arteries of rat have a mean membrane capacitance of 10.8 ± 0.4 pF (n = 47) (*8*), corresponding to a membrane surface area of about 1080 μm^2. The fraction of the surface area experiencing a spark was estimated as the surface area of a hemisphere (about 9 μm^2) with a radius (1.19 μm) of the mean spread of a spark divided by the cell surface area (1080 μm^2). The volume of a single cell was estimated to be about 1 pl.

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18. Very few K^+ channels need to open to cause a membrane hyperpolarization of arterial smooth muscle (*3*), because arterial smooth muscle cells have high input resistances (10 gigohms) at physiological membrane potentials in the absence of STOCs. Therefore, the membrane potential would hyperpolarize towards the K^+ equilibrium potential (−85 mV) during an average STOC (*16*). STOCs in smooth muscle cells in the arterial wall would sum to cause a graded membrane potential hyperpolarization. The average membrane potential would reflect the contribution of K_{Ca} conductance caused by Ca^{2+} sparks and other conductances.
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25. A single Ca^{2+} spark at its peak occupies about 7 fl with an average Ca^{2+} of about 300 nM, and occurs in 0.7% of the volume of a smooth muscle cell. A Ca^{2+} spark would thus have an insignificant effect on spatially averaged $[Ca^{2+}]_i$. The highest STOC rate that we observed was 9/s, which would also have little effect on $[Ca^{2+}]_i$, assuming that this corresponded to 9 sparks/s. Even if the increase of $[Ca^{2+}]_i$ lasted 100 ms, the average Ca^{2+} in the cell would still change by <2 nM.
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28. Our results are consistent with the ryanodine- and thapsigargin-induced constrictions of the artery being caused by membrane depolarization, which increases Ca^{2+} entry through voltage-dependent Ca^{2+} channels (*3*) (Fig. 4D), particularly because the iberiotoxin-, ryanodine-, and thapsigargin-induced constrictions were similar and not additive. In support of this mechanism, we observed that ryanodine and thapsigargin had no effect on arterial diameter in the presence of the Ca^{2+} channel blocker, nisoldipine (n = 6). Further, nifedipine has been shown to block ryanodine- and cyclopiazonic acid–induced increases in spatially averaged $[Ca^{2+}]_i$ and constrictions of myogenic skeletal muscle arterioles [J. Watanabe *et al.*, *Circ. Res.* **73**, 465 (1993)].
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Localization of Protein Implicated in Establishment of Cell Type to Sites of Asymmetric Division

Fabrizio Arigoni, Kit Pogliano, Chris D. Webb, Patrick Stragier, Richard Losick*

Asymmetric division in *Bacillus subtilis* generates progeny cells with dissimilar fates. SpoIIE, a membrane protein required for the establishment of cell type, was shown to localize near sites of potential polar division. SpoIIE initially localizes in a bipolar pattern, coalescing at marks in the cell envelope at which asymmetric division can take place. Then, during division, SpoIIE becomes restricted to the polar septum and is lost from the distal pole. Thus, when division is complete, SpoIIE sits at the boundary between the progeny from which it dictates cell fate by the activation of a cell-specific transcription factor.

A fundamental challenge in developmental biology is to understand how cells of one type differentiate into other, more specialized types of cells (*1*). One way specialization occurs is by asymmetric cell division in which a progenitor cell gives rise to two dissimilar progeny that follow different pathways of differentiation. A simple system in which the relation between cell fate and asymmetric division has been investigated is spore formation in *Bacillus subtilis* (*2*). Spore formation involves an asymmetric cell division in which a septum is formed near one pole of the developing cell (the "sporangium"), partitioning it into unequal-sized progeny called the forespore (the small cell) and the mother cell. Crucial to the establishment of the dissimilar fates of the progeny is a putative integral membrane protein (*3*) called SpoIIE (*4*), whose synthesis commences shortly before asymmetric division (*5*).

SpoIIE is not needed for the formation of the polar septum (*6*, *7*) but is required for the activation in the forespore of a transcription factor called σ^F (*4*). The σ^F factor is present in the predivisional sporangium but is held in an inactive complex prior to septation by the inhibitory protein SpoIIAB (*8*). After the polar septum is formed, σ^F continues to be held in an inactive complex in the mother cell, while SpoIIE triggers the release of σ^F from SpoIIAB in the forespore. The mechanism by which SpoIIE activates σ^F has

F. Arigoni and P. Stragier, Institut de Biologie Physico-Chimique, 75005, Paris, France.
K. Pogliano, C. D. Webb, R. Losick, Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed.

been unknown until now (9) but is inferred to involve an additional protein (SpoIIAA) to which SpoIIAB binds in order to release σ^F (*4*, *8*). Freed from inhibition in the forespore as a result of the action of SpoIIE, σ^F sets in motion a chain of events that determine the subsequent fate of the two progeny cells (*2*).

Because of its role in the establishment of cell type, we investigated the subcellular localization of SpoIIE. Polyclonal antibodies were raised against the COOH-terminal region of SpoIIE (*10*) and used to examine the spatial distribution of the protein by immunofluorescence microscopy (*11*). Cells were collected at various times early after the onset of sporulation, fixed, and permeabilized for labeling with antibodies. In some sporangia (designated class i; Fig. 1A) antibody labeling was bipolar, with zones of fluorescence (red color) located near both ends of the cells (*12*). Sporangia with the bipolar pattern of SpoIIE immunostaining were at a very early stage of development because (as revealed by DAPI staining) (*13*) they lacked a condensed forespore chromosome. As sporulation progresses and a polar septum is formed, one chromosome becomes tightly packed into the forespore and is recognized as a region of intense DAPI staining near one pole of the sporangium (*14*). An additional indication that class i sporangia were at a very early stage was that σ^F-directed gene expression had not yet commenced. This was determined by also staining the sporangia, which contained *lacZ* fused to a gene under the control of σ^F, with antibodies to β-galactosidase (β-Gal) (Fig. 1B, representing the same field of cells as in Fig. 1A). The absence of β-Gal staining from class i sporangia indicated that little or no σ^F-directed gene expression had occurred.

Class ii sporangia contained immunostaining of SpoIIE preferentially at one pole (Fig. 1, A, C, and D). Class ii sporangia are more advanced than class i cells because a condensed forespore chromosome was evident (*13*). Also, a low level of β-Gal from σ^F-directed synthesis of the enzyme could be detected (seen as white-to-pink color in Fig. 1B and, as a result of the absence of DAPI fluorescence in this photograph, the yellow color in Fig. 1D). The bipolar pattern (class i) of SpoIIE staining was observed with high frequency among sporangia that had not yet formed a condensed forespore chromosome, and the forespore-specific pattern (class ii) was frequent among sporangia that contained a condensed chromosome (Fig. 2). Finally, class iii sporangia represented a third, more advanced stage of development. In these sporangia, little or no SpoIIE could be detected at either pole. Rather, class iii sporangia exhibited a fully condensed forespore chromosome (evident from photographs of DAPI staining alone) and substantial accumulation of β-Gal (green-blue color; Fig. 1B).

Thus, SpoIIE localizes near both ends

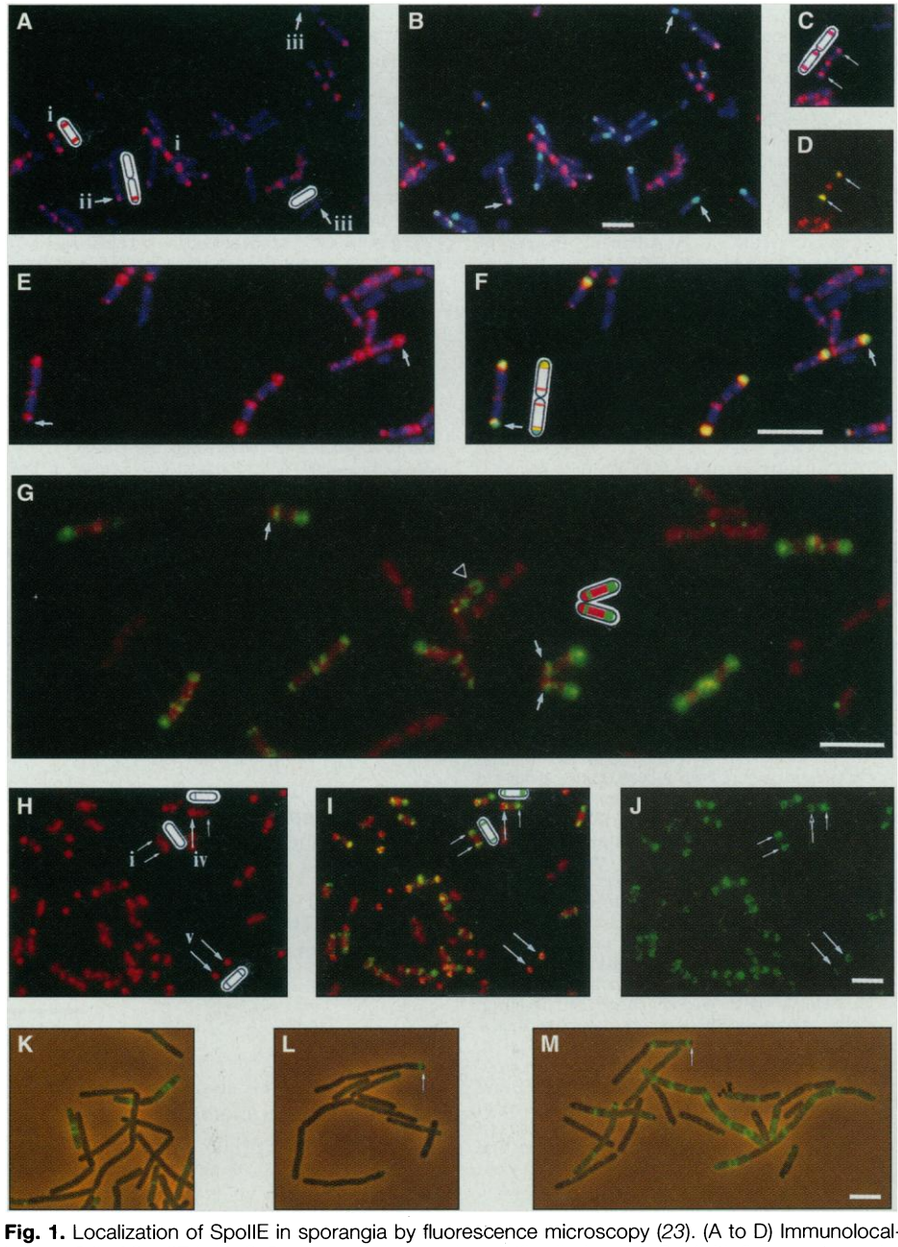

Fig. 1. Localization of SpoIIE in sporangia by fluorescence microscopy (*23*). (A to D) Immunolocalization (*24*) of SpoIIE (with Cy3-conjugated secondary antibodies) and β-Gal (with fluorescein-conjugated secondary antibodies) produced under the control of σ^F (*25*). (**A**) Doubly exposed micrograph showing immunostaining of SpoIIE (red) and DAPI staining of DNA (blue) (*13*). (**B**) Triply exposed micrograph of the same field with immunostaining of β-Gal (green-blue). White-to-pink corresponds to regions in which SpoIIE (red) and β-Gal (green-blue) coincide. (**C**) Doubly exposed micrograph showing SpoIIE (red) and the DAPI-stained DNA (blue) in a pair of sporangia with adjoining mother cells. (**D**) Doubly exposed micrograph of the same field as in (C) showing SpoIIE (red) and β-Gal (green) (*22*). Yellow color corresponds to regions in which SpoIIE and β-Gal are nearly coincident. (E and F) Immunolocalization of SpoIIE (Cy3) and β-Gal (fluorescein) produced under the control of σ^F in *spoIIIE* mutant sporangia (*26*). (**E**) shows SpoIIE (red) and the DAPI-stained DNA (blue), and (**F**) additionally shows β-Gal (green) (*27*). Yellow color occurs where SpoIIE and β-Gal overlap. (**G**) Immunolocalization of SpoIIE (fluorescein) in propidium iodide–stained (red) *spoIIIE* mutant sporangia (*28*). (H to J) Immunolocalization of SpoIIE (fluorescein) in a disporic mutant (*spoIIGAB*::*erm*) (*29*). (**H**) Propidium iodide–stained DNA (red). (**I**) Multiply exposed micrograph showing SpoIIE (green) and DNA (red); when the two are in close proximity, yellow is apparent. (**J**) SpoIIE alone (green). (**K** to **M**) Phase and fluorescence micrographs of living cells producing SpoIIE-GFP (*18*) at hour 2 of sporulation (*30*).

of the cell very early in development, probably before a septum has formed. Then, when the septum forms and the chromosomes segregate, SpoIIE disappears from the forespore-distal pole but remains localized near the forespore pole (*15*). Finally, after a chromosome is fully packaged into the forespore and σ^F is maximally active, SpoIIE disappears entirely from the sporangium.

A brighter image was obtained when immunostaining was carried out in a mutant (*spoIIIE*) defective in chromosome segregation in which class i sporangia persisted longer (Fig. 1E). The *spoIIIE* gene product has been implicated in the process of translocating one chromosome into the forespore (*16*). In studies with the *spoIIIE* mutant, it was apparent that SpoIIE is not located at the extreme ends of sporangia but instead is slightly internal from the poles, at or near the sites of asymmetric septation. This result was seen in mutant sporangia that were additionally stained for β-Gal produced under the control of σ^F. Fluorescence from immunostaining of β-Gal (green color in Fig. 1F, representing the same field as in Fig. 1E) in some of the sporangia (identified with arrows) extended beyond the region of SpoIIE immunostaining (red color) toward the cell pole. The overlap in the staining pattern of SpoIIE and β-Gal produced a yellow color. Furthermore, in some of the sporangia (identified with arrows) stained both for DNA (red color in Fig. 1G) and SpoIIE (green color), fluorescence from DNA extended out beyond the zone of SpoIIE immunostaining. Similar results were obtained with wild-type sporangia. Thus, SpoIIE coalesces at sites near to, but not at the ends of, cells. We sometimes observed SpoIIE immunostaining in the shape of a ring in the *spoIIIE* mutant (open triangle; Fig. 1G). This finding suggests that SpoIIE assembles into a collar-like structure on the inside surface of the cell at or near sites of potential septum formation (*17*).

We also determined the subcellular localization of SpoIIE with an additional approach. We constructed a strain producing a fusion protein of SpoIIE with green fluorescent protein (GFP) of *Aequorea victoria* (*18*). GFP autocatalytically generates a fluorophore that exhibits green fluorescence in unfixed, living cells (*19*). Sporangia producing the SpoIIE-GFP fusion protein displayed fluorescence patterns (Fig. 1, K to M) similar to that observed with immunostaining. Furthermore, phase contrast and fluorescence microscopy demonstrated that the SpoIIE-GFP fusion was localized in a sharp zone close to, but set back from, the poles of the sporangia (Fig. 1, L and M).

Normally, asymmetric division occurs at only one pole. Nonetheless, both poles are capable of undergoing division, as inferred from the existence of mutants that divide at both ends of the sporangium (*7*). Bipolar division in these mutants produces aberrant, "disporic" sporangia with chromosome-containing forespores in which σ^F is active (*20*) at both poles, and produces an empty mother cell in the middle (Fig. 3). We examined the localization of SpoIIE in a disporic mutant (*spoIIG*) (Fig. 1, H to J, representing the same field of cells). A bipolar pattern of SpoIIE immunostaining was observed in class i sporangia. At later stages of development, a class of sporangia (designated class iv) was observed with strong immunostaining at the forespore-distal pole. In these class iv sporangia, a condensed chromosome was visible at one pole of the cell (Fig. 1H), and a strong SpoIIE signal was detected at the opposite pole (Fig. 1, I and J), the site at which a second septum was presumably forming. Such class iv sporangia were abundant (76%; Fig. 2) among mutant sporangia at the stage of a condensed forespore chromosome but uncommon (2%; Fig. 2) among wild-type sporangia. Later in development, when the disporic mutant phenotype was fully manifest (class v sporangia), condensed chromosomes were visible at both poles (Fig. 1H), but relatively little SpoIIE could be detected at either end of the sporangia (Fig. 1, I and J). Therefore, in disporic sporangia, SpoIIE localizes near both poles during early stages of development, persists near each division site until a septum is formed at each pole, and finally disappears entirely from the sporangia (Fig. 3).

These observations suggest that the cell envelope contains "marks" at medial and

SpoIIE pattern	Chromosome pattern	
	Wild type	
	68% class i	12%
	32%	86% class ii
		2%
	Disporic mutant	
	83% class i	16%
	17%	8%
		76% class iv

Fig. 2. Tabulation of SpoIIE immunostaining pattern in wild-type and in a disporic mutant. Immunostaining and DNA staining were done as described in Fig. 1 legend. Micrographs of the stained sporangia were scored according to the pattern of chromosome condensation and the pattern of SpoIIE localization. The percentages indicate the percentage of wild-type and disporic (*spoIIGAB::erm*) mutant sporangia exhibiting (right-hand column of percentages) or not exhibiting (left-hand column) a condensed forespore chromosome with the indicated pattern of SpoIIE immunostaining. The figure distinguishes two kinds of SpoIIE patterns: strong staining at both poles and preferential staining at one pole. [Note that for sporangia without a condensed forespore chromosome (left-hand column), the future forespore pole could not be distinguished from the future distal pole.] The percentages are based on 225 and 218 wild-type sporangia with or without a condensed chromosome, respectively, and 61 and 77 mutant sporangia with or without a condensed chromosome, respectively. Not included are wild-type sporangia that had reached the stage of development (when σ^F was fully active) at which little or no immunostaining could be detected. Also not included are disporic mutant sporangia that had reached the stage at which condensed, forespore chromosomes were present at both poles. Such sporangia in which the mutant phenotype was fully manifest (corresponding to class v in the text) displayed little immunostaining or weak staining, often preferentially at one pole.

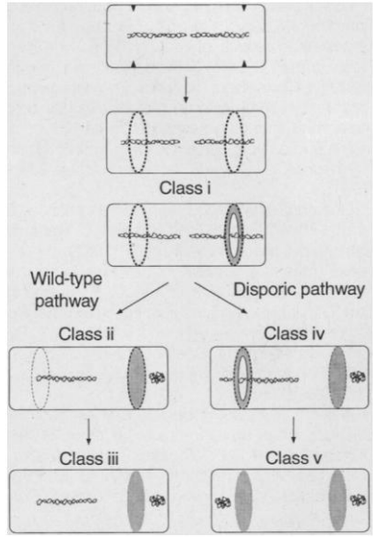

Fig. 3. Model for the subcellular localization of the cell-type determining protein SpoIIE in wild-type and disporic mutant bacteria. The cartoons represent cells at early stages of sporulation in wild-type and disporic mutant bacteria. The wavy horizontal lines indicate chromosomes; the chromosomes are extended when in the predivisional sporangium or the mother cell and compact when present in the forespore. The arrowheads in the top cartoon signify hypothetical marks for potential polar division. The incomplete, shaded disks represent still-forming septa, and the complete, shaded disks represent completed septa. The dotted oval lines represent SpoIIE, with the thin dotted lines indicating the presence of SpoIIE in low abundance and the dark dotted line indicating high abundance. In the wild-type pathway (on the left) a division septum is formed at only one pole, whereas in the disporic pathway of mutant bacteria, septa for m successively at both poles.

polar positions, and that at the start of sporulation the medial mark is masked or destroyed whereas marks at both poles are created or rendered accessible to the septation machinery of the cell (Fig. 3). The finding that SpoIIE initially localizes in the vicinity of both sites of potential polar division suggests that SpoIIE recognizes the hypothetical polar marks (Fig. 3) and hence could serve as a tool for verifying their existence and determining their molecular nature.

Finally, the finding that SpoIIE comes to be sequestered at the position of the polar septum indicates that SpoIIE sits at the boundary between the small cell in which σ^F is active and the large cell in which it is inactive. A simple model for how the septal location of SpoIIE could contribute to the selective activation of σ^F in the forespore, and the assignment of a biochemical function to SpoIIE, are presented in the accompanying report (9).

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13. The blue fluorescence from DAPI (4,6-diamidino-2-phenylindole propidium iodide) is difficult to see because the double-exposure photographs were designed to optimize fluorescence from immunostaining and, in the case of fluorescein, the band-pass filter sets required to separate blue from green fluorescence produced blue images of low contrast. However, the blue fluorescence could be clearly seen in single exposures of DAPI staining alone with the same fields of cells (F. Arigoni and K. Pogliano, unpublished data).
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17. The finding that SpoIIE localizes at or near sites of septum formation, combined with the observation that septum formation is aberrant in *spoIIE* mutant sporangia (*6*, *7*), indicates that SpoIIE could play a direct role in maturation of the polar septum.
18. The *spoIIE-gfp* fusion was constructed by polymerase chain reaction (PCR)–mediated amplification of *spoIIE* sequences from chromosomal DNA. The amplified DNA was cut at a Sac I site within the gene [located 1375 base pairs (bp) downstream of the start codon] and at an artificial Bam HI site located beyond the 3′ end of the open reading frame that had been created by a primer used in the PCR. This 1.1-kb Sac I–Bam HI fragment was cloned into the polylinker region located just upstream of *gfp* in plasmid pCW8 [C. D. Webb, A. Decatur, A. Teleman, R. Losick, *J. Bacteriol.*, in press]. This created pCW27, which contained the 3′ half of *spoIIE* joined in-frame to *gfp* by means of 13 codons of polylinker sequence. Next, a kanamycin-resistance gene was inserted in pCW27 to create pCW28 which was, in turn, integrated into the chromosomal *spoIIE* locus of wild-type strain PY79 by single, reciprocal (Campbell-like) recombination to create strain CW314. Integration of pCW28 created in the chromosome an intact *spoIIE* ORF that was joined in-frame to *gfp*. CW314 sporulates at nearly wild-type levels.
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21. KJP86 [*amyE::sspE(2G)-lacZ, spoIIIGΔ1*] is a derivative of strain PY79. *sspE(2G)* is a modified promoter that is recognized by σ^F-RNA polymerase [D. Sun *et al.*, *J. Bacteriol.* **173**, 7867 (1991)]. Similar results were obtained without *spoIIIGΔ1*, which inactivates σ^G, a sigma factor that also recognizes the *sspE(2G)* promoter.
22. The upper sporangium of the pair is an example of a class ii sporangium (it has unipolar SpoIIE and β-Gal), whereas the lower sporangium is intermediate between class i (it has bipolar SpoIIE) and class ii (it also has β-Gal).
23. The sporangia are frequently in chains or pairs with the mother cells adjoining one another. The scale bars are 5 μm. Sporulation was induced by the resuspension method [J. M. Sterlini and J. Mandelstam, *Biochem. J.* **113**, 29 (1969)]. Arrows in (A to F) point to forespores. The interpretative cartoons in (A, C, F, G, and I) illustrate the pattern of immunostaining of SpoIIE or β-Gal or both.
24. Immunofluorescence microscopy was done as described (*11*) with the following modifications. The bacteria were fixed with a final concentration of 2.6% paraformaldehyde and either 0.005% or 0.013% glutaradehyde, directly in resuspension medium, to which sodium phosphate buffer (pH 7.5) was added to a final concentration of 30 mM. The methanol/acetone treatment was omitted, and the slides were incubated at 4°C overnight with the primary antibodies. Antibodies to SpoIIE were produced in rabbits, affinity-purified as described (*10*), and used at a 1:5 dilution. Mouse antibodies directed against β-Gal were obtained from Promega and used at a 1:1500 dilution. For the simultaneous localization of SpoIIE and β-Gal in Fig. 1, A to F, the slides were incubated overnight in a mixture of these two antibodies, washed in phosphate-buffered saline (PBS), and then incubated for ~2 hours in a solution containing DAPI (0.2 mg/ml), and a mixture of two secondary antibodies (both at 0.75 mg/ml)—multiple-labeling grade, Cy3-conjugated goat antibodies to rabbit immunoglobulin G (IgG), and multiple-labeling grade, fluorescein-conjugated goat antibodies to mouse IgG (Jackson ImmunoResearch Laboratories). When only SpoIIE was localized (Fig. 1G), the slides were incubated overnight with antibodies to SpoIIE, washed, and incubated in a solution of fluorescein-conjugated goat antibodies to rabbit IgG (0.75 mg/ml) and propidium iodide (0.1 mg/ml). The samples were then washed with PBS containing propidium iodide (0.1 mg/ml).
25. Cells of strain KJP86, which contains *lacZ* fused to a promoter under the control of σ^F (*21*), were collected either 3 hours (A and B) or 2 hours (C and D) after the start of sporulation. Class i sporangia have bipolar staining of SpoIIE and no detectable β-Gal (examples of a single sporangium and a chain of four sporangia are indicated); class ii stain weakly for both SpoIIE and β-Gal (one forespore of a pair of sporangia is indicated); class iii have little or no detectable SpoIIE but stain strongly for β-Gal (two single sporangia are identified).
26. Cells of strain KJP123 [*spoIIIE::spc, amyE::sspE(2G)-lacZ, spoIIIGΔ1*], which contains a *spoIIIE::spc* null mutation (constructed by P. A. Levin) and *lacZ* fused to a promoter under the control of σ^F (*22*) were collected 2 hours after the start of sporulation (*23*) and processed for immunofluorescence microscopy (*24*).
27. In the two sporangia identified by arrows, β-Gal extends beyond SpoIIE, as demonstrated by the green region on the mother cell–distal side of the forespore compartment and the red region on the mother cell–proximal side of the forespore.
28. Cells of strain PL412 (*spoIIIE::spc*) were collected 2 hours after the start of sporulation and processed for immunofluorescence. The arrows indicate sporangia in which the propidium iodide–stained DNA (red) projects beyond SpoIIE (green). The open triangle indicates a sporangium with a ring of SpoIIE.
29. Indicated are a class i sporangium in which chromosome segregation is incomplete; a class iv disporic sporangium in which one forespore contains a completely segregated chromosome but the second does not; and a class v sporangium in which both forespore compartments contain a fully segregated chromosome. The long arrows point to forespores with a fully segregated chromosome, and the short arrows point to forespores with an incompletely segregated chromosome. The interpretative cartoons in (H) signify the presence of a forespore with a fully segregated chromosome (as inferred from the pattern of DNA staining) by a septal crossline.
30. The arrows point to two sporangia in which the forespore pole extends beyond SpoIIE-GFP. (M) contains many chains of sporangia; the circular pattern apparent in such cells results from the adjacent crescents of SpoIIE-GFP in neighboring sporangia, not from a SpoIIE ring in a single sporangium.

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