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Asymmetric Cell Division in *B. subtilis* Involves a Spiral-like Intermediate of the Cytokinetic Protein FtsZ

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Summary

A fundamental feature of development in the spore-forming bacterium *Bacillus subtilis* is the switch from medial to asymmetric division. The switch is brought about by a change in the location of the cytokinetic Z ring, which is composed of the tubulin-like protein FtsZ, from the cell middle to the poles during sporulation. We report that the medial Z ring is replaced by a spiral-like filament of FtsZ that grows along the long axis of the cell. We propose that the filament mediates the switch by redeploying FtsZ to the poles. Spiral formation and the switch to polar Z rings are largely caused by a sporulation-specific increase in transcription of the gene for FtsZ and activation of the gene for the FtsZ-associated protein SpoIIE.

Introduction

The establishment of cell type in development is often associated with a visibly asymmetric process of cell division (Horvitz and Herskowitz, 1992). Asymmetric division gives rise to dissimilar progeny that follow different pathways of differentiation. A primitive organism in which cell fate is closely tied to asymmetric division is spore formation in *Bacillus subtilis*. *B. subtilis* exhibits two modes of cell division. When growing vegetatively, the bacterium divides by forming a septum at the midcell (binary fission), which generates equal-sized progeny. However, upon entry into sporulation, the bacterium divides asymmetrically, switching the division site from the middle to a position close to one pole of the developing cell (or sporangium). Asymmetric division gives rise to unequal-sized progeny called the forespore (the small compartment) and the mother cell, which initially lie side-by-side in the sporangium. The forespore ultimately becomes the spore, whereas the mother cell is discarded by lysis when morphogenesis is complete. Here we address the question of how the site of cytokinesis switches from the medial to the polar position.

Cell division in bacteria is mediated by the tubulin-like protein FtsZ (Lutkenhaus and Addinall, 1997; Margolin, 2000). Molecules of FtsZ assemble into a ring, designated the Z ring, across the short axis of the cell at the future site of cell division. The Z ring directs division by recruiting other proteins that participate in septum formation, such as the actin-like protein FtsA and several integral membrane proteins (Lutkenhaus and Addinall, 1997; Margolin, 2000). The Z ring is normally found at the midcell in growing cells, but upon entry into sporulation, it switches its position to sites near both poles (Levin and Losick, 1996). The formation of polar Z rings is governed by Spo0A and σ^H, regulatory proteins that control entry into sporulation (Levin and Losick, 1996; this study). However, the identity of the targets of these transcription factors in effecting the switch is unknown. Whereas some evidence indicates that the FtsZ-associated protein SpoIIE is involved in the switch, a null mutant of *spoIIE* exhibits only a mild defect in polar septation (Arigoni et al., 1995; Barak and Youngman, 1996; Feucht et al., 1996; Levin et al., 1997; Khvorova et al., 1998; Lucet et al., 2000). Both polar Z rings have the potential to trigger cytokinesis, but a regulatory pathway involving three septation-inhibiting proteins ensures that only one Z ring is converted into a septum (Pogliano et al., 1999; Eichenberger et al., 2001; Piggot and Losick, 2001).

The discovery of a switch in the localization of FtsZ from the cell middle to the poles suggested a model for Z ring switching in which the formation of the medial Z ring is blocked and sites of FtsZ polymerization near the poles are activated (Levin and Losick, 1996; Barak et al., 1998). Here we provide evidence for an alternative model that involves a hitherto unrecognized, spiral-like assembly of FtsZ in sporulating cells. We propose that the spiral is a transient intermediate in the switch and that it is responsible for redeploying FtsZ from the cell middle to the poles. A key feature of our model is that formation of the medial Z ring is not blocked upon entry into sporulation. Rather, its formation is an initial step in the formation of polar Z rings via the spiral intermediate. Finally, we show that Spo0A and σ^H govern the switch from medial to bipolar Z rings and that they do so, at least in part, by activating *spoIIE* and stimulating the transcription of the operon that contains the gene for FtsZ.

Results

Spirals of FtsZ in Cells Undergoing Sporulation

To investigate Z ring switching, we monitored the subcellular localization of FtsZ using a fusion of GFP to the cell division protein. FtsZ-GFP is not fully functional, and cells that contain the *ftsZ-gfp* gene fusion in place of wild-type *ftsZ* are temperature sensitive (Levin et al., 1999). We therefore used a strain that harbored both unmodified *ftsZ* and a copy of the *ftsZ-gfp* fusion under the control of an inducible promoter. When grown in the presence of a low level of inducer (under which conditions, as judged by Western blot analysis, relatively little of the fusion protein was being produced [data not shown]), the merodiploid strain (JDB401) exhibited no measurable defect in growth or sporulation (Levin et al., 1999). When growing cells of JDB401 were examined by fluorescence microscopy, Z rings could be readily detected, and these were located at the midcell. This can be seen in image 1 of Figure 1A and in the overlay of image 1' in which green fluorescence from FtsZ-GFP was superimposed on red fluorescence from the mem-

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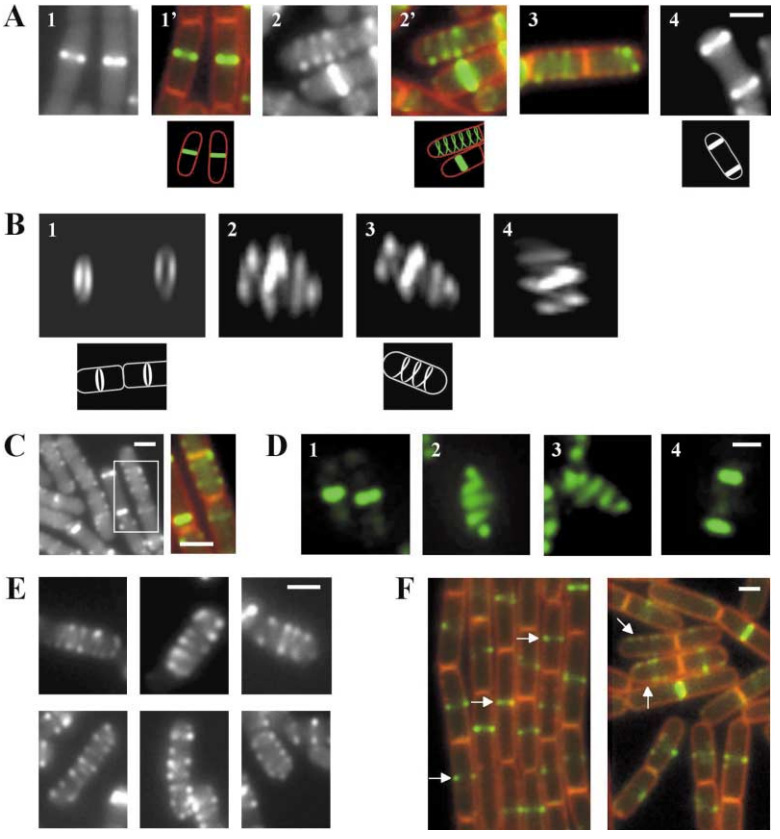

Figure 1. A Spiral-like Intermediate in Sporulation

(A) Fluorescence in cells of strain JDB401 (*amyE*::P_{spac}-*ftsZ-gfp*) in which the synthesis of FtsZ-GFP is under the control of an inducible promoter and was induced with a low concentration of inducer. Image 1 shows growing cells with medial Z rings. Image 2 shows a cell with a spiral (upper cell) at 90 min of sporulation (as well as a cell with a medial ring). Images 1' and 2' are overlays of the green fluorescence of images 1 and 2, respectively, with red fluorescence from the membrane stain FM4-64. The overlay of image 3 shows another example of cells with spirals at 90 min of sporulation. Image 4 shows a cell with bipolar Z rings at 120 min of sporulation. Cartoon interpretations are shown below images 1', 2', and 4.

(B) Three-dimensional images of FtsZ-GFP in cells of JDB401 reconstructed by deconvolution microscopy (see Experimental Procedures). Image 1 shows medial Z rings in two adjacent growing cells. Images 2–4 show three different spiral-like structures in cells at 90 min of sporulation. Cartoon interpretations are shown below images 1 and 3.

(C) Fluorescent spiral in cells of strain SB169 (*amyE*::*ftsAZ-gfp*) at 90 min of sporulation in which the synthesis of FtsZ-GFP is under the control of the wild-type promoter for the *ftsAZ* operon. The right-hand image is a magnification of the framed region (green) in the left panel, overlayed with staining from FM4-64 (red).

(D) Immunofluorescence staining of fixed cells of strain PY79 (wild-type) using anti-FtsZ antibodies. Image 1 shows growing cells with medial Z ring. Images 2 and 3 show cells with spiral-like structures at 90 min of sporulation. Image 4 shows a cell with bipolar Z rings at 120 min of sporulation.

(E) Fluorescent spiral-like structures in cells of the FtsA-GFP-producing strain SB168 at 90 min of sporulation.

(F) Overlays of the green fluorescence from cells of the EzrA-GFP-producing strain SB127 (*ezrA-gfp*) with red fluorescence from the membrane stain FM4-64 at 30 min (left) and at 90 min (right) of sporulation. The arrows point to medial rings of EzrA-GFP in the 30-min time point and spiral-like structures in the 90-min time point.

The scale bars correspond to 1 μm.

brane stain FM4-64, which demarcates the boundaries of the cell.

We then examined fluorescence from FtsZ-GFP in cells that had entered sporulation. Many sporangia had a Z ring at one or both poles, a characteristic of cells that are about to undergo polar division (Levin and Losick, 1996). An example of bipolar Z rings is shown in image 4 of Figure 1A. In addition, however, we readily observed sporangia in which FtsZ-GFP had assembled into an elongated filament that extended lengthwise across the cell. Two examples are displayed in images 2, 2', and 3 of Figure 1A. Because of their zigzag-like appearance, the images appear to represent spiral-like filaments of FtsZ-GFP. Spiral-like filaments were also observed in sporulating cells of a merodiploid strain (SB169) in which the gene fusion was under the control of the normal promoter for the *ftsAZ* operon (rather than the inducible promoter; Figure 1C).

Next, we carried out deconvolution microscopy to help clarify the three-dimensional nature of the spiral-like structures. Stacks of optical sections were collected, and a deconvolution program was used to reconstruct three-dimensional images. Image 1 of Figure 1B shows two reconstructed images of Z rings from growing cells, which can be seen to be circular. Images 2–4 (Figure 1B) show reconstructions of the structures observed in sporulating cells, which appear to be spirals with 3–3.5 helical turns. In other examples we frequently observed as many as 7–9 turns.

Do elongated filaments of FtsZ arise normally during sporulation, or are they a consequence of fusing GFP to FtsZ? It seemed unlikely that the spirals were an aberrant feature of the fusion protein, because very few such elongated filaments were observed during vegetative growth (see below). Nonetheless, to address this issue further, we carried out immunostaining using anti-FtsZ antibodies on cells producing unmodified, wild-type FtsZ. As expected, we were able to detect medial Z rings during vegetative growth as well as bipolar rings during sporulation (Figure 1D, images 1 and 4). In addition, we observed curlicue-like structures during sporulation that, at least in some cases, appeared to correspond to the spiral-like structures we had observed with FtsZ-GFP (taking into account the lower limits of resolution of immunostaining; Figure 1D, images 2 and 3). Moreover, the appearance of these curlicues correlated in time of appearance with the spirals observed with FtsZ-GFP (see below).

FtsZ-Associated Proteins Also Form Spirals

To examine whether spiral formation during sporulation was unique to FtsZ and as a further test of the possibility that spirals were an idiosyncratic feature of FtsZ-GFP, we used fusions to GFP to visualize two other cell division proteins, FtsA and EzrA. FtsA is a cytokinetic protein that colocalizes with FtsZ in a FtsZ-dependent manner (Ma et al., 1996, 1997; Addinall and Lutkenhaus, 1996a; Feucht et al., 2001). We constructed a merodiploid strain bearing *gfp* fused to a copy of *ftsA* under the control of its normal promoter. (Cells harboring *ftsA-gfp* and lacking an unmodified copy of *ftsA* are impaired in growth and sporulation; data not shown). The images with FtsA-GFP were not as bright as those obtained with FtsZ-GFP. Nonetheless, we readily observed zigzag-like assemblies of FtsA-GFP that extended a considerable distance along the long axis of the sporangium, several examples of which are shown in Figure1E.

EzrA is a dispensable component of the *B. subtilis* septasome and is known to form a ring-like structure that colocalizes with FtsZ (Levin et al., 1999). We used a strain (SB127) in which *ezrA-gfp* was transcribed from its normal promoter (Levin et al., 1999). EzrA-GFP is functional, and the *ezrA-gfp* gene fusion was present in SB127 in place of the wild-type gene. Figure 1F (left) shows that shortly (30 min) after the start of sporulation, EzrA-GFP was principally present in the form of rings (which appear as lines across the short axis of the cells) and that these rings were located at a midcell position. However, by 90 min, EzrA-GFP was often present in the form of apparently helical structures that extended to a greater or lesser extent across the long axis of the cells (Figure 1F, right). Thus, spiral-like filaments were observed during sporulation for FtsZ-GFP as well as for two FtsZ-associated proteins.

Spirals Are Intermediates in the Transition from Medial to Polar Z Rings

Time course and time lapse experiments were carried out to investigate the timing of the appearance of spirals and to ask whether their formation preceded the appearance of polar Z rings. In the time course analysis, samples were collected at 30, 60, 90, and 120 min after suspension of the cells in sporulation medium at 30°C. Thirty minutes after suspension, most of the cells exhibited medial Z rings (Figure 2). At 60 min, however, many of the cells exhibited nascent spirals, which had an N-like appearance and were located at the midcell position. By 90 min after suspension, mature spiral structures could be seen that extended along the long axis of the cells. Finally, at 120 min, bipolar and unipolar Z rings could be observed (yellow arrows) as well as polar septa (green arrows). Quantitation indicated that the percentage of cells exhibiting spiral forms increased sharply between 30 min (6%) and 90 min (67%), a time at which only 7% of the cells exhibited at least one polar Z ring (Table 1). Meanwhile, the percentage of cells with medial Z rings decreased between 30 and 90 min.

Similar results were obtained with EzrA-GFP. As noted above, sporulating cells principally exhibited medial rings of EzrA-GFP at 30 min after suspension (Figure 1F, left), but by 90 min, spiral structures were readily observed (Figure 1F, right). The results of quantitative analysis showed that the percentage of cells with spirals of EzrA-GFP increased from 2% at 30 min to 63% at 90 min (data not shown). Thus, with both FtsZ-GFP and EzrA-GFP, sporangia with spirals appeared to represent an intermediate stage of sporulation between cells with medial Z rings and cells with polar Z rings.

The simplest interpretation of these results is that spirals arise from medial Z rings and are an intermediate in the redistribution of FtsZ from the midcell toward the poles, where FtsZ assembles into polar rings. As a test of this hypothesis, we carried out time lapse microscopy to follow the fate of FtsZ structures in individual sporulating cells. FtsZ-GFP-producing cells were collected after the start of sporulation, applied to an agarose pad on a glass slide at room temperature, and viewed at intervals. Figures 3A and 3B show representative fields displaying cells in which FtsZ-GFP was present as medial Z rings at the first time point. It can be seen that over time the medial rings were replaced with spirals that grew out from the cell middle toward the poles. In some cases the spiral grew symmetrically, extending toward both poles of the cells (for example, the bottom cell in Figure 3A), whereas in other cases an asymmetrical pattern was observed (Figure 3B). In the latter example, a unipolar Z ring was observed at late time points in the same half of the cell in which the spiral had formed. The time-lapse experiment of Figure 3C shows an example of a sporangium in which the medial Z ring, which was present at the earliest time point, was replaced with two short polar spirals (presumably through an intermediate stage in which a single spiral extended across the cell), which were ultimately replaced by mature polar Z rings (by the final time point).

Taken together, the results are consistent with a model in which medial Z rings are a precursor in the formation of polar Z rings. Rather than causing cytokinesis, the medial Z ring undergoes remodeling so as to form spiral structures that extend to one or both poles of the cell. These spiral structures could serve to redistribute (presumably through depolymerization and repolymerization) FtsZ from the midcell to the poles so as to create polar Z rings. Finally, one of the two polar Z rings (perhaps the first to form or the one having the greatest concentration of FtsZ) allows cytokinesis to take place, resulting in the formation of a polar septum.

Spirals Are Intermediates in the Transition from Polar to Medial Z Rings

Sporulating cells do not become committed to spore formation until, or after, polar division (Parker et al., 1996). We took advantage of this reversibility to ask whether polar Z rings would be converted back to medial Z rings when cells at an early stage of sporulation were transferred to growth medium. If so, would the spiral be an intermediate in the reappearance of medial rings? To address these questions, FtsZ-GFP-producing cells were collected at 120 min after the start of sporulation and transferred to growth medium.

As seen in Supplemental Figure S1 (see Supplemental Data at http://www.cell.com/cgi/content/full/109/2/257/DC1) and in quantitative analysis (data not shown), most of the cells displayed spirals (63%) or polar Z rings

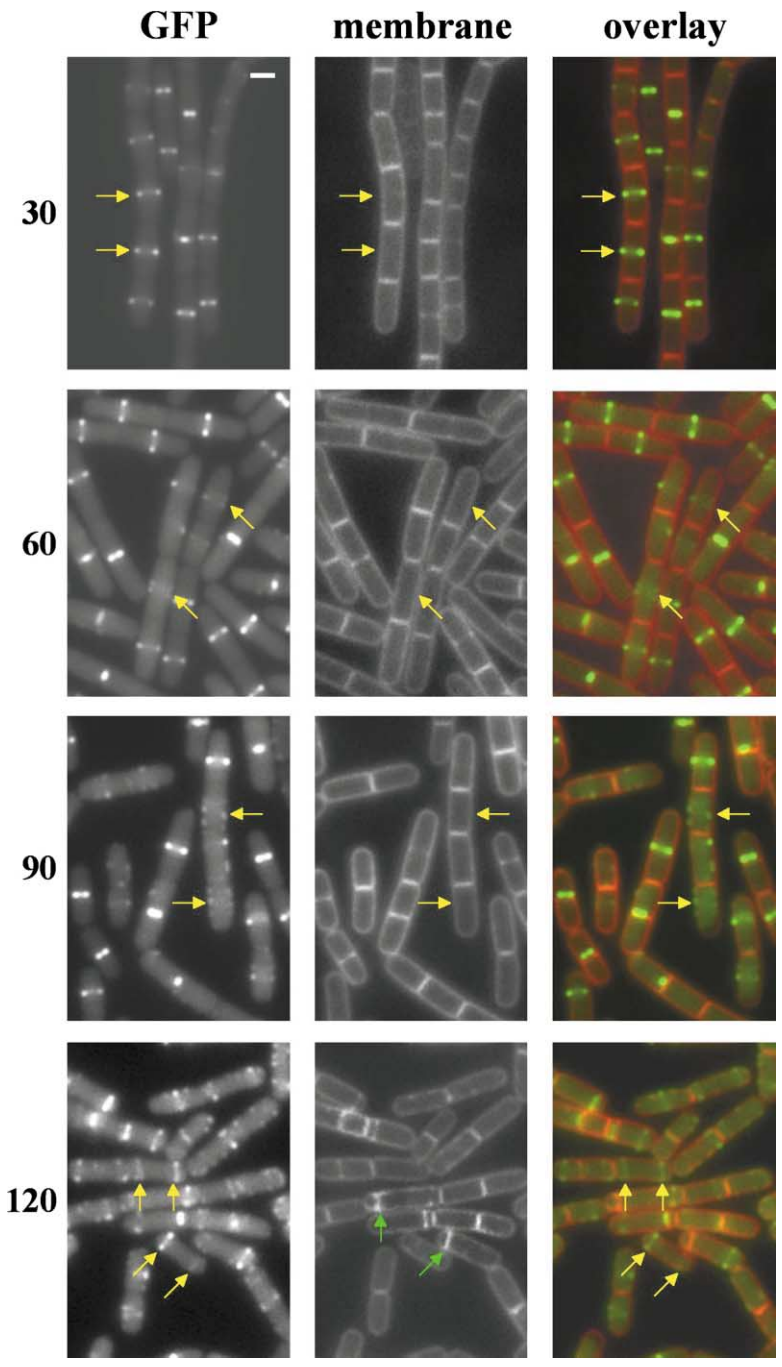

Figure 2. Time Course Microscopy with FtsZ-GFP-Producing Cells

Fluorescence microscopy was carried out on cells of strain JDB401 (*amyE*::P_{spac}-*ftsZ-gfp*) at the indicated times (in minutes) of sporulation. The columns show, as indicated, fluorescence from FtsZ-GFP, fluorescence from the membrane stain FM4-64, and an overlay of the two. The yellow arrows indicate the positions of medial Z rings (30 min), nascent spirals (60 min), mature spirals (90 min), and polar Z rings (120 min). The green arrows in the 120-min time point (center image) identify polar septa. The scale bar corresponds to 1 μm.

(25%) just after the time of the transfer to growth medium (time 0). A similar pattern was still evident 15 min later, when the percentage of sporangia with spirals was 59% and the percentage with polar Z rings was 25%. However, by 30 min after transfer, the percentage of sporangia with polar Z rings had decreased to 3%, and the percentage with spirals had increased to 75%. By 45 min after transfer to growth medium, most of the cells (72%) had medial Z rings, the percentage with spirals had decreased to 26%, and only 2% exhibited polar Z rings. Finally, by 60 min (when the cells had just started to resume growth as judged by the optical density of the culture), 80% of the cells exhibited medial Z rings, 19% showed spiral structures, and only 1% had polar rings. The decrease in the percentage of polar Z rings could not be attributed to their conversion into polar septa because the percentage of sporangia with polar septa (15%) did not measurably increase over the course of the experiment. Rather, the simplest interpretation of these findings is that the polar Z rings were being converted into medial Z rings and that spirals were an intermediate in the conversion. This interpretation is supported by time lapse experiments in which we were able to follow the conversion of polar Z rings into spiral structures and finally to medial rings in the same sporangium over time (data not shown).

Table 1. Time Course of Changes in the Relative Abundance of rings and Spirals during Sporulation

Time (min)	Medial	Spiral	Polar
30	94	6	0
60	79	21	0
90	26	67	7
120	12	63	25

Cells of FtsZ-GFP-producing strain JDB401 (*amyE*::P_{spac}-*ftsZ-gfp*) that had been grown in the presence of a low concentration of inducer (see Experimental Procedures) were visualized by fluorescence microscopy at the indicated times after the start of sporulation. The cells were scored for the presence of medial Z rings, spirals, and polar Z rings by visualizing fluorescence from GFP and by superimposing the GFP images on corresponding fluorescent images from the membrane stain FM4-64. The "polar" column includes cells with either unipolar or bipolar Z rings. At least 250 cells were scored for each time point, with the numbers indicating the percentage of the total represented by the indicated structure.

The Formation of Spirals Is Impaired in Mutants Blocked at the Start of Sporulation

The master regulator for entry into sporulation is Spo0A, mutants of which are blocked both in the formation of polar septa and in the formation of polar Z rings (Dunn et al., 1976; Levin and Losick, 1996). As expected, *spo0A* mutant cells that had been suspended in sporulation medium exhibited medial Z rings and few, if any, polar Z rings (see Supplemental Figure S2 at http://www.cell.com/cgi/content/full/109/2/257/DC1). We were able to detect a small number of spirals in the mutant, but they were less prominent and shorter than those observed in the wild-type. Another regulator for entry into sporulation is σ^H, which is encoded by *spo0H*. Mutants of σ^H are blocked in sporulation prior to the formation of the polar septum. It was previously reported that *spo0H* mutant cells display bipolar Z rings, similar to the pattern observed in wild-type sporangia (Levin and Losick, 1996). However, we found that FtsZ-GFP was almost entirely localized in medial Z rings in a *spo0H* null mutant. This was true both at 120 min after the start of sporulation (see Supplemental Figure S1) and as late as 180 min (data not shown). Similar results were obtained using EzrA-GFP fusion and in immunoflourescence experiments using anti-FtsZ antibodies with cells producing unmodified, wild-type FtsZ (data not shown). Whereas *spo0H* produced almost no polar Z rings, we did observe a low level of spiral formation in the mutant, but at later times and in lower abundance than in the wild-type (data not shown). We conclude that both Spo0A and σ^H are required for the efficient formation of spirals as well as for the formation of polar Z rings.

Identification of the Targets of Spo0A and σ^H that Mediate the Formation of Polar Septa and Polar Z Rings

What are the targets of Spo0A and σ^H that are responsible for the switch to polar division? One candidate is *spoIIE*, which is under Spo0A control. The product of *spoIIE* colocalizes with FtsZ in a FtsZ-dependent manner (Levin et al., 1997), forming polar "E rings" (Arigoni et al., 1995; King et al., 1999). Indeed, *spoIIE* mutations are known to delay polar septation during sporulation and to prevent polar septation during growth in cells engineered to produce a constitutively active form of Spo0A (Barak and Youngman, 1996; Feucht et al., 1996; Khvorova et al., 1998). Nonetheless, under conditions of sporulation, a substantial proportion of *spoIIE* mutant cells are eventually capable of forming polar septa (Barak and Youngman, 1996; Feucht et al., 1996). Likewise, an attractive candidate for the target of σ^H is the P2 promoter of the *ftsAZ* operon, which contains the genes for FtsA and FtsZ. The *ftsAZ* operon is transcribed from two σ^A-controlled promoters and a σ^H-controlled promoter (P2), which boosts transcription of the operon

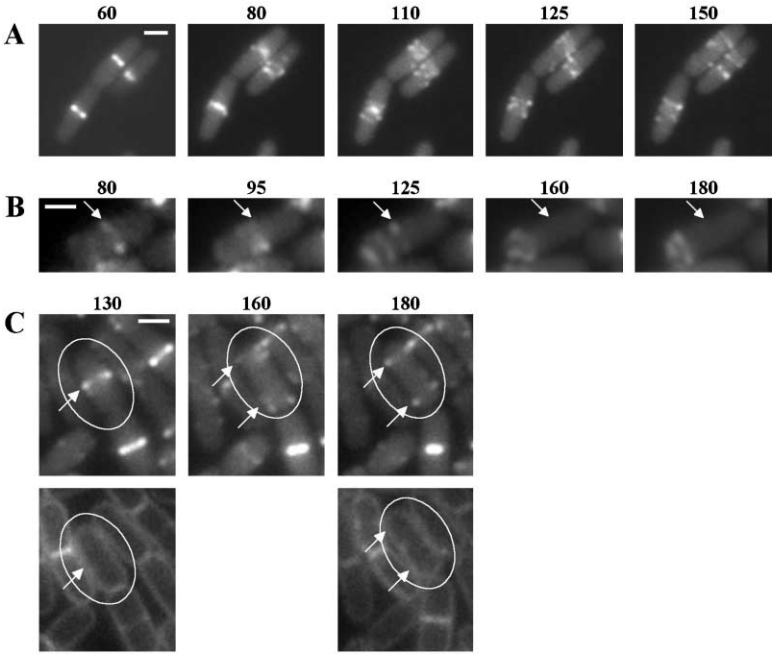

Figure 3. Time Lapse Microscopy with FtsZ-GFP-Producing Cells

The same field of cells of strain JDB401 (*amyE*::P_{spac}-*ftsZ-gfp)* was visualized by fluorescence microscopy at the indicated times (in minutes) of sporulation at room temperature (see Experimental Procedures).

(A) Conversion of medial Z rings into nascent spirals.

(B) Replacement of a medial Z ring with a nascent spiral, which elongates asymmetrically into a mature spiral and then into a unipolar Z ring. Note that because of the angle at which the image was taken, the polar ring looks almost circular. The arrows indicate the position of the medial Z ring observed at the first time point.

(C) Conversion of a medial Z ring into two polar Z rings. The upper images show fluorescence from FtsZ-GFP, whereas the lower images show fluorescence from FM4-64 of the same cell (highlighted by the ovals) at the same time point. The arrows indicate the position of the FtsZ-GFP. Notice from the FM4-64 staining that the medial Z ring was not converted into a medial septum.

The scale bars correspond to 1 μm.

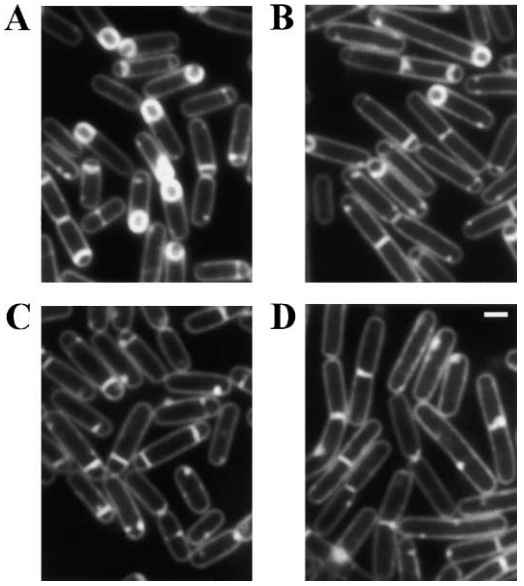

Figure 4. A Double Mutant of *spoIIE* and the Sporulation Promoter for the *ftsAZ* Operon Is Blocked in Polar Septation

Cells of strains FG17 ([A] *spoIIE*$^+$ *ftsAZ*$^+$), SB206 ([B] *spoIIE*$^+$, P2$_{ftsAZ::Xmnl}$-*ftsAZ*), SB120 ([C] *spoIIE*Δ::*kan ftsAZ*$^+$), and SB128 ([D] P2$_{ftsAZ::Xmnl}$-*ftsAZ, spoIIE*Δ::*kan)* were visualized with the membrane stain FM4-64 at 180 min of sporulation at 37°C. The scale bar corresponds to 1 μm.

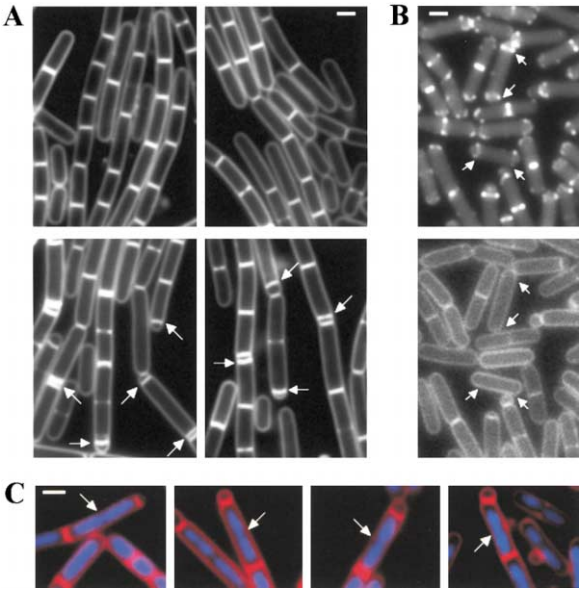

Figure 5. Polar Division and Polar Z Ring Formation in Growing Cells Expressing *spoIIE* and Harboring an Extra Copy of *ftsAZ*

(A) Cells of strain SB161 (*amyE*::*ftsAZ* P$_{xyl}$-*spoIIE*) were grown in CH medium at 30°C in the presence (bottom) or absence (upper) of inducer (0.5% xylose). The cells were visualized with the membrane stain FM1-43 at 180 min after the addition of inducer (OD$_{600}$~0.5). The arrows identify polar septa.

(B) Cells of the FtsZ-GFP-producing strain SB185 (*amyE*::*ftsAZ-gfp* P$_{xyl}$-*spoIIE*) were grown in the presence of inducer (0.5% xylose). Fluorescence from FtsZ-GFP is shown in the upper image, and fluorescence from the membrane stain FM4-64 is shown in the lower image at 180 min after the addition of inducer. Arrows show the positions of polar Z rings.

(C) Examples of cells of strain SB161 in which staining of DNA with DAPI (blue) was superimposed with fluorescence from the membrane stain FM4-64 (red). Growth conditions were as described for (A), and the cells were visualized at 180 min after the addition of the inducer. The arrows show the boundary between nucleoids. The scale bars correspond to 1 μm.

3- to 4-fold at the onset of sporulation (Gholamhoseinian et al., 1992; Gonzy-Treboul et al., 1992) as well as the FtsZ levels (as demonstrated by Western blot analysis; data not shown). However, cells mutant for the P2 promoter are capable of sporulation, albeit at a 3-fold reduced efficiency. Thus, neither *spoIIE* alone nor the P2 promoter for *ftsAZ* alone provides a sufficient explanation for the tight requirement for Spo0A and σ^H in asymmetric division.

We reasoned, however, that induction of *spoIIE* and enhanced transcription of *ftsAZ* might be partially redundant pathways for effecting the switch to polar division. In support of this idea, a *spoIIE* P2$_{ftsAZ}$ double mutant exhibited a strong defect in asymmetric division, with only 0.6% of the sporangia producing polar septa by 180 min of sporulation (Figure 4D; data not shown). For comparison, cells singly mutant for *spoIIE* produced polar septa at efficiency of 30% at the same time (Figure 4C; data not shown). Likewise, 37% of the cells singly mutant for P2$_{ftsAZ}$ displayed polar septa or reached a later stage of sporulation (Figure 4B; data not shown). At the same time point, 55% of the wild-type cells had reached the stage of polar septation or beyond (Figure 4A; data not shown). Sporulating cells of the double mutant not only exhibited a strong defect in septation but also were impaired in the formation of spirals and produced few polar Z rings (data not shown).

Polar Division and Polar Z Ring Formation in Vegetative Cells Harboring an Extra Copy of the *ftsAZ* Operon and Expressing *spoIIE*

We infer that the formation of polar Z rings results, at least in part, from enhanced synthesis of FtsZ and the appearance of SpoIIE. As a test of whether elevated FtsZ levels and SpoIIE are sufficient to explain the switch to polar septation, we asked whether cells engineered to produce SpoIIE in response to an inducer and harboring an extra copy of the *ftsAZ* operon would undergo asymmetric division under conditions of vegetative growth. Growing cells of the wild-type strain (PY79) showed only very low levels of polar septation (<0.2%; see Supplemental Table S1 at http://www.cell.com/cgi/content/full/109/2/257/DC1). Likewise, growing cells of a strain (SB152) harboring an inducible copy of *spoIIE* showed little or no increase in polar septation upon the addition of inducer (~0.5%; see Supplemental Table S1). A somewhat higher (but still modest) level of polar septation was observed in growing cells of a strain (SB150) harboring an extra copy of the *ftsAZ* operon (~2%; see Supplemental Table S1). However, growing cells of a strain (SB161) that both harbored an inducible copy of *spoIIE* and an extra copy of the *ftsAZ* operon exhibited a substantial level of polar septation (20%–27%) and did so in a manner that was dependent upon the presence of inducer (Figure 5A; see Supplemental Table S1). (Sometimes the small compartment created

by polar division was pinched off as a minicell.) We obtained similar results with a strain bearing a *spo0A* null mutation (data not shown), indicating that no Spo0A-controlled gene other than *spoIIE* was required for polar septation.

DAPI staining of SB161 frequently revealed two nucleoids in the same cell in which a polar septum was observed (Figure 5C). These results suggest that polar division was occurring in cells in which medial septation (which ordinarily is expected to occur between nucleoids) had not taken place. Evidently, then, induction of SpoIIE synthesis in growing cells of SB161 is frequently able to bring about a switch from medial to polar division. At the same time, the results with DAPI staining revealed the presence of little or no DNA in the minicell-like chamber generated as a result of polar division. Thus, growing cells of SB161 are able to mimic the switch to asymmetric division observed during sporulation but not the chromosome translocation process that is observed during development.

Finally, we investigated the localization of Z rings in FtsZ-GFP-producing cells that had been engineered to generate polar septa during growth. For this purpose, we used a strain (SB185) that harbored a wild-type copy of the *ftsAZ* operon, a second copy of the operon in which *gfp* was fused to *ftsZ*, and an inducible copy of *spoIIE*. We were able to detect spirals in mid-exponential phase cells of this strain even without the addition of inducer but few polar Z rings (data not shown). However, when SpoIIE synthesis was induced, unipolar and bipolar Z rings were readily detected (Figure 5B). Analogous experiments revealed the presence of polar and bipolar E rings in growing cells of a SpoIIE-GFP-producing strain that harbored an extra copy of the *ftsAZ* operon and an inducible copy of *spoIIE-gfp* (data not shown). The formation of polar E rings was dependent upon the presence of the extra copy of *ftsAZ*, because the E rings were found at a medial position in cells of a strain that harbored a single copy of *ftsAZ* (data not shown). These findings are consistent with the idea that enhanced levels of FtsZ (and/or FtsA) and the presence of SpoIIE are able to bring about a switch to polar Z ring and E ring formation.

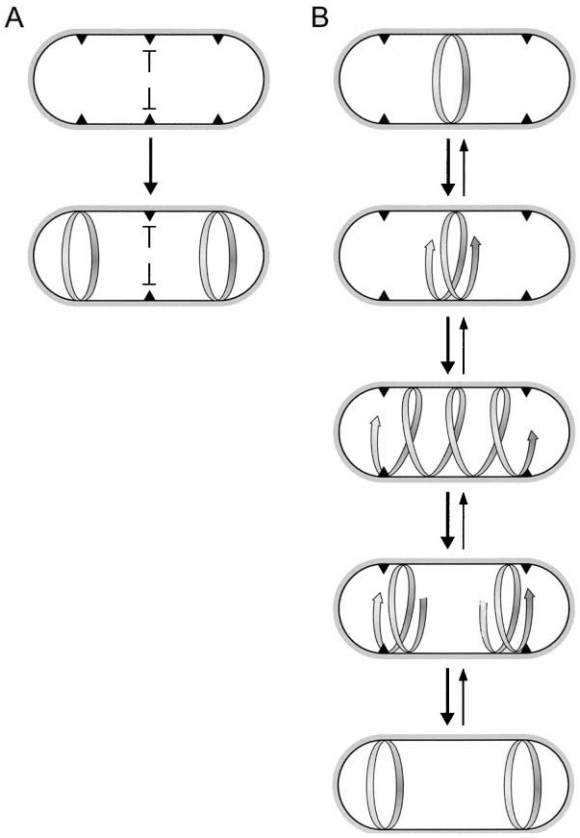

Figure 6. Z Ring Switching during Sporulation

(A) A previously proposed model for Z ring switching in which the cell has potential division sites (filled triangles) at the midcell position and near both poles. Upon entry into sporulation, formation of a Z ring at the midcell position is blocked, and polar sites of Z ring formation are activated.

(B) A new model for Z ring switching in which a Z ring is formed at the midcell position as an initial step in the switch. Next, the medial Z ring is converted into a spiral, which grows transversely toward both poles. After elongating, the spiral splits into two polar spirals, each of which eventually coalesces into a polar Z ring. For simplicity, the medial Z ring is shown as undergoing conversion into a single long spiral, but it is equally possible that two separate spirals grow out from the midcell position. Also for simplicity, the spiral is shown as being symmetric, but the spiral is frequently seen as growing faster in one direction than the other (see the text). The arrows are intended to convey that the sequence is reversible and that polar Z ring can undergo conversion back to a medial Z ring via a spiral intermediate in sporulating cells that have been transferred to growth medium.

Discussion

Asymmetric division is mediated by a switch in the localization of FtsZ from the midcell to positions near both poles of the developing cell (Levin and Losick, 1996). It is generally assumed that this switch occurs by inhibition of FtsZ polymerization at the cell middle and activation of latent polymerization sites near the poles (e.g., Levin and Losick, 1996; Barak et al., 1998; Figure 6A). Here we present evidence for a new model for Z ring switching (Figure 6B), involving a hitherto unrecognized sporulation-specific intermediate in the formation of polar Z rings. This intermediate is a spiral-like filament that is composed of FtsZ and at least two other FtsZ-associated proteins. The spiral appears to arise from a medial Z ring and grows progressively toward the cell poles, where it is eventually replaced by polar Z rings. Sometimes, as depicted in Figure 6B, the spiral splits in two before the polar Z rings are formed. A key feature of our model is that formation of the medial Z ring is not suppressed during sporulation. Rather, sporulating cells do form a medial Z ring, but this medial Z ring does not mature into a cytokinetic structure. Instead, it is converted into a spiral, which we propose helps to redistribute FtsZ from the cell middle to sites near the ends of the cell. While favoring the model of Figure 6B, we cannot exclude an alternative possibility in which the medial ring is disassembled (or degraded) and the spiral is entirely created de novo from the cytoplasmic pool of unpolymerized FtsZ molecules.

Evidence that spiral formation is an intermediate stage in the transition from medial to polar Z rings came from

time course and time lapse experiments. These experiments showed that cells with spirals appeared at an intermediate time between cells with medial Z rings and cells with polar Z rings. Furthermore, time lapse microscopy directly showed that medial rings are replaced by spirals and that spirals are, in turn, replaced by polar Z rings. Complementary evidence for a spiral intermediate came from the discovery that sporulating cells that have formed polar Z rings but have not yet undergone polar division can revert to the formation of medial Z rings when shifted to rich medium. This transition was accompanied by the formation of spirals, once again representing an intermediate step between polar and medial Z rings. Thus, not only is the spiral involved in the replacement of medial rings with polar rings, but also the reverse. A simple interpretation of these results is that the spiral is responsible for redeploying FtsZ both from the middle to the poles and, upon a nutrient shift, back again. Verification of this model will require a direct demonstration that FtsZ molecules present in the medial Z ring contribute to the formation of polar Z rings and visa versa.

Spirals of FtsZ and FtsA have been observed in cells of *E. coli* that have been engineered to overexpress *ftsZ* (Ma et al., 1996). This is, of course, an artificial circumstance, but these experiments show that even in a distantly related bacterium, FtsZ and FtsA are capable of polymerizing into spiral structures. Spirals of FtsZ have also been observed in *E. coli* cells producing a mutant form (FtsZ26) of the cytokinetic protein (Addinall and Lutkenhaus, 1996b) and in cells deprived of phosphatidylethanolamine (Mileykovskaya et al., 1998). FtsZ spirals were also observed in *B. subtilis* cells mutant for the cell shape control gene *mbl* (Jones et al., 2001). Moreover, FtsZ, which like tubulin is a GTPase, assembles into curved and spiral structures in vitro when polymerization is carried out in the presence of GDP in place of GTP (Erickson, 1997, 1998, 2000; Lu et al., 2000, 2001). Evidently the capacity to assemble into spiral-like structures is an intrinsic property of FtsZ, which can be revealed under a variety of circumstances. (Conceivably, even Z rings are actually tight spirals that can expand under conditions of high FtsZ levels.) Whereas spirals have previously been observed only under abnormal conditions, we propose that the process of sporulation has exploited this property of FtsZ as a mechanism for switching from medial to polar Z rings.

The switch to polar division is known to be under the control of Spo0A and σ^H. Indeed, cells engineered to produce an activated form of Spo0A during growth produce polar septa when growing (Levin and Losick, 1996). An important challenge has been to identify the targets of Spo0A and σ^H that are responsible for the switch. One attractive candidate is *spoIIE*, which encodes a FtsZ-associated protein whose synthesis is activated by Spo0A (York et al., 1992; Arigoni et al., 1995; Levin et al., 1997; Hatt and Youngman, 1998; Lucet et al., 2000). Supporting the idea that *spoIIE* is involved in the switch are the findings of Khvorova et al. (1998), who showed that polar Z rings formation is markedly impaired in a *spoIIE* mutant engineered to produce activated Spo0A during growth. A second candidate is the P2 promoter for the *ftsAZ* operon, which helps to boost FtsZ levels during sporulation (Gholamhoseinian et al., 1992; Gonzy-Treboul et al., 1992; Feucht et al., 2001; data not shown).

Nonetheless, the contribution of *spoIIE* and *ftsAZ* P2 to polar Z ring formation has been uncertain because cells singly mutant for one or the other are only mildly impaired in asymmetric division during sporulation (Gonzy-Treboul et al., 1992; Barak and Youngman, 1996; Feucht et al., 1996; this study). A significant contribution of the present work is the discovery that a double mutant of *spoIIE* and the P2 promoter is severely impaired in polar Z ring formation and polar division. Evidently, SpoIIE and elevated levels of FtsZ (and/or FtsA) play important roles in effecting the switch to polar Z ring formation, but each partially masks the contribution of the other. Only when both are eliminated is a severe defect in polar ring formation and polar division observed. Conversely, increasing FtsZ and FtsA levels in growing cells (through the presence of an extra copy of the *ftsAZ* operon) and artificially inducing the synthesis of SpoIIE causes the formation of polar Z rings, polar E rings, and polar septa that closely resemble those of cells undergoing sporulation. Thus, elevated levels of FtsZ (and/or FtsA) and induction of SpoIIE could be sufficient to explain the roles of Spo0A and σ^H in effecting the switch to polar Z ring formation. At the same time, we do not exclude the possibility that additional genes whose transcription is under sporulation control contribute to the switch, but if so, their effect is masked by the contributions of *ftsAZ* and *spoIIE*.

The discovery that the P2 promoter for the *ftsAZ* operon contributes to the switch dovetails with the findings of Ma et al. (1996) who, as discussed above, observed that overexpression of *ftsZ* causes the formation of spirals in *E. coli*. We propose that heightened accumulation of FtsZ during sporulation contributes to the formation of spirals, which in turn bring about the switch to polar Z ring formation. Transcriptional regulation of *ftsZ* is also observed in other differentiating bacteria. Transcription of *ftsZ* is subject to cell cycle control in the dimorphic bacterium *Caulobacter crescentus* (Kelly et al., 1998) and to developmental control in the filamentous bacterium *Streptomyces* in which FtsZ assembles into ladder-like structures during the process of aerial mycelium formation (Schwedock et al., 1997; Flardh et al., 2000; Kwak et al., 2001).

The contribution of SpoIIE to polar Z ring formation is unclear beyond the obvious fact that it is a FtsZ-associated protein (Levin et al., 1997; Lucet et al., 2000). In localization experiments, we were able to observe SpoIIE-GFP in a spiral-like structure prior to the formation of polar E rings (data not shown). Thus, SpoIIE may be a component of the spiral and may facilitate its formation (perhaps by helping it to compete against MinCD, an inhibitor of FtsZ polymerization [Lutkenhaus and Addinall, 1997; Margolin, 2000]). It may be noteworthy that SpoIIE is an integral membrane protein. Conceivably, SpoIIE contributes to the formation of polar Z rings by anchoring the spiral along the entire inner surface of the cell membrane. Also, SpoIIE might help to specify the precise site at which polar Z ring formation takes place, perhaps through the recognition of unknown topological marks near the ends of the cell.

Finally, we come to the question of how septation is confined to one pole even though Z rings are frequently

observed at both poles. In wild-type cells, the formation of a septum at one pole of the sporangium triggers a pathway that blocks cytokinesis at the other pole (Stragier and Losick, 1996; Pogliano et al., 1999; Eichenberger et al., 2001). In mutants in which this pathway is abrogated, both polar Z rings are converted into a septum (Piggot and Coote, 1976). Evidence indicates, however, that septum formation takes place more rapidly at one pole than the other (Lewis et al., 1994; Pogliano et al., 1999). Thus, both polar Z rings are potential division sites, but the formation of a septum at one pole blocks the formation of a septum at the distal pole. The key question, therefore, is one of timing: why does division take place asynchronously rather than at both poles simultaneously? As noted above, the spiral intermediates we observed are frequently asymmetric, being longer and thicker in one half of the cell than the other. If, as we propose, spirals are responsible for redeploying FtsZ to the cell poles, then perhaps this asymmetry allows cytokinetic proteins to accumulate to a threshold level more rapidly at one end of the cell than the other. If so, future investigations into the structure of the spiral and the mechanism of its formation might provide insights into the underlying basis for the generation of cellular asymmetry.

Experimental Procedures

Strains

Strains were derivatives of PY79 (Youngman et al., 1984) and are listed in Supplemental Table S2 (http://www.cell.com/cgi/content/full/109/2/257/DC1). Plasmid constructions are described in the Supplemental Data.

General Methods

Cells of *B. subtilis* were grown in hydrolyzed casein (CH) growth medium unless indicated otherwise. The cultures were inoculated at an OD_{600} of 0.05 from an overnight culture in the same medium. Sporulation was induced by transferring cells growing in CH medium to the resuspension medium of Sterlini and Mandelstam (Sterlini and Mandelstam, 1969; Harwood and Cutting, 1990). Growth and sporulation were carried out at 30°C unless indicated otherwise. Time of sporulation was measured from the time of suspension in sporulation medium. Sporulation efficiency was determined as described previously (Rudner et al., 1999). For experiments in which sporulation was reversed by transfer to growth medium, cells at 120 min of sporulation were transferred to CH medium by centrifugation and suspension. For induction of the P_{Xyl}-*spoIIE* construct, the final xylose concentration was 0.5%. For induction of the P_{spac}-*ftsZ-gfp* construct, the final IPTG concentration was 5 μM (Levin et al., 1999).

Competent cells were prepared as described previously (Dubnau and Davidoff-Abelson, 1971; Sambrook et al., 1989). Genomic DNA was extracted using the Genomic DNA Extraction Kit (Promega, Madison, WI). Plasmid preparation from *E. coli* grown in LB medium in the presence of ampicillin (50 μg/ml) was performed by using the QIAprep Spin Miniprep Kit (Qiagen).

Time Course and Time Lapse Fluorescence Microscopy

Samples (0.5 ml) of culture were removed, centrifuged briefly, and resuspended in 10 μl of 1× PBS (phosphate-buffered saline) supplemented with the membrane stain FM4-64 or FM1-43 (Molecular Probes, Eugene, OR) at 1 μg/ml. This concentrated cell suspension (3 μl) was placed on a microscope slide. A freshly prepared poly L-lysine (Sigma, Saint-Louis, MO) -treated coverslip was used to immobilize the cells for membrane visualization.

To visualize FtsZ-GFP, EzrA-GFP, and FtsA-GFP structures and for time lapse observations, a chambered slide (VWR Scientific Inc., West Chester, PA) filled with the sporulation medium containing 1% agarose was used. For vegetative experiments, CH medium containing 1% agarose was used. Samples (0.5 ml) of sporulating or growing culture were collected and centrifuged briefly. For time course microscopy experiments, the cells were resuspended in 10 μl of 1 μg/ml FM4-64. For time lapse microscopy, the cells were resuspended in 10 μl of the original culture supernatant, and the dye was added directly to the sporulating culture or to the growing culture (0.1 μg/ml). The concentrated bacteria were applied to the agarose bed in the chambered slide, and then an untreated coverslip was used to immobilize the cells for time lapse microscopy experiments at room temperature (approximately 25°C). Fluorescence microscopy was performed as described previously (Eichenberger et al., 2001).

Immunofluorescence Microscopy

Strains were sporulated by resuspension at 30°C, and samples were collected at various times. The cells were fixed in methanol (1 ml cells and 10 ml 100% methanol) for 60 min before proceeding with immunofluorescence as described previously (Levin and Losick, 1996). Affinity-purified anti-FtsZ antibodies from a rabbit were used at 1:150 dilution and FITC secondary antibodies were used at 1:100 dilution.

Deconvolution Microscopy

JDB401 strain was induced to produce FtsZ-GFP, as described above. Cells in samples of 0.1 ml were concentrated and applied to agarose pads, as describe above, and subjected to deconvolution microscopy. The microscopy was carried out in an inverted DeltaVision microscope. Between 20 and 40 images of optical sections of fluorescence from GFP were collected at spacings of 0.05–0.1 μm. The images were deconvolved through 15 iterations using the Delta Vision deconvolution software. The deconvolved images were then projected using the Volume-Viewer function.

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