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The ATPase SpoIIIE Transports DNA across Fused Septal Membranes during Sporulation in *Bacillus subtilis*

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SUMMARY

The FtsK/SpoIIIE family of ATP-dependent DNA transporters mediates proper chromosome segregation in dividing bacteria. In sporulating *Bacillus subtilis* cells, SpoIIIE translocates much of the circular chromosome from the mother cell into the forespore, but the molecular mechanism remains unclear. Using a new assay to monitor DNA transport, we demonstrate that the two arms of the chromosome are simultaneously pumped into the forespore. Up to 70 molecules of SpoIIIE are recruited to the site of DNA translocation and assemble into complexes that could contain 12 subunits. The fusion of the septal membranes during cytokinesis precedes DNA translocation and does not require SpoIIIE, as suggested by analysis of lipid dynamics, serial thin-section electron microscopy, and cell separation by protoplasting. These data support a model for DNA transport in which the transmembrane segments of FtsK/SpoIIIE form linked DNA-conducting channels across the two lipid bilayers of the septum.

INTRODUCTION

Chromosome segregation is a fundamental step in the division of all cells. In bacteria, replicated chromosomes are not always completely segregated to the two daughter cells prior to cytokinesis. In these cases, the division septum closes around the chromosome and an ATPase of the FtsK/SpoIIIE family is required to translocate the DNA into one of the two daughter cells (Errington et al., 2001). An extreme example of this type of chromosome segregation is provided by sporulating *Bacillus subtilis* cells. During sporulation a polar septum divides the cell into two unequally sized compartments: a larger mother cell and a smaller forespore. Approximately 30% of the forespore chromosome, roughly centered on the origin of replication, is always positioned within the forespore compartment (Wu and Errington, 1998). This leaves nearly 70% of the forespore chromosome in the mother cell. The ATPase SpoIIIE is required to actively translocate the remaining 70% of the chromosome (~3 Megabases) into the forespore. Cells lacking SpoIIIE are unable to move the chromosome into the spore compartment and are therefore deficient for sporulation (Wu and Errington, 1994).

How the FtsK/SpoIIIE proteins transport DNA from one cell to the other is only poorly understood. In all current models, the FtsK/SpoIIIE proteins translocate the DNA prior to the completion of cytokinesis (Figure 1A). Thus, transport would occur through an aqueous channel that connects the dividing cells (Wu and Errington, 1997; Errington et al., 2001; Sharp and Pogliano, 2003; Bartosik and Jagura-Burdzy, 2005; Liu et al., 2006; Lau et al., 2003; Bernhardt and de Boer, 2005; Bigot et al., 2007). Here we present evidence for a new model of DNA transport in which FtsK/SpoIIIE translocates DNA *across* the two membranes of the division septum. In this model, the cell first undergoes cytokinesis, including membrane fusion, and then translocates the DNA. Although DNA transport across membranes occurs in other systems (Chen et al., 2005), it is generally poorly understood and has not been considered in the context of chromosome segregation.

The FtsK/SpoIIIE proteins are membrane-bound ATPases. They have N-terminal transmembrane segments that are required for proper septal localization (Wu and Errington, 1997; Yu et al., 1998; Sharp and Pogliano, 1999; Sharp and Pogliano, 2002). The C-terminal regions of these proteins form cytoplasmic motor domains belonging to the family of RecA-like ATPases (Iyer et al., 2004). SpoIIIE is present in vegetative cells where, like FtsK, it plays a role in DNA segregation when replicated chromosomes are not completely segregated prior to cell division (Sharpe and Errington, 1995; Britton and

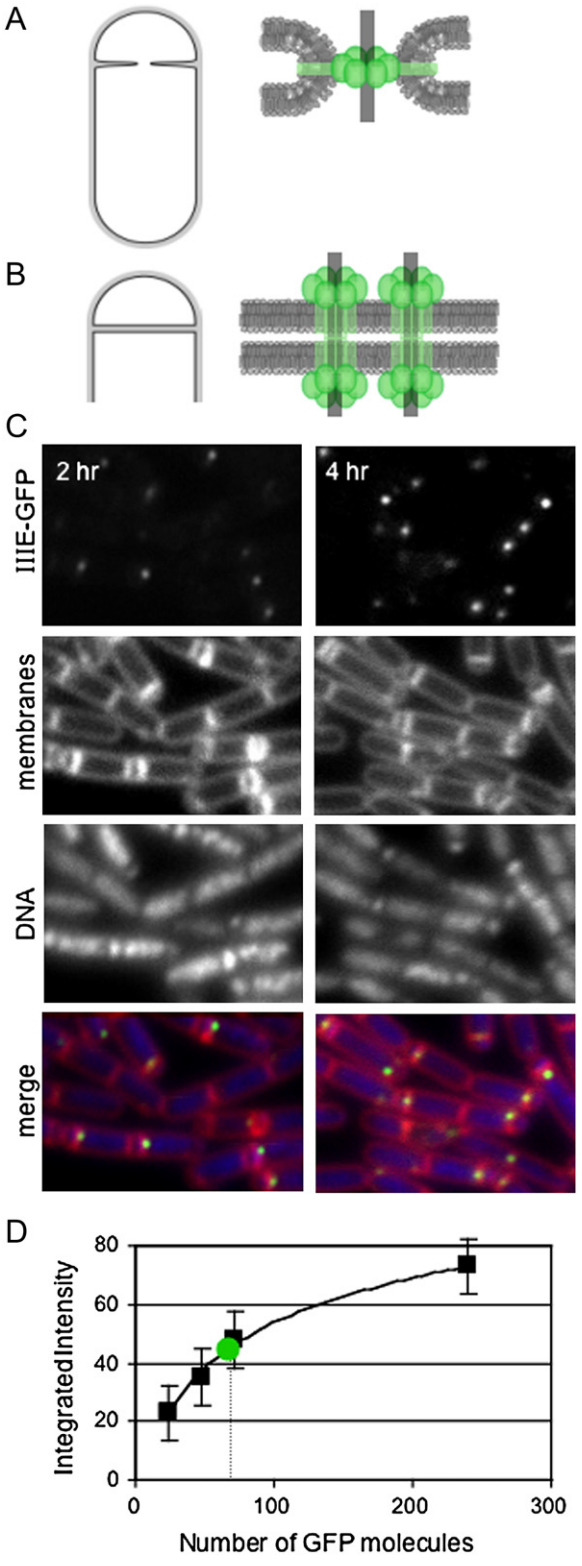

Figure 1. A Large Number of SpoIIIE Molecules Are Recruited to the Polar Septum

(A) Models for SpoIIIE assembly at the septum. The cytoplasmic ATPase domains form an aqueous channel, and the transmembrane segments anchor the complex in the invaginating septal annulus. The hexameric SpoIIIE ring accommodates a single double-stranded DNA (gray line). A single ring is depicted in the septal annulus, but the complex could also be formed by paired or stacked rings.

(B) SpoIIIE forms separate DNA-conducting channels for each chromosome arm in the septal membranes. The ATPase domains *and* the transmembrane segments form these channels.

(C) SpoIIIE-GFP was monitored by fluorescence microcopy in sporulating cells (strain BDR1626) grown at 30°C. The intensity of the GFP foci increased between hour two and hour four of sporulation. Membranes and DNA from the same fields were visualized using the fluorescent dyes FM4-64 and DAPI, respectively. Similar results were obtained from cells sporulated under more rapid conditions (Figure S4).

(D) Calibration and quantitation of the number of GFP molecules. Sporulating cells harboring arrays of 12, 24, 36, and 120 *tetO* operators and saturating amounts of TetR-GFP were analyzed by fluorescence microscopy. The intensities of the flourescent foci (Figure S3) were plotted against the expected number of bound TetR-GFP molecules (error bars represent one standard deviation). TetR binds the *tetO* operator as a dimer. The green circle indicates the median integrated intensity of SpoIIIE-GFP foci at hour 4.

Grossman, 1999). In vegetative cells that have segregated their chromosomes completely, SpoIIIE is distributed uniformly throughout the cytoplasmic membrane (Wu and Errington, 1997). At the onset of sporulation, SpoIIIE redistributes to a focus at the polar septum. The generation of this SpoIIIE focus requires the presence of DNA in the septal annulus (Ben-Yehuda et al., 2003), and once localized, the SpoIIIE focus remains at this site until the entire chromosome has been pumped into the forespore (Sharp and Pogliano, 1999).

The soluble ATPase domains of SpoIIIE and FtsK can translocate along DNA in an ATP-dependent manner in vitro (Bath et al., 2000; Pease et al., 2005). Moreover, electron microscopy of the FtsK motor domain in the presence of DNA suggests that the protein forms rings around the DNA (Massey et al., 2006; Aussel et al., 2002). The crystal structure of the soluble ATPase domain of FtsK indicates that it assembles into a hexameric ring with a central pore that is large enough (30 Å) to accommodate a double-stranded DNA molecule (Massey et al., 2006).

Based on the crystal structure and biochemical experiments, it is likely that the cytoplasmic ATPase domains of FtsK/SpoIIIE form a hexameric ring around the DNA. Given the size of the pore in the structure, the hexamer would allow the transport of only one double-stranded DNA molecule. However, ultimately, SpoIIIE and FtsK must transport both arms of the double-stranded circular chromosome from one cell to the other. These considerations raise problems for the currently favored model, in which FtsK/SpoIIIE is anchored in the membranes of the constricting annulus with the cytoplasmic ATPase domains forming a single aqueous channel connecting the two cells (Figure 1A). In this case, the chromosome arms would have to be transported sequentially through the pore of the hexamer; the circular chromosome would have to be cleaved and translocated by the hexamer into the forespore; and finally the two ends would need to be rejoined. There is currently no evidence for this

type of cleavage and rejoining. An alternative to DNA cleavage would be a model in which more than one aqueous channel is present in the septum (Errington et al., 2001; Bigot et al., 2007). However, the formation of multiple aqueous channels around double-stranded DNAs would require temporally and topologically distinct fusion events (Figure S1 available online).

Here we present evidence for a new model in which SpoIIIE transports DNA *across* the two membranes of the division septum after the completion of cytokinesis. First, we show that a large number of SpoIIIE molecules are recruited to the site of DNA translocation, many more molecules than expected if a single hexameric ring were the active pump. Second, we demonstrate that the two chromosome arms are translocated at the same time into the forespore compartment. Finally, we show by independent methods that fusion of the septal membranes occurs prior to DNA transport. We propose that SpoIIIE assembles hexamers in the mother cell and forespore membranes. The conduit for the DNA would be formed by the cytoplasmic ATPase domains, as well as by the transmembrane segments (Figure 1B). Consistent with this model, SpoIIIE is present on both sides of the fused septum at the site of DNA transport and assembles into complexes that could contain 12 SpoIIIE molecules. The proposed model in which FtsK/SpoIIIE forms DNA-conducting channels in the lipid bilayer provides a new paradigm for DNA transport across membranes with implications for chromosome translocation and bacterial conjugation.

RESULTS

Many SpoIIIE Molecules Are Recruited to the Polar Septum

Fluorescence microscopy experiments indicate that SpoIIIE-GFP accumulates as a focus at the septum when it transports DNA into the forespore (Wu and Errington, 1997; Ben-Yehuda et al., 2003). To rigorously assess the accumulation of SpoIIIE, we performed a quantitative analysis. To this end, cultures of *B. subtilis* expressing a functional *spoIIIE-gfp* from its native promoter were induced to sporulate by resuspension at 30°C under conditions where sporulation proceeds slowly (see Experimental Procedures). The cells were analyzed by fluorescence microscopy at different time points during sporulation. At the onset of sporulation, SpoIIIE-GFP was evenly distributed in the peripheral membranes (Wu and Errington, 1997; Figure S2). At hour two of sporulation, a population of the cells had polar septa and many of them contained dots, which correspond to nascent SpoIIIE foci (Figure 1C). At hour four, essentially all cells contained septa with foci. Interestingly, the foci not only appeared over time but also increased in intensity. Importantly, the total level of SpoIIIE-GFP remained unchanged, as monitored by immunoblot analysis using anti-GFP antibodies (data not shown), in agreement with previous observations (Wu and Errington, 1997).

To quantitate the increase in intensity of the foci during sporulation, we analyzed several fields of sporulating cells ($n > 1000$ foci) from images collected at hours two and four of sporulation. Representative images are shown in Figure 1C. Quantitation revealed a 4- to 5-fold increase in the mean focus intensity between hours two and four (Figure S3A). Cells analyzed at hour four had about the same focus intensity regardless of the extent of DNA transport into the forespore, as assessed by DAPI staining of the DNA (Figure S3B). These data suggest that at the beginning of DNA transport, between hours 2 and 4, multiple SpoIIIE hexamers accumulate at the septum. Moreover, once localized, the number of SpoIIIE molecules remains constant until DNA transport is completed.

To estimate the actual number of SpoIIIE-GFP molecules in the foci, we developed a calibration method based on the binding of the Tet repressor GFP fusion (TetR-GFP) to arrays of the *tet* operator (*tetO)* (Michaelis et al., 1997). Binding of TetR-GFP to these arrays, which binds as a dimer (Hillen and Berens, 1994), produces a fluorescent focus. We introduced arrays containing 12, 24, 36, and 120 copies of *tetO* (Lau et al., 2003) into the *B. subtilis* chromosome. The TetR-GFP fusion was synthesized at the onset of sporulation and in excess of these binding sites. The integrated fluorescence of the foci for each array was analyzed (Figure S3C) and plotted to generate a calibration curve (Figure 1D). With the smaller arrays, the fluorescence increased linearly. The largest *tetO* array was less fluorescent than predicted, perhaps due to self-quenching. Extrapolation of the curve suggests that foci containing as few as 10 molecules of GFP could be detected. Comparison of the SpoIIIE-GFP fluorescence with the calibration curve indicates that the number of SpoIIIE molecules at the septum increased from 10–12 at hour two of sporulation to as many as 70 at hour four (Figures 1D and S3). We conclude that the number of SpoIIIE molecules assembled at the site of DNA translocation greatly exceeds a hexamer, suggesting that SpoIIIE translocates more than one double-stranded DNA molecule.

SpoIIIE Transports Both Chromosome Arms Simultaneously

Next we investigated whether SpoIIIE moves one or both double-stranded chromosome arms across the division septum at the same time. Previous experiments assessing the importance of gene position on the chromosome during sporulation suggested that the terminus is the last region of the chromosome to be transported into the forespore (Frandsen et al., 1999; Khvorova et al., 2000; Zupancic et al., 2001; Dworkin and Losick, 2001). However, these experiments tested populations of cells and therefore did not address whether, in a given cell, the two chromosome arms are transported at the same time. To analyze chromosome transport directly, we developed a quantitative, fluorescence-based assay. We fused the genes encoding cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to a promoter (P_{spoIIQ}) that is

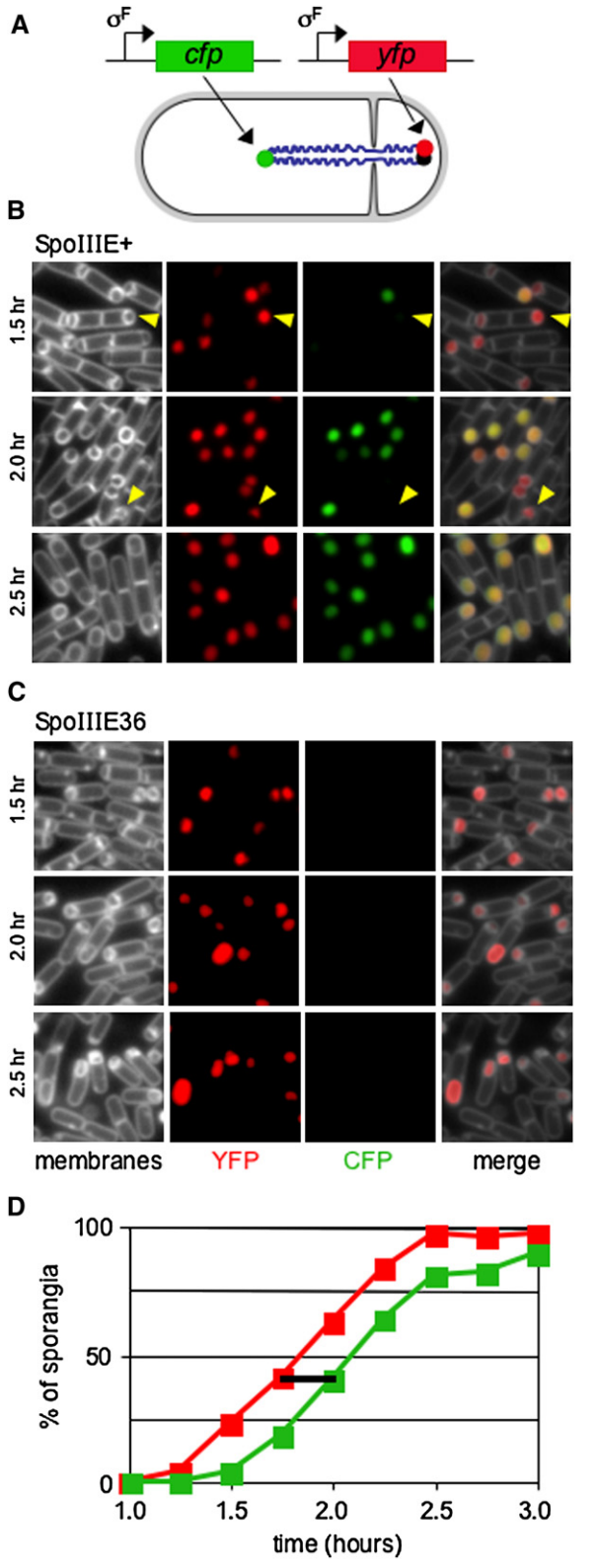

Figure 2. In Vivo DNA Transport Assay

(A) Schematic of a sporulating cell used in this assay. For simplicity, only the chromosome transported by SpoIIIE is diagrammed. Red and green circles represent the location of the sigmaF-controlled reporter genes, *yfp* and *cfp*. The origin of the chromosome is marked with a black dot.

(B) Sporulation (SpoIIIE+, strain BBB128) was induced by resuspension, and YFP (pseudo-colored red) and CFP (pseudo-colored green) fluorescence were monitored over time. Examples of forespores with YFP fluorescence but not CFP fluorescence are indicated (yellow carets).

(C) Sporulation (SpoIIIE36, strain BNS276) was induced by resuspension, and YFP (pseudo-colored red) and CFP (pseudo-colored green) fluorescence were monitored over time.

(D) Three fields of several hundred sporangia each were scored per time point (strain BBB128). The percent of sporangia expressing each reporter was plotted against time. The black bar denotes the average time (~20 min) between visualization of the origin and terminus reporters.

recognized by a forespore-specific transcription factor (sigmaF) immediately upon septation (Khvorova et al., 2000; Zupancic et al., 2001). By placing these promoter fusions at various locations on the chromosome, we could assay for the presence of the marked chromosomal region in the forespore. To demonstrate the validity of the assay, we first placed the fluorescent reporters at the extreme ends of the chromosome. The *yfp* reporter was placed near the origin (−7°), a region of the chromosome that is always trapped in the forespore at the onset of sporulation (Wu and Errington, 1998), and *cfp* was placed near the terminus (174°) in the region of DNA that must be actively transported by SpoIIIE to reach the forespore compartment (Figure 2A). Synchronous sporulation was induced at 37°C, and CFP and YFP fluorescence were monitored over time. As expected, YFP fluorescence was detectable in the forespores of all sporulating cells soon after septation, whereas CFP fluorescence was not yet detectable (Figure 2B, 1.5 hr). At later time points, all cells expressed both the origin-proximal *yfp* and the origin-distal *cfp* (Figure 2B, 2.5 hr), indicating that the rest of the chromosome had been transported into the forespore. As a control, we used a SpoIIIE mutant (SpoIIIE36) that is defective in DNA transport (Wu et al., 1995) (Figure 2C). In this case, all the forespores contained YFP but not CFP, even at late time points.

To determine the rate of DNA translocation in wild-type cells, the percentage of cells containing YFP or CFP was plotted versus time (Figure 2D). The difference in the presence of YFP and CFP provides a population average for the time it takes to transport the *cfp*-containing chromosomal region into the spore. For wild-type SpoIIIE, we measured an average transport time of 20 min (Figure 2D, black bar), which corresponds well with the previously published estimates (Pogliano et al., 1999).

To test whether one arm of the chromosome is transported before the other, we placed *yfp* and *cfp* at −100° and +90° from the origin, respectively (Figure 3A). Since the asymmetric septum always traps the origin-proximal region of the chromosome (approximately −50° to +40°) in the forespore (Wu and Errington, 1998; N.L.S. and D.Z.R., unpublished data), the two markers will be present

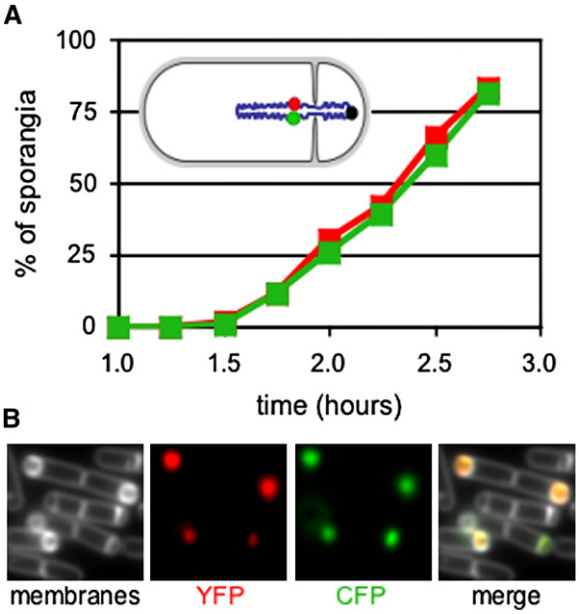

Figure 3. Simultaneous Transport of the Two Chromosome Arms into the Forespore

(A) Analysis of fluorescence from a strain (BB162) that contains the *cfp* and *yfp* reporters on the left and right arms of the chromosome. The appearance of YFP and CFP in the forespore is similar over time. Three fields of several hundred sporangia each were scored for YFP and CFP fluorescence in the forespore. The percent of sporangia expressing each reporter was plotted against time. Inset shows a schematic of a cell and the location of the origin (black dot), the sigmaF-controlled *yfp* gene (red dot), and the sigmaF-controlled *cfp* gene (green dot).

(B) Individual cell analysis demonstrates that essentially all forespores that contain one fluorescent marker contain the other. Thus, both arms are translocated simultaneously. A strain in which the positions of the two markers were swapped produced identical results (data not shown).

in the mother cell at the time of polar division. Importantly, there will be roughly the same amount of DNA on each chromosome arm between the marker sites and the SpoIIIE complex at the septum. Consistent with the idea that the two arms are translocated simultaneously, in all experiments, the percentage of forespores with YFP fluorescence increased with the same kinetics as the percentage of forespores with CFP fluorescence (Figure 3A). Furthermore, at every time point essentially all forespores (97%–99%) that contained YFP also contained CFP, confirming that the two markers entered the forespore at the same time in each cell (Figure 3B).

DNA Transport with a Slow-Pumping SpoIIIE Mutant

Our experiments exclude the extreme scenario in which one chromosome arm is stationary or cleaved while the other is translocated. However, we were concerned that subtle differences in transport of the two arms might be obscured by the fact that DNA translocation is quite rapid in wild-type cells. We therefore wished to generate a SpoIIIE mutant with a reduced transport rate. After screening several mutants in and around the ATPase motifs, we identified one with the desired property. This mutant has an Asp to Ala substitution at residue 584, close to the Walker B motif of the ATPase domain. The SpoIIIE(D584A) mutant sporulates near wild-type levels (80%–90% compared to wild-type). In addition, cells containing a GFP fusion to SpoIIIE(D584A) produced regular-sized foci at septa and were normal for other visual markers of sporulation, such as the engulfment of the forespore (data not shown). However, using our in vivo DNA transport assay with *yfp* and *cfp* located near the origin and terminus, DNA transport was significantly slower in the SpoIIIE(D584A) strain compared to wild-type (Figure 4A). Quantitation showed that the SpoIIIE(D584A) mutant transports ~2.5-fold slower than wild-type SpoIIIE (Figure 4B), with CFP fluorescence lagging behind YFP by ~50 min. We note that at the latest time points, there were still some cells that did not contain CFP in this mutant (Figure 4B). This can be explained by the fact that sigmaF activity (required for expression of the fluorescent reporters) is reduced once the late-acting forespore sigma factor sigmaG is activated (Li and Piggot, 2001). Indeed, in a sigmaG mutant, in which sigmaF activity persists, eventually almost all forespores contained CFP and YFP in the slow-pumping mutant (data not shown). We further verified the slow DNA transport rate of the SpoIIIE(D584A) mutant using a second quantitative assay, in which a sigmaF-controlled gene essential for sporulation was placed at increasing distance from the origin and its movement into the forespore was assessed by monitoring sporulation efficiency (Figure S5).

We used the slow-pumping SpoIIIE mutant to evaluate transport of the two chromosome arms into the forespore. The *yfp* and *cfp* genes were placed at −100° and +90° from the origin and the expression of the reporters was monitored in the SpoIIIE(D584A) mutant. As with the wild-type strain, YFP and CFP were produced in the forespore at the same time (Figure 4C). Quantitation revealed that the percentage of cells containing YFP or CFP increased with the same kinetics (Figure 4D). Again, nearly every forespore that contained one reporter contained the other (97% at hour 1.75). Thus, there was no detectable difference in the rate of transport of the two chromosome arms. We conclude that the two arms of the forespore chromosome are translocated simultaneously by SpoIIIE. We also confirmed that two reporters placed on the same chromosome arm were transported sequentially into the forespore; a reporter at position +90° was expressed in the forespore earlier than a reporter at position +174° (Figure S6).

Septal Membrane Fusion Occurs prior to the Completion of DNA Transport and Is Independent of SpoIIIE

We next addressed whether SpoIIIE transports the chromosome before or after membrane fusion occurs between the mother and forespore cells. If DNA translocation

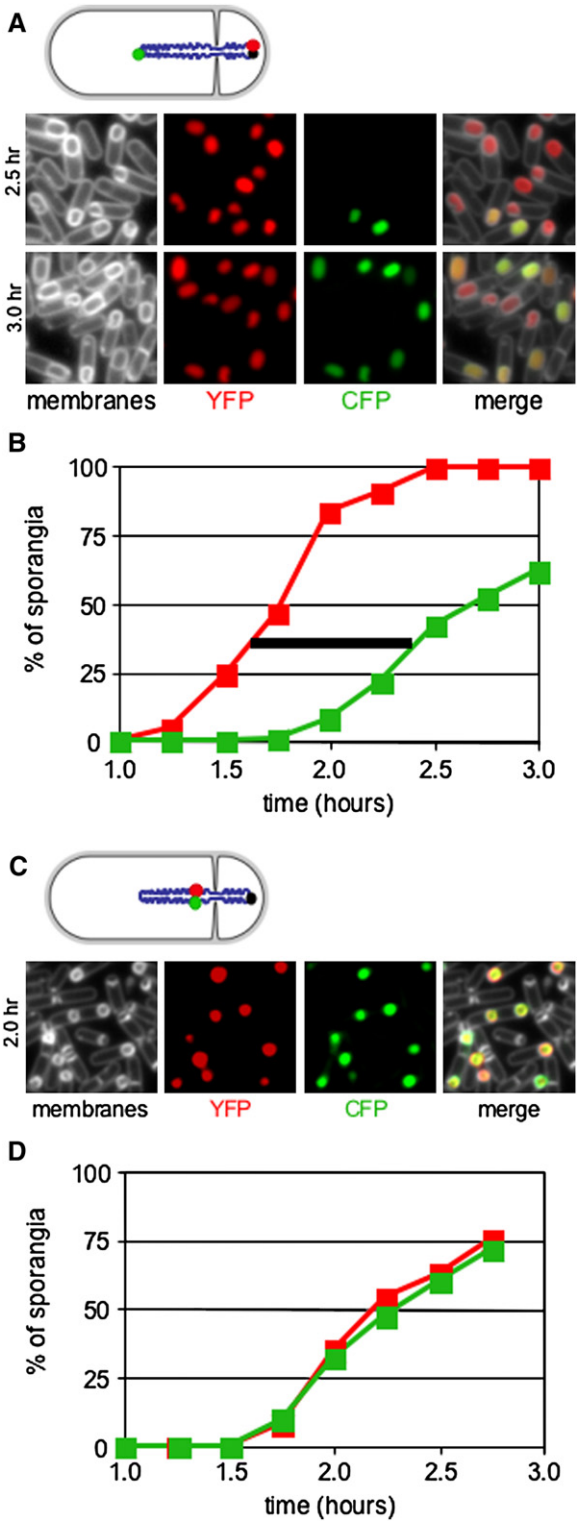

Figure 4. A Slow-Pumping Mutant of SpoIIIE Pumps Both Arms Simultaneously

(A) Red and green dots represent the location of the sigmaF-controlled reporter genes, *yfp* and *cfp*. Sporulation in the SpoIIIE(D584A) mutant (strain BBB131) was induced by resuspension, and YFP (pseudo-colored red) and CFP (pseudo-colored green) fluorescence were monitored over time.

(B) Determination of the DNA transport rate for the slow-pumping mutant. Three fields of several hundred sporangia each were scored per time point. The percent of sporangia expressing each reporter was plotted against time. The black bar denotes the average time (~50 min) between visualization of the origin and terminus reporters.

(C) Individual cell analysis from a strain (BBB213) harboring reporters on the left and right arms of the chromosome. The fluorescence micrographs demonstrate that nearly all forespores that contain one fluorescent marker contain the other.

(D) In a population of sporulating cells, the appearance of YFP and CFP occurs at the same time. Three fields of several hundred sporangia each were scored for YFP and CFP fluorescence in the forespore. The percent of sporangia expressing each reporter was plotted against time.

occurs at sites where fusion has not yet occurred, then SpoIIIE would transport the chromosome through an aqueous channel. In this scenario, the membranes of the mother cell and forespore would form a continuous lipid bilayer. On the other hand, if DNA translocation occurs after membrane fusion is complete, then SpoIIIE would move the chromosome arms across two separate lipid bilayers and the membranes of the mother cell and forespore would form independent compartments. To distinguish between these possibilities, we used the lipophilic dye FM4-64 and fluorescence recovery after photobleaching (FRAP) to assess lipid movement between the two compartments. To ensure that we were photobleaching cells in which the DNA was crossing the septum, we first used the transport-deficient mutant SpoIIIE36 (Wu and Errington, 1994). Sporulating cells were incubated with FM4-64 and then washed extensively to remove unincorporated dye molecules. The forespore membranes were photobleached and then monitored for recovery of fluorescence over time (Figure 5A). Of 12 forespores bleached, none showed significant recovery of fluorescence. The mother cell fluorescence decreased only slightly during the recovery period, corresponding to the rate of photobleaching during image acquisition (Figure 5A, graph).

Similar results were obtained with cells that contain wild-type SpoIIIE. To ensure that SpoIIIE had not yet completed DNA transport, we visualized the +130° position using an array of *tet* operators and TetR-GFP. Sporulating cells with two foci (one for each chromosome) in the mother cell were selected for photobleaching, as these must be actively transporting DNA and still have to move the marked chromosome region into the forespore. As before, upon bleaching of the forespore compartment, there was no recovery of fluorescence (Figure S7), indicating that fusion of the septal membranes had occurred in cells actively transporting the DNA.

To rule out the possibility that the transmembrane segments of SpoIIIE form a lipid diffusion barrier at the septal constriction, we carried out the same FRAP experiment in a *spoIIIE* null mutant. In the absence of SpoIIIE, the septum still forms around the chromosome arms but DNA pumping does not occur (Wu and Errington, 1994). Again,

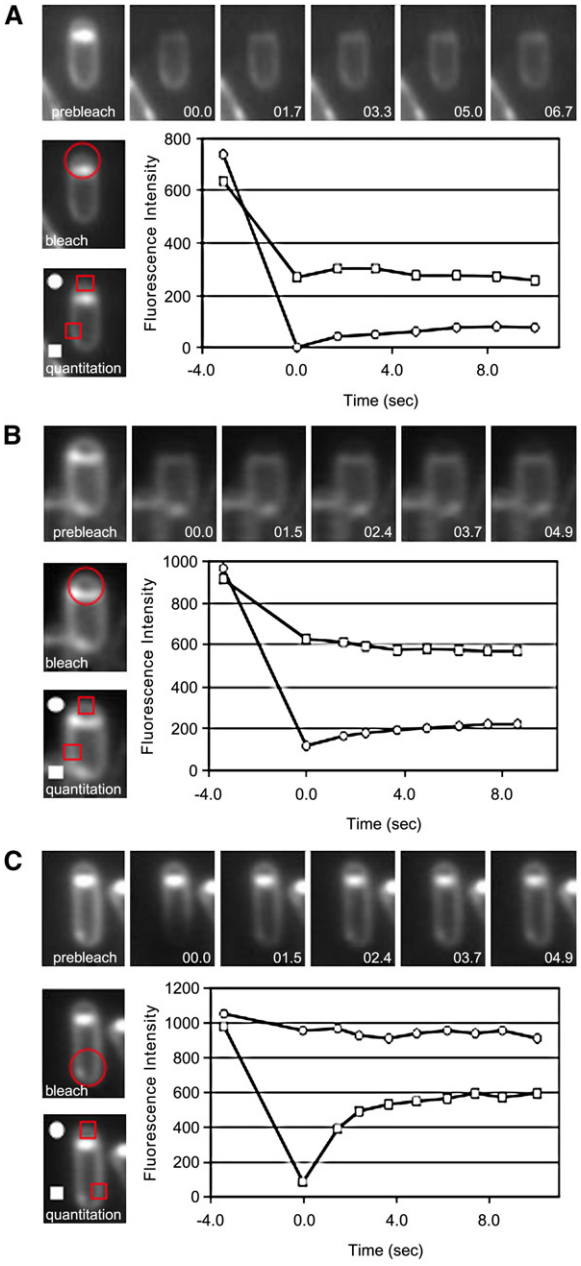

Figure 5. Membrane Fusion at the Sporulation Septum Occurs in the Absence of SpoIIIE and DNA Transport

Analysis of lipid dynamics using FM4-64 and fluorescence recovery after photobleaching (FRAP). Image series above the graphs shows the photobleaching time course. Images to the side of the graphs indicate the region of photobleaching (top images, red circles) and the regions used for fluorescence quantitation (bottom images, red boxes). Graphs show forespore membrane (circles) and mother cell membrane (squares) fluorescence over the course of the experiment. (A) Photobleaching of the forespore membranes in a *spoIIIE36* mutant (strain BDR1050). (B) Photobleaching of the forespore membranes in a *spoIIIE* null mutant (strain BDR1832). (C) Photobleaching of the mother cell membranes in a *spoIIIE* null mutant.

we did not observe any significant recovery of fluorescence in the forespore after photobleaching, and the fluorescence in the mother cell decreased only slightly (Figure 5B).

To demonstrate that the FM4-64 dye diffuses efficiently within continuous membranes, we photobleached part of the mother cell membranes and followed fluorescence recovery (Figure 5C). The recovery of fluorescence in the mother cell membrane was greater than 85% complete within 2 seconds. The fluorescence of the forespore membranes in this experiment decreased only at the rate of photobleaching during image acquisition (Figure 5C, graph), further suggesting that the two cellular compartments have physically distinct membranes. The difference between the forespore and mother cell bleaching experiments was highly statistically significant ($p = 0.02$, Figure S8). These results suggest that the mother cell and forespore membranes fuse while the DNA is still in the septum and that SpoIIIE does not participate in membrane fusion during cytokinesis.

The idea that the membranes fuse while DNA is still in the septum is further supported by electron microscopy experiments in which serial sections, each 60 nm thick, of sporulating cells expressing SpoIIIE-GFP were stained with gold-labeled anti-GFP antibodies. The images show continuous membranes between the mother cell and forespore in every section, including and surrounding the ones that contained the gold particles marking the location of SpoIIIE (Figures 6A and S9A). Although in the particular cell shown the gold particles were only seen on the mother cell side of the septum, in other examples gold particles were visualized on the forespore side (Figure S9B), indicating that SpoIIIE is present at the site of DNA transport in both membranes.

To provide independent evidence that the membranes between the mother cell and forespore fuse before DNA transport, we performed protoplasting experiments. A population of cells at early stages of sporulation was treated with lysozyme and the membranes were monitored every 30 s by timelapse fluorescence microscopy. In most cells, the mother and forespore rounded up into two distinct protoplasts (Figure 6B, yellow arrows), indicating that in these cells septal fusion had occurred. In some cells, the septal membranes retracted during the incubation with lysozyme, eventually resulting in single, rounded protoplasts (Figure 6B, red arrows). This class of cells demonstrates that the division apparatus cannot hold the invaginating septal membranes in place once the septal peptidoglycan is removed and argues that lysosome treatment does not artificially induce membrane fusion. With this assay, we determined that at a time point when DNA transport was just beginning, ~70% of the sporulating cells had completed septal fusion. Furthermore, similar results were obtained with a *spoIIIE* null mutant (Figure 6B, right panel), supporting the idea that SpoIIIE is not required for fusion. All together, these results support the model shown in Figure 1B, in which membrane fusion occurs prior to DNA translocation.

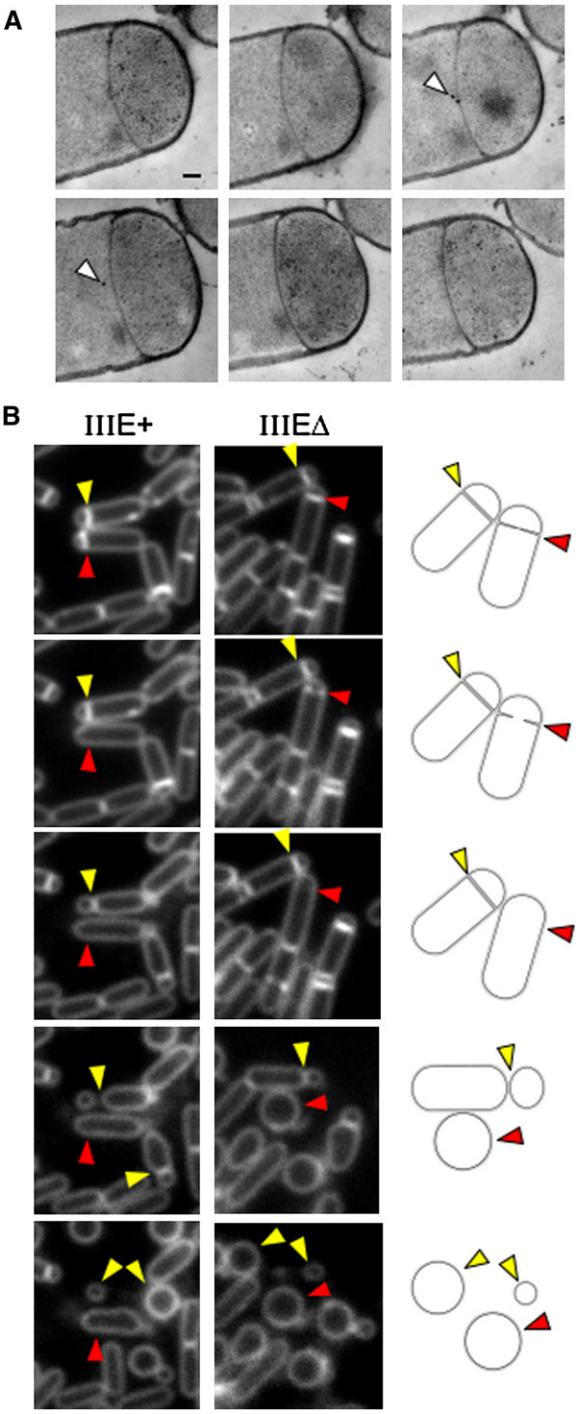

Figure 6. The Mother and Forespore Compartments Are Separated by Two Complete Septal Membranes at the Time of DNA Transport

(A) Serial thin section electron micrographs of a sporulating cell expressing a functional *spoIIIE-gfp* fusion (strain BDR1626), immunostained with gold-conjugated anti-GFP antibodies. The gold particles (carets in two middle sections) identify SpoIIIE-GFP. Scale bar is 100 nm. Larger versions of these images can be found in Figure S9A. In the cell shown, gold particle labeling is only observed on the mother cell side of the septum. In other cells gold particle labeling of SpoIIIE-GFP can be observed on both sides of the septum (See Figure S9B). (B) Timelapse images of sporulating cells treated with lysozyme. The protoplasted strains were wild-type *spoIIIE* (SpoIIIE+) (BTD1559) or a *spoIIIE* null mutant (BBB339). Both strains contained a *spoIIQ* mutation to prevent protoplast engulfment (Broder and Pogliano, 2006). The timelapse movies can be found in Supplemental Data (Movie S1A and S1B). Representative cells in which the polar septa have fused are indicated (yellow carets). Septa that have not yet fused and reverse upon lysozyme treatment are also highlighted (red carets).

SpoIIIE Forms Large, Detergent-Stable Complexes during Sporulation

The model in Figure 1B implies that the SpoIIIE channels associate across the two membranes during sporulation, forming a complex that would contain 12 SpoIIIE molecules. To investigate whether SpoIIIE assembles into complexes of this size during chromosome translocation, we performed sucrose gradient centrifugation experiments. A membrane fraction was isolated from sporulating cells and solubilized with the mild detergent digitonin. Clarified extracts were subjected to centrifugation in a 10%–40% linear sucrose gradient, and fractions were analyzed by SDS-PAGE, followed by immunoblot analysis with anti-GFP antibodies. At the beginning of sporulation (hour zero), when SpoIIIE-GFP was distributed evenly in all membranes (see Figure S2), SpoIIIE-GFP was found exclusively in low-molecular-weight fractions (Figure 7A). After 2 hr of sporulation, when a small subset of cells contained SpoIIIE-GFP foci at their septa (see Figure 1C), a fraction of the SpoIIIE-GFP migrated at a larger molecular weight (Figures 7A). Finally, after 4 hr of sporulation, when the majority of cells had SpoIIIE-GFP foci but had not yet completed DNA transport, most of the SpoIIIE-GFP migrated at the high-molecular-weight position. Membranes harvested from the same time point but treated with SDS to disrupt protein complexes produced a single peak of protein at the top of the gradient, which is consistent with the expected position of monomeric SpoIIIE-GFP (115 kDa). The size of the high-molecular-weight SpoIIIE-GFP complex was estimated to be approximately 1 MDa by comparison to ribosomal subunits analyzed in parallel gradients (data not shown). The increase in size is not likely to be caused by the association of SpoIIIE with DNA since extensive DNase treatment was carried out during lysis and detergent solubilization. These experiments do not indicate whether SpoIIIE forms homo- or hetero-oligomers during sporulation. However, if the large complexes seen in the sucrose gradients consisted of only SpoIIIE, they could contain 12 molecules, consistent with our model of linked transmembrane channels (Figure 1B).

DISCUSSION

Our data indicate that SpoIIIE transports the DNA after the membranes of the mother cell and forespore have fused, implying that the DNA is transported across two lipid

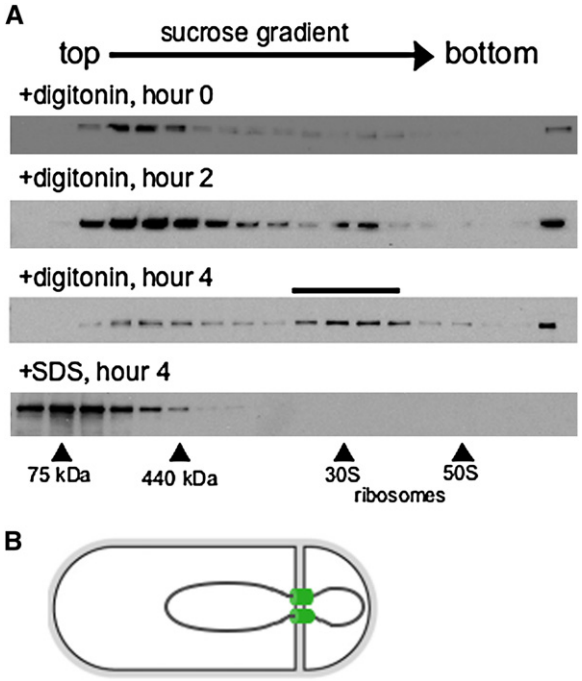

Figure 7. SpoIIIE Assembles into Large Complexes

(A) Analysis of SpoIIIE-GFP complexes from sporulating cells. Samples were harvested at the initiation of sporulation (hour zero) and after 2 and 4 hr. Detergent-solubilized extracts from membrane fractions were loaded onto linear (10%–40%) sucrose gradients. Fractions were analyzed by immunoblot with anti-GFP antibodies. The high-molecular-weight peak of SpoIIIE in the hour four gradient is indicated (bar). Molecular weight standards of 75 and 440 kDa, as well as 30S and 50S *E. coli* ribosomes, were analyzed in a parallel gradient. The peak fraction for each size marker is indicated with a caret. Differences in intensities between blots are due to variation in lysis efficiency and protein transfer.

(B) Schematic diagram of two DNA-conducting channels at the sporulation septum.

bilayers. Consistent with this idea, we show that the two chromosome arms are translocated at the same time into the forespore compartment and that a large number of SpoIIIE molecules are recruited to the site of DNA transport. Moreover, we show that SpoIIIE assembles into complexes that could contain two hexameric SpoIIIE rings. Finally, our results indicate that SpoIIIE is not required for membrane fusion during cytokinesis.

Altogether, our results are most consistent with a novel model for DNA transport in which SpoIIIE forms linked DNA-conducting channels across two lipid bilayers after the completion of cell division (Figure 1B). The conduit for DNA would consist of rings of the cytoplasmic ATPase domains in the mother cell and forespore compartments, as well as of membrane channels in both the forespore and mother cell membranes. Based on the structural data, the rings would contain six ATPase molecules, implying that each arm of the chromosome is transported through a separate conduit (Figures 1B and 7C). It is possible that, once formed, these conduits remain in contact with each other because we rarely observe more than one SpoIIIE focus in the sporulation septum. Each hexameric membrane channel would contain 24 transmembrane segments, four from each monomer, sufficient to transport a double-stranded DNA molecule. In support of this idea, point mutations and insertions in the transmembrane domain of SpoIIIE abolish DNA transport (Liu et al., 2006; Wu et al., 1995). Some of these mutations are located in the extracellular loops, which might be involved in linking the channels in the mother cell and forespore membranes.

Upon the onset of sporulation, the distribution of SpoIIIE changes from an even localization around the cell membrane to a focus at the septum. Concomitantly, SpoIIIE assembles into a larger complex, as indicated by the sucrose gradient experiments. The SpoIIIE oligomers likely assemble around the DNA. In fact, previous experiments have shown that the DNA is required for SpoIIIE assembly at the division septum (Ben-Yehuda et al., 2003) and that in vitro the FtsK ATPase domains assemble around the DNA (Massey et al., 2006). The assembly could occur during the membrane fusion event. However, it is also possible that the fused bilayers form transient micelles to cap the lipid ends before SpoIIIE assembles around the DNA. These micelle caps would likely resemble natural lipid micelles (Nesmeyanova, 1982) and would prevent direct contact between the hydrophilic DNA molecules and the hydrophobic region of the membrane. Our model implies that the active translocation complex contains 12 molecules of SpoIIIE with a hexamer in each lipid bilayer (Figures 1B and 7B), consistent with the size of SpoIIIE in the sucrose gradients and the presence of SpoIIIE on both sides of the division septum at the site of DNA transport. The sucrose gradient experiments do not exclude the possibility that other proteins associate with SpoIIIE during translocation, but genetic screens have failed to identify additional components involved in DNA transport. Moreover, our experiments indicate that cell division proteins, the only proteins known to be present at the constricting annulus, have left the septum at the time when SpoIIIE transports the DNA (Figure S10). We therefore favor a model in which the translocation complexes consist exclusively of SpoIIIE. The same considerations make it unlikely that the diffusion barrier between the mother and forespore membranes that we observed in FRAP experiments was generated by proteins, rather than by the fusion of the septal lipid bilayers.

In Figures 1B and 7B, we have depicted the simplest case, in which the septum closes around only two chromosome arms. However, it is conceivable that the septum can trap several DNA strands. When the septum forms, the region between positions −50° and +40° of the chromosome is trapped (Wu and Errington, 1998; and our own results), and it could contain several loops crossing the two membranes. In this scenario, additional SpoIIIE channels would be needed to transport these loops. The presence of these channels might explain why we observed up to 70 molecules of SpoIIIE in the septum.

However, the bulk of the chromosome outside the trapped region would be moved linearly into the forespore, with each chromosome arm employing its own SpoIIIE channel.

The directionality of DNA transport from the mother cell to the forespore implies that the ATPase domains in the two compartments are not equivalent. In fact, there is evidence that the ATPase domains in only one compartment are active. For example, DNA transport that is blocked in the SpoIIIE36 mutant can be restored by expression of wild-type SpoIIIE in the mother cell (Sharp and Pogliano, 2002; Chary and Piggot, 2003). In addition, some translocation in the reverse direction can be seen when the wild-type protein is overexpressed in the forespore (Sharp and Pogliano, 2002). Recent data indicate that the directionality of chromosome translocation of *E. coli* FtsK is largely determined by short DNA sequences dispersed throughout the chromosome (Levy et al., 2005; Corre and Louarn, 2005; Bigot et al., 2006). Homologous sequences in the *B. subtilis* chromosome are predominantly in the permissive orientation, which could direct the ATPase domains in the mother cell to move the DNA into the forespore. The ATPases in the forespore would have the opposite orientation and would therefore ignore these signals.

Our data, which indicate that membrane fusion takes place prior to DNA transport and independently of SpoIIIE, are different from previous suggestions (Ben-Yehuda et al., 2003; Liu et al., 2006; Errington, 2001). Since soluble GFP can move between the two cellular compartments in a SpoIIIE deletion mutant, it was thought that the membranes had not yet fused (Hilbert et al., 2004; Liu et al., 2006). We hypothesize that, in the absence of SpoIIIE, the micelle caps (see above) would persist and allow GFP to diffuse between the two compartments. Alternatively, in a SpoIIIE mutant the DNA might simply span a distorted lipid bilayer. Either explanation is consistent with the observation that diffusion of GFP between the two compartments is prevented in the *spoIIIE* null when mutations are introduced that prevent peptidoglycan from retracting between the two cells (Hilbert et al., 2004).

Fusion of the membranes prior to DNA translocation is also supported by the observation that two tested cell division proteins (FtsL and FtsW) are no longer present at the polar septum when SpoIIIE is transporting DNA (Figure S10). In addition, analysis of SpoIIIE-GFP indicates that it does not always remain at the septal midpoint during DNA translocation (Sharp and Pogliano, 1999), suggesting that it can laterally diffuse in the membrane. Our data indicate that SpoIIIE is assembled in the plane of the septal membrane and therefore lateral diffusion would be expected. However, to explain the movement of the SpoIIIE-GFP focus with the previous models in which SpoIIIE formed an aqueous pore would require ''treadmilling'' of the lipid bilayer, a phenomenon without precedent. Finally, models in which SpoIIIE participates in septal fusion (Liu et al., 2006) would place the ATPase domains in the mother cell and forespore and the transmembrane segments in the aqueous connection between them, which seems topologically and energetically unlikely. All together, our data strongly suggest that FtsK/SpoIIIE transporters are not involved in membrane fusion during cytokinesis.

One interesting question raised by the proposed model is how the final loop of DNA would be translocated across the double membrane. Breakage of the circular DNA appears unlikely since the enzymes that catalyze chromosome end-joining (YkoU and YkoV) are not essential for sporulation (Wang et al., 2006). Cleavage by a site-specific endonuclease (and subsequent ligation in the forespore) could explain translocation of the terminus but would not accommodate situations in which multiple loops traverse the septal membrane. Instead, we propose that upon encountering a loop of DNA the independent channels open laterally to transiently form a larger central channel allowing the loop to move across the septum. Opening the complex in this way would be a reversal of the assembly process. This would allow the loop of chromosomal DNA to pass uncleaved across the septal membrane. The lateral opening of the channels could be driven by the mechanical force that the DNA loop exerts on the two hexameric complexes, as the FtsK/SpoIIIE ATPases can act against a force of up to 60 pN (Pease et al., 2005).

The FtsK/SpoIIIE transporters are part of a superfamily that includes membrane proteins, such as TraJ and TraG, involved in DNA conjugation (Gunton et al., 2007; Iyer et al., 2004). The structure of the soluble ATPase domain of one of these proteins (TrwB) indicates that it forms a hexameric ring that can accommodate a single-stranded DNA molecule (Gomis-Rüth and Coll, 2001; Gomis-Rüth et al., 2001). Many proteins including several with transmembrane segments are required for translocation of DNA during conjugation; however, which protein actually forms the channel is unknown (Christie et al., 2005; Chen et al., 2005; Cabezon and de la Cruz, 2006). By analogy to SpoIIIE, we propose that the transmembrane segments in the SpoIIIE-like DNA transporter form the channel for the single-stranded DNA.

EXPERIMENTAL PROCEDURES

General Methods

All *B. subtilis* strains were derived from the strain PY79 (Youngman et al., 1983). Sporulation was induced by resuspension at either 30°C or 37°C as described (Harwood and Cutting, 1990). Cells sporulated at 30°C were grown in 1 l cultures for biochemical analysis. This significantly slowed down the sporulation process compared to cultures of smaller volumes. Sporulation efficiency was determined in 36 hr cultures as the percentage of heat-resistant (80°C for 20 min) colony-forming units (CFU). The Supplemental Data contain tables of strains (Table S1), plasmids (Table S2), and oligonucleotide primers (Table S3) used in this study. It also includes detailed protocols for fluorescence microscopy and electron microscopy, which were performed essentially according to published procedures (Rudner and Losick, 2002; Preble et al., 2001).

GFP Focus Quantitation

To quantitate SpoIIIE-GFP foci, images were corrected for background fluorescence obtained from images acquired from isogenic strains lacking GFP, which were grown and treated identically. The

integrated intensities of the foci were obtained using the Integrated Morphometry application in Metamorph v 6.1. The intensities were compared with a calibration curve, obtained by expressing TetR-GFP in cells harboring arrays of 12, 24, 36, or 120 copies of *tetO*. Only foci from cells with cytoplasmic TetR-GFP signal were used for quantitation to ensure that the arrays were saturated for binding. The integrated intensities of these foci were determined after subtracting background from the cytoplasmic signal.

Sucrose Gradients

One liter cultures were induced to sporulate by resuspension at 30°C. One hundred milliliters of cells were harvested, protoplasted, and lysed as described (Rudner et al., 2002). Harvested membranes were solubilized and homogenized in Buffer A (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM PMSF). Membrane proteins were solubilized with digitonin or SDS (final concentration of 1%) for 1 hr at either 4°C or room temperature, respectively. The extracts were clarified by centrifugation at 40K rpm in a Beckmann Ti35 rotor and loaded onto linear sucrose gradients (10%–40% w/v), containing Buffer A and either 0.5% digitonin or 0.1% SDS. Centrifugation was performed for 3900 $\omega^2 t \times 10^5$ in a TLS-55 rotor (Beckman) at 4°C. The gradients were fractionated, and samples were precipitated with a combination of methanol and chloroform and analyzed by immunoblotting using anti-GFP antibodies.

Protoplasting Assay

Cell cultures were induced to sporulate by resuspension at 37°C and collected at hour 1.5. Cell pellets from 600 μl were resuspended in SMM buffer (Harwood and Cutting, 1990) supplemented with 1 mg/ml lysozyme and 5 μg/ml FM4-64. Cells were immediately adhered to poly-L-lysine coverslips and imaged by fluorescence microscopy. Images were acquired every 30 or 60 s over 45 min. All protoplasting was conducted at 22°C. Quantitation of the retracting septa was done by counting the number of septa that reversed during the time course as compared to the total number of cells with asymmetric septa at the initial time point.

Fluorescence Recovery after Photobleaching

Cells were induced to sporulate by resuspension at 30°C, collected at hour 3, pelleted, and resuspended in FM4-64 at a final concentration of 15 μg/ml. The cells were washed three times in 1× PBS before imaging. FM4-64 staining was imaged using a Nikon TE2000U inverted microscope equipped with a 100× Plan Apo 1.4 NA objective lens, a Chroma Wide Green filter set (Ex 535/50x, Em 590LP, Part # 11007v2), and a Hamamatsu ORCA II camera. A Photonic Instruments Micropoint nitrogen pulse laser tuned to 515 nm and focused to a near diffraction limited spot (approximately 0.7 micron) was used for photobleaching. The focused beam was positioned in the field of view using galvometer steering optics controlled with MetaMorph 7.1 software. The target regions were photobleached with five pulses at 80% attenuation of laser power. Metamorph v 7.1 software was used for quantitation of the photobleaching experiments.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, ten figures, three tables, and two movies and can be found with this article online at http://www.cell.com/cgi/content/full/131/7/1301/DC1/.

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