* gene, which is required for septation in both vegetative and sporulating cells (Harry et al. 1993). This mutation was chosen because it reduces septation severely even at the normal growth temperature, 37°C. During vegetative growth at this temperature, the mutant produced many long filaments that were devoid of septa (Fig. 5A), but, as expected, chromosome partitioning occurred more or less normally; the nucleoids were regularly distributed throughout the cell filaments (Fig. 5B). Thus, chromosome partitioning in vegetative *B. subtilis* does not require septation. After the induction of sporulation, chromosome partitioning was more irregular (Fig. 5C,D) and no condensed prespore nucleoids were evident. Similar results have been obtained with mutants affected in other cell division genes (*ftsZ* and *pbpB*; P.J. Lewis, R.A. Daniel, and J. Errington, unpubl.). These results strongly suggest that proper prespore nucleoid partitioning requires formation of the spore septum, although it is formally possible that the septum is needed to maintain the prespore chromosome in a condensed state.

Discussion

Seven class I *spoIIIE* mutations, which specifically prevent prespore chromosome partitioning, were all found to affect the carboxy-terminal half of SpoIIIE. Mutagenesis of the amino-terminal coding portion of *spoIIIE* gave

numerous new alleles of *spoIIIE*, but all were class II except for two mutations that lay close to the distal end of the mutagenized segment. Thus, the domain responsible specifically for prespore chromosome partitioning probably does not extend far into the amino-terminal half of the protein. The carboxy-terminal region of SpoIIIE could be aligned over most of its length with four proteins that are essential for conjugative plasmid DNA transfer in *Streptomyces* (Figs. 1 and 2). Furthermore, the most highly conserved portions of SpoIIIE, defined by comparison with the proteins encoded by two closely related genes from other bacteria, coincided generally with the regions of highest conservation among the Tra proteins (Fig. 2). It is thus tempting to speculate that the functions of these proteins are related.

Although conjugation is widespread in bacteria, little is known about the mechanisms of conjugative DNA transfer other than those of F and related systems of *E. coli* (Wilkins and Lanka 1993), which require the products of many genes (Frost et al. 1994). Conjugation mediated by plasmids in *Streptomyces* is likely to be quite different from that of F because only a single plasmid-encoded function is needed for DNA transfer (Hopwood and Kieser 1993). Perhaps the need for complicated mechanisms with which to bring together donor and recipient *E. coli* is unnecessary in *Streptomyces* because of the sedentary mycelial habit of these organisms; donor and recipient presumably interact only when the growing mycelia from adjacent colonies overlap. DNA transfer during sporulation almost certainly involves double-stranded molecules, unlike the single-stranded transfer of DNA mediated by F. However, there is no reason to suppose that other conjugation systems might not work in a double-stranded manner (Wilkins and Lanka 1993). The apparent simplicity of the *Streptomyces* system, including the absence of DNA helicases, nicking enzymes, and so forth, would be compatible with a double-stranded DNA transfer process.

We showed previously that *spoIIIE* mutants were deficient in prespore chromosome partitioning, but our observations gave no clue as to the relative timing of partitioning and septation. The similarity between SpoIIIE and the Tra proteins suggested that the later stages of partitioning might proceed essentially through the septum, by analogy to the process of conjugative DNA transfer. In wild-type sporulating cultures, we found that the DNA content of cells with visible asymmetric septa was highly variable. At the lower end of the range, the DNA content resembled that of *spoIIIE* mutant cells (~30% of a chromosome equivalent). A range of values intermediate between 30% and fully segregated chromosomes was visible in both a wild-type strain and in a *spoIIIG* mutant. The *spoIIIE* phenotype, with the prespore chromosome bisected by the polar septum thus represents an intermediate in the normal partitioning process. The idea that prespore chromosome translocation can occur through a nearly completed septum was tested directly. Sporulating cells of a *spoIIIE* mutant were allowed to accumulate in a state in which their chromosomes were trapped in the partially segregated

Figure 5. Effect of the *divIB105* septum initiation mutation on DNA partitioning during vegetative growth and sporulation. (A,C) Phase-contrast images of *B. subtilis* SU105 cells grown at 37°C, taken at the time of initiation of sporulation and 3 hr later, respectively. (B,D) Fluorescence micrographs of the same cells stained with DAPI. Microscopic magnification, 1250 $\times$.

state by polar septa. Previous electron microscopic examination of such cells had shown that the septa were complete within the limits of detection (Wu and Errington 1994). The septum probably closes to the point where it completely seals off the large and small compartments because we showed previously that σ^F activity is correctly restricted to the prespore compartment in the *spoIIIE36* mutant (Wu and Errington 1994). Following infection with a phage carrying the wild-type *spoIIIE* gene, prespore chromosome partitioning was rescued in a substantial proportion of the cells, confirming that the chromosome can pass through a septum that is effectively completed. The dependence of prespore chromosome partitioning on septation (Fig. 5) suggests that an interaction with the septum is an important function of SpoIIIE, presumably mediated by its hydrophobic, amino-terminal domain.

Figure 6 shows a schematic model for prespore chromosome partitioning and the action of SpoIIIE. We suggest that partitioning is a two-step process. In the first step, the septum forms, trapping a segment of DNA in the small compartment. The remainder of the chromosome is then translocated through the septum in a SpoIIIE-dependent manner. It is evident that the second step in partitioning bears a striking resemblance to plasmid-mediated conjugation: DNA is transferred between two bacteria that are joined by a narrow cytoplasmic pore. The functional and morphological similarities between these processes have interesting evolutionary implications, and comparative studies of the two processes are likely to be enlightening. Moreover, the finding of apparent chromosomal homologs of SpoIIIE in diverse bacteria suggests that the postseptational partitioning machinery may have functions aside from the specialized situation in sporulating cells.

Figure 6. Proposed mechanism of chromosome partitioning into the prespore. Formation of the asymmetric septum traps ~30% of the prespore chromosome in the small compartment. The remainder of the chromosome is then translocated across the septum, in a SpoIIIE-dependent manner (Wu and Errington 1994), producing small compartments with increasing amounts of DNA. Toward the end of the DNA translocation process or after translocation is complete continued development of the spore (involving engulfment by the mother cell) results in the disappearance of polar septa.

Materials and methods

Bacterial strains, plasmids, and media

The *B. subtilis* strains used in this study are listed in Table 3, along with the plasmids used in this work and their construction. *E. coli* strain DH5 α [F^- *endA1 hsdR17 supE44 λ^- thi-1 recA1 gyrA96 relA1 Δ (*lacZYA-argF*)U169 ϕ 80d*lac* Δ (*lacZ*)M15; GIBCO-BRL] was used for plasmid constructions. A *spoIIIG'*-*'lacZ* fusion was introduced into *spoIIIE* mutant strains by transformation with plasmid pSG139 and selection for chloramphenicol resistance.*

Solid media used for growing *B. subtilis* were nutrient agar (Oxoid) and lactose glutamate minimal (LGM) agar (Piggot 1973). Chloramphenicol (5 μ g/ml), kanamycin (5 μ g/ml), erythromycin (1 μ g/ml), and lincomycin (25 μ g/ml), or 0.01% X-gal, were added as required. Media for growing *E. coli* were 2 $\times$ TY (Sambrook et al. 1989) and nutrient agar (Oxoid), supplemented with ampicillin (100 μ g/ml), as required.

General methods

B. 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 *llv⁺ Leu⁺* transformants, and screening for Spo $^-$ congressants on LGM agar plates. The congressants were checked for complementation with a recombinant transducing phage ϕ 105J115 (East and Errington 1989). *B. subtilis* chromosomal DNA was prepared by a scaled-down method based on the one described by Errington (1984). DNA manipulations and *E. coli* transformations were carried out using standard methods (Sambrook et al. 1989).

Induction of sporulation

B. subtilis cells grown in hydrolyzed 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 (hr) after resuspension of cells in the starvation medium were denoted t_0 , t_1 , and so forth.

Cloning and sequencing *spoIIIE* alleles

To sequence the *spoIIIE* mutations, each mutation was transferred from the *B. subtilis* chromosome to integration plasmids by the method of plasmid integration and excision (Niaudet et al. 1982; Errington 1990). Mutant strains were transformed with integration plasmids containing either the promoter-proximal (pSG255) or promoter-distal (pSG801) portions of the wild-type gene, selecting for the appropriate antibiotic resistance. Chromosomal DNA was then prepared from suitable transformants (either Spo $^+$ or Spo $^-$, depending on the restriction endonuclease used for plasmid recovery), and the integrated plasmids were recovered by digestion with appropriate restriction endonucleases, end-filling (when necessary), religation, and transformation into *E. coli* with selection for ampicillin resistance. Plasmid DNA from *E. coli* transformants was subjected to restriction endonuclease analysis. Individuals with the correct restriction patterns were then transformed into both the wild-type strain, SG38, and the parental *spoIIIE* mutant to determine whether or not they contained the mutant allele. PCR amplification of the *spoIIIE913* mutant allele was done with oligonucleotides 5'-AAGGTGATGCATATGGCAAAGAAA-3' and 5'-GTTAGCCCCTACAGG-3'.

Double-stranded plasmid DNA or PCR-amplified DNA was

Table 3. Bacterial strains and plasmids

Strain	Relevant genotype ^a	Construction, source, or reference ^b
<i>B. subtilis</i>		
CU267	<i>leuB16 ilvB2 trpC2</i>	S.A. Zahler (Genetics and Development Department, Cornell University, Ithaca, NY)
SG10	<i>purA16 divIVB1</i>	Reeve et al. (1973)
SG38	<i>trpC2 amyE</i>	Errington and Mandelstam (1986)
SU105	<i>trpC2 thyA thyB divIB105</i>	Harry et al. (1993)
36.3 [NG1.67] ^c	<i>trpC2 spoIIIE36</i>	Piggot (1973)
47.4 [NG8.17] ^c	<i>trpC2 spoIIIE47</i>	Piggot (1973)
82.1	<i>trpC2 spoIIIE82</i>	Hranueli et al. (1974)
601.6	<i>trpC2 spoIIIE601</i>	Deadman (1986)
602.6	<i>trpC2 spoIIIE602</i>	Deadman (1986)
603.6	<i>trpC2 spoIIIE603</i>	Deadman (1986)
604.6	<i>trpC2 spoIIIE604</i>	Deadman (1986)
629 (A3) ^c	<i>trpC2 spoIIIE2^d</i>	Piggot (1973)
644	<i>trpC2 spoIIIE644</i>	Foulger and Errington (1989)
647	<i>trpC2 Ω (spoIIIE::aphA-3)647</i>	Wu and Errington (1994)
649	<i>trpC2 spoIIIE649</i>	J. Errington (unpub.)
687	<i>trpC2 Ω (spoIIIG'-lacZ ermC)687</i>	Partridge et al. (1991)
901	<i>trpC2 Ω (spoIIIGA::aph-A3)901</i>	pSG814 DNA → SG38 (K)
902	<i>trpC2 spoIIIE36</i> <i>Ω (spoIIIGA::aph-A3)901</i>	901 DNA → SG38 (K)
911	<i>trpC2 spoIIIE911</i>	923 DNA → CU267 (ILV)
913	<i>trpC2 spoIIIE913</i>	921 DNA → CU267 (ILV)
921	<i>trpC2 spoIIIE::pSG255 spoIIIE913 cat</i>	NTG-mutagenized pSG255 → SG38 (C)
923	<i>trpC2 Ω (spoIIIG'-lacZ ermC)687</i> <i>spoIIIE::pSG255 spoIIIE911 cat</i>	NTG-mutagenized pSG255 → 687 (C)
Plasmids		
pSG2	<i>bla cat</i>	Fort and Errington (1985)
pSG91	<i>bla cat spoIIIGAB spoIIIG646</i>	Foulger and Errington (1989)
pSG122	<i>bla aph-A3</i>	Daniel and Errington (1993)
pSG139	<i>bla cat spoIIIG'-lacZ</i>	Foulger and Errington (1989)
pSG203	<i>bla cat spoIIIE644 (1-3491)</i>	Butler and Mandelstam (1987)
pSG209	<i>bla cat spoIIIE (3040-3491)</i>	Butler and Mandelstam (1987)
pSG253	<i>bla cat spoIIIE644 (2265-3491)</i>	Foulger and Errington (1989)
pSG255	<i>bla cat spoIIIE (598-2264)</i>	1.75 kb <i>EcoRV</i> fragment from pSG203 cloned into <i>SmaI</i> -digested pSG2 (D. Foulger, unpub)
pSG801	<i>bla cat spoIIIE (2265-3491)</i>	<i>EcoRI</i> , <i>EcoRV</i> , double digest of chromosomal DNA from SG38::pSG253 (<i>Spo</i> ⁻ transformant), end-repaired and religated
pSG814	<i>bla cat Ω (spoIIIGA::aph-A3)901</i>	<i>aph-A3</i> gene from pSG122 inserted in place of <i>PstI</i> fragment internal to <i>spoIIIGA</i> gene in pSG832
pSG832	<i>bla cat spoIIIGA</i>	pSG91, digested with <i>HindIII</i> and religated to eliminate <i>spoIIIG</i> and most of <i>spoIIIGAB</i>

^aNumbers in parentheses after *spoIIIE* refer to the first and last nucleotides of the insert, numbered according to Butler and Mandelstam (1987) as corrected by Foulger and Errington (1989).

^bFor 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; (K) kanamycin resistance.

^cOriginal isolation numbers of strains containing the *spoIIIE* mutation.

^dPreviously assigned to the *spoIIB* locus (Piggot and Coote 1976).

sequenced directly by the chain termination method of Sanger et al. (1977) with Sequenase (v. 2.0), using the Reagent Kit for Sequencing with Sequenase (U.S. Biochemical) and ³⁵S-labeled nucleotides (Amersham), essentially as described by the suppliers. Synthetic oligonucleotides were made to a final concentration of ~3.3 ng/ml dH₂O. In addition to the oligonucleotides used for the PCR reactions, the following oligonucleotides were used for sequencing: M13 universal forward sequencing primer, 5'-ACTGGCCGTCGTTTAC-3'; M13 universal reverse sequencing primer, 5'-AACAGCTATGACCATG-3'; 5'-GGTC-

ATAGGAGGAGCAG-3'; 5'-GCGGGCGAAACCGCATGAA-GTGA3-'; 5'-CCTGTAAACGGCTGC-3'. Two independently amplified products carrying the *spoIIIE913* mutation were sequenced.

Localized mutagenesis of *spoIIIE*

E. coli strain DH5α containing plasmid pSG255, harboring the amino-terminal coding region of *spoIIIE*, was mutagenized with NTG. A culture containing cells from a single colony of *E. coli*

strain DH5 α (pSG255) was grown overnight at 37°C in 2 $\times$ TY containing ampicillin. A 1-ml portion of the overnight culture was used to inoculate 20 ml of the same medium and grown at 37°C with shaking until the OD₆₀₀ was 1.0 (~90 min). Then, 2.5-ml portions of the culture were removed and mixed with equal volumes of warm 2 $\times$ TY containing 400–600 μ g of NTG. The cultures were incubated for 15 min at 37°C with shaking and then centrifuged to collect the cells. The cell pellets were washed twice in warm 2 $\times$ TY containing ampicillin and resuspended in 2.5 ml of the same medium. The cells were grown for an additional 4.5 hr with shaking; then, a 2-ml portion of the culture was used for isolating plasmid DNA, and the rest was frozen in 10% glycerol and stored at –70°C. The plasmid mixtures containing mutagenized pSG255 were then transformed into the wild-type strain SG38, with selection for resistance to chloramphenicol, and Spo[–] transformants were isolated and purified. The plasmid mixtures were also transformed into strain 687, containing a *spoIIIG'*–*lacZ* fusion, with selection for resistance to both erythromycin and chloramphenicol. Transformants that were white in the presence of X-gal were isolated.

Sequence similarity searches and alignments

Proteins similar to SpoIIIE were identified in protein sequence data bases using the programs BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988). Sequence alignments were generated manually, based on output from the MACAW program (Schuler et al. 1991).

Visualization of spore septa and DNA

Samples of sporulating cultures (0.65 ml), taken 75–90 min after starvation, were prepared for visualization of septa as follows: The sample was chilled, and NaN₃ was added (final concentration 0.1 % wt/vol). The cells were harvested (microcentrifuge, 3 min), resuspended in ice-cold 70% (vol/vol) ethanol, and stored at 4°C overnight. The cells were harvested as above, resuspended in sterile distilled water, and viewed immediately by spreading a 2- to 5- μ l sample of the suspension on a coverslip and inverting this onto a glass slide carrying a 5- μ l drop of 50% (vol/vol) glycerol containing 1 μ g/ml of 4,6-diamidino-2-phenylindole (DAPI; Sigma). Coverslips were pretreated with 0.01% polylysine (Sigma) and air-dried. The preparations were examined on a Zeiss Axiophot photomicroscope, equipped with an Optovar, and digital images were obtained and analyzed as described previously (Wu and Errington 1994). The minicell strain, SG10, was grown in Oxoid Penassay broth to late exponential phase. Samples were treated and viewed as for sporulating cells.

Postseptational partitioning of prespore DNA by phage infection

The phage cloning vector ϕ 105J106 and its *spoIIIE*⁺ derivative ϕ 105J115 (East and Errington 1989) were purified and titered by standard methods (Errington 1990). Strain 902 was induced to sporulate as described above. After 3.5 hr, the culture was divided into three aliquots. Two aliquots were infected with phage preparations (ϕ 105J106 or ϕ 105J115) at a multiplicity of infection of ~5. The third aliquot was uninfected. Samples taken at the time of infection or at intervals thereafter were fixed with ethanol and prepared for phase-contrast and fluorescence microscopy as described above. A standard preparation of cells with single completed nucleoids was mixed with each sample to provide an internal standard with which to measure the DNA content of the prespore compartment (Hauser and Errington 1995). Sporulating cells were defined as those in

which prespore septa were present (as judged by phase-contrast microscopy). Cells in which the DNA fluorescence of the prespore compartment was brighter than that of the central compartment were considered to have been at least partially rescued following phage infection, a deliberately conservative definition.

Acknowledgments

We thank Dr. A.J. Day for assistance with the amino acid alignments, David Foulger for plasmid constructions, Tobias Keiser for helpful discussions and for communicating unpublished amino acid sequences, and R.G. Wake and M.D. Yudkin for helpful discussions and comments on the manuscript. This work was supported by grants from the Science and Engineering Research Council. L.J.W. was the recipient of a K.C. Wong Scholarship and an EPA Cephalosporin Trust award. R.A. was supported by a Postdoctoral Fellowship of the European Community. P.J.L. was supported by a Science and Engineering Research Council/North Atlantic Treaty Organization fellowship. P.M.H. was supported by fellowships from the Swiss National Fund for Scientific Research and the European Molecular Biology Organization.

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L J Wu, P J Lewis, R Allmansberger, et al.

Genes Dev. 1995, **9**:

Access the most recent version at doi:[10.1101/gad.9.11.1316](https://doi.org/10.1101/gad.9.11.1316)

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