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MinCD-dependent regulation of the polarity of SpoIIIE assembly and DNA transfer

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During *Bacillus subtilis* sporulation, the SpoIIIE DNA translocase moves a trapped chromosome across the sporulation septum into the forespore. The direction of DNA translocation is controlled by the specific assembly of SpoIIIE in the mother cell and subsequent export of DNA into the forespore. We present evidence that the MinCD heterodimer, which spatially regulates cell division during vegetative growth, serves as a forespore-specific inhibitor of SpoIIIE assembly. The deletion of *minCD* increases the ability of forespore-expressed SpoIIIE to assemble and translocate DNA, and causes otherwise wild-type cells to reverse the direction of DNA transfer, producing anucleate forespores. We propose that two distinct mechanisms ensure the specific assembly of SpoIIIE in the mother cell, the partitioning of more SpoIIIE molecules into the larger mother cell by asymmetric cell division and the MinCD-dependent repression of SpoIIIE assembly in the forespore. Our results suggest that the ability of MinCD to sense positional information is utilized during sporulation to regulate protein assembly differentially on the two faces of the sporulation septum.

Keywords: DNA segregation/MinCD/polarity/protein assembly/sporulation

Introduction

The generation of asymmetry is essential for development in both eukaryotes and prokaryotes, since it allows the products of a single cell division event to adopt dissimilar developmental programs. The sporulating bacterium *Bacillus subtilis* provides a simple developmental system to investigate the generation of asymmetry. A key step in sporulation is the asymmetrically positioned cell division event that gives rise to two progeny of differing size and developmental fate: a smaller forespore, which ultimately becomes the spore, and a larger mother cell, which eventually lyses after contributing to spore development (Piggot and Losick, 2002). Two dramatic alterations in cellular architecture prepare the cell for polar division: reorganization of the chromosomes into an elongated structure known as the axial filament (Ryter, 1965) and relocalization of the cell division machinery from midcell to the poles (Levin and Losick, 1996). Recent studies indicate that axial filament formation results from the anchoring of both chromosomes to the cell poles (Thomaides *et al*., 2001) and the asymmetric partitioning of the future forespore chromosome, with the origin-proximal 30% condensed near one cell pole (Pogliano *et al*., 2002). Relocalization of the cell division machinery occurs when the medial ring of the key cell division protein FtsZ is converted into a spiral intermediate through which it relocalizes to two rings, one near each cell pole (Ben-Yahuda and Losick, 2002) (Figure 1B and C). This event requires elevated expression of both FtsZ and the SpoIIE phosphatase, a bifunctional protein required for both polar division and activation of the first cell-specific transcription factor, σ^F. Although the two polar division sites are formed concurrently, they are activated sequentially, producing a single forespore (Lewis *et al*., 1994; Pogliano *et al*., 1999). Cell-specific gene expression commences first in this smaller cell and subsequently in the larger mother cell, which produces three proteins responsible for repressing division at the second polar division site (Pogliano *et al*., 1999; Eichenberger *et al*., 2001). Mutants lacking mother cell-specific gene expression therefore divide at both polar sites, producing abortively disporic sporangia that contain two forespores and an anucleate mother cell (Setlow *et al*., 1991). Thus, during *B.subtilis* sporulation, the establishment of asymmetry requires a dramatic reorganization of cellular architecture, while the maintenance of asymmetry requires cell-specific gene expression.

In addition to its asymmetric position, the sporulation septum differs from a vegetative septum as it bisects the forespore chromosome, thereby trapping the origin-proximal 30% of the chromosome in the forespore. The remaining 70% of the trapped chromosome is translocated subsequently across the sporulation septum by SpoIIIE (Wu and Errington, 1994; Wu *et al*., 1995), which assembles into a focus at the septal midpoint and probably serves as a DNA channel (Wu and Errington, 1997; Bath *et al*., 2000; Errington *et al*., 2001). This post-septational chromosome segregation causes transient genetic asymmetry, since the forespore lacks the bulk of its genetic complement prior to the completion of chromosome translocation. This genetic asymmetry facilitates activation of the first cell-specific transcription factor (σ^F), by allowing the proteolytic depletion of the anti-σ^F factor SpoIIAB from the forespore during the period in which its gene remains trapped in the mother cell (Dworkin and Losick, 2001). Genetic asymmetry also impacts activation of mother cell-specific transcription, since the gene encoding the forespore-expressed signaling protein necessary for activation of mother cell-specific gene expression (*spoIIR*) must be located in the chromosomal domain initially trapped in the forespore so that it can be expressed immediately after polar septation, thereby activating mother cell-specific transcription before the production of abortively disporic sporangia (Khvorova *et al*., 2000; Zupancic *et al*., 2001).

A more subtle asymmetry is necessary for the completion of chromosome segregation into the forespore, a step that requires SpoIIIE to act in a vectoral manner, to specifically move the trapped chromosome into the forespore. Recently, we have provided evidence that the direction of DNA transfer is ensured by the specific assembly of SpoIIIE on the mother cell face of the septum, where it acts as a DNA exporter (Sharp and Pogliano, 2002b). Our results demonstrate that SpoIIIE exports DNA from the cell in which it is synthesized, and suggest the existence of a regulator of SpoIIIE assembly capable of discriminating between the forespore and mother cell faces of the sporulation septum to ensure the correct polarity of SpoIIIE assembly and DNA transfer (Sharp and Pogliano, 2002b).

Little is known about how positional information is encoded or sensed in bacterial cells. Two conserved proteins with well-established roles in spatial regulation are MinC and MinD (hereafter MinCD), which act in concert to restrict division to the cell midpoint during vegetative growth (de Boer *et al*., 1989; Levin *et al*., 1992; Varley and Stewart, 1992; Marston *et al*., 1998; Marston and Errington, 1999). These proteins prevent assembly of FtsZ at non-medial positions, thereby inhibiting polar division; in their absence, division takes place near the cell poles, producing small anucleate cells (minicells) and longer multinucleate filaments. In *Escherichia coli*, MinCD oscillate from pole to pole (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a,b), while in *B.subtilis*, MinCD appear to localize stably to both cell poles (Marston *et al*., 1998; Marston and Errington, 1999). The ability of MinCD to spatially regulate cell division and to disrupt FtsZ polymerization (Hu *et al*., 1999) prompted us to explore the possibility that they also regulate SpoIIIE assembly during sporulation. Our results suggest that MinCD contribute to the forespore-specific inhibition of SpoIIIE assembly, and thereby play a key role in establishing cellular polarity during sporulation.

Results

Effects of MinCD on SpoIIIE localization

To determine whether MinCD regulates SpoIIIE assembly, we first tested the effects of a *minCD* deletion on the localization of SpoIIIE–green fluorescent protein (GFP). When expressed from its native promoter, SpoIIIE–GFP localized as a focus at the middle of the sporulation septum (Figure 2A, arrowhead), where it probably forms a channel through which the forespore chromosome is translocated (Wu and Errington, 1997). Subsequently, this protein focus moved around the forespore to the cell pole, where it participated in the final step of engulfment, membrane fusion (Sharp and Pogliano, 1999). However, when expressed in the forespore from the *spoIIQ* promoter, SpoIIIE–GFP failed to assemble foci in most sporangia, instead localizing as a line along the polar septum (Figure 2B, arrow) (Sharp and Pogliano, 2002b). In wild-type cells expressing P_{spoIIQ}-*spoIIIE*, only 8% of sporangia with flat polar septa show normal focus assembly (Figure 2B, arrow); however, when *minCD* are deleted, this frequency rises to 43% (Figure 2C, arrowhead, and F). Similar results were obtained when SpoIIIE was expressed in the forespore at lower levels from the *spoIIR* promoter, where SpoIIIE–GFP foci were seen in 18% of wild-type sporangia (Figure 2D, arrow) compared with 56% of Δ*minCD* mutant sporangia (Figure 2E, arrowhead, and F). The absence of MinCD had no effect on focus assembly when *spoIIIE* was expressed from either its native promoter or the mother cell-specific *spoIID* promoter (data not shown; scored in Figure 2F). These results suggest that MinCD are necessary for the forespore-specific inhibition of SpoIIIE DNA translocase assembly.

Deletion of MinCD improves the efficiency of reverse chromosome translocation

If the reduced assembly of foci by forespore-produced SpoIIIE explains its reduced ability to support chromosome translocation (Sharp and Pogliano, 2002b), then the Δ*minCD* mutation should improve the ability of forespore-produced SpoIIIE to translocate DNA. In order to test this prediction, we needed to discriminate between anucleate minicells produced during growth of Δ*minCD* strains and anucleate forespores resulting from reverse chromosome translocation. This was accomplished by fusing either *gfp* or *spoIIIE-gfp* to the forespore-specific *spoIIQ* promoter. These GFP reporters were integrated in the origin-proximal region of the chromosome, which is located in the forespore immediately after division and prior to chromosome translocation. Anucleate cells containing

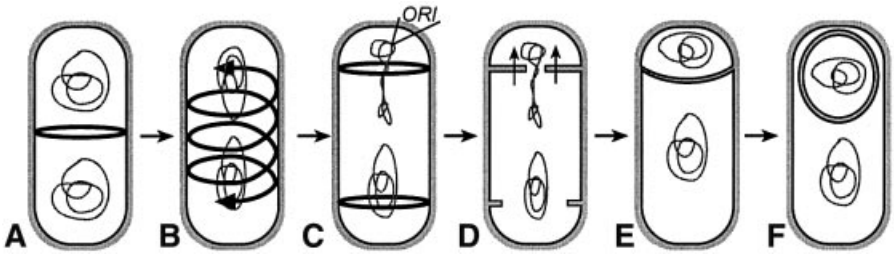
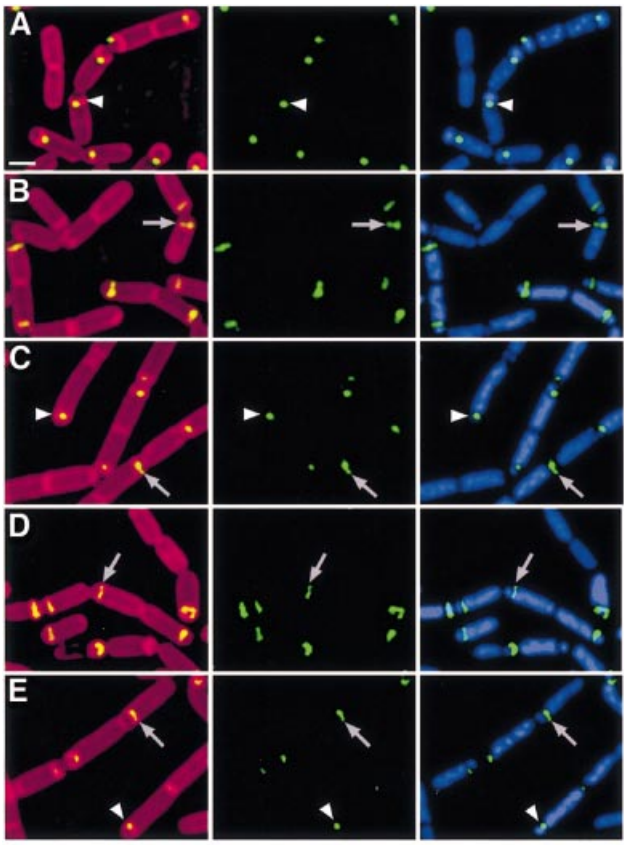


Fig. 1. The sporulation pathway of *B.subtilis*. (**A**) Vegetative *B.subtilis* cells divide at midcell, assembling medial rings of the cell division protein FtsZ (rings) and separately condensing their chromosomes prior to cytokinesis. (**B**) At the onset of sporulation, production of the SpoIIE phosphatase and higher levels of FtsZ allow FtsZ to form a spiral intermediate, and (**C**) relocalize to two rings, one near each cell pole. At the same time, the axial filament forms, with the chromosomes anchored to the cell poles and the future forespore chromosome partitioned to condense the origin-proximal 30% near the cell pole. (**D**) Division occurs at one FtsZ ring, thereby trapping the forespore chromosome in the septum, from which it is cleared by the SpoIIIE DNA translocase (arrows). Three proteins produced in the mother cell inhibit division at the second division site, thereby ensuring the maintenance of asymmetry. (**E**) During the phagocytosis-like process of engulfment, the mother cell membrane migrates around the forespore, (**F**) which ultimately is fully enclosed in the mother cell cytoplasm.

GFP fluorescence were forespores that must have contained DNA, activated forespore gene expression (Figure 3C) and have undergone reverse chromosome translocation (Figure 3D). In contrast, anucleate cells lacking GFP must have never contained DNA or activated forespore-specific gene expression (Figure 3A and B), and therefore are either minicells or anucleate forespores resulting from the defective anchoring of the chromosome to the cell pole (which has been reported to occur in certain minicell-producing mutants; Mulder *et al*., 1990; Äkerlund *et al*., 1992; Thomaides *et al*., 2001).

Deletion of *minCD* increased the efficiency with which SpoIIIE produced by the forespore-specific *spoIIQ* promoter catalyzed reverse chromosome translocation, from 32% in the wild type to 61% in Δ*minCD* 4 h after the onset of sporulation (t_4) (Figure 4A, B and E). We also noted that in Δ*minCD* strains, the frequency of anucleate cells lacking GFP fluorescence (minicells) declined from 10% at $t_{1.5}$ to <0.5% at t_4 (Figures 4E and 5C). Therefore, by later times of sporulation, the contribution of minicells to the total population of anucleate cells was negligible. This observation allowed us to also test the effect of the Δ*minCD* mutation on reverse chromosome translocation when SpoIIIE–GFP is expressed from the weaker *spoIIR* promoter, although we could not readily detect the low level of GFP fluorescence produced by this fusion after the chemical fixation employed to ensure uniform permeability to 4′,6-diamidino-2-phenylindole (DAPI). The Δ*minCD* mutation also increased the number of anucleate cells produced by P_{spoIIR}-*spoIIIE*, from 28% in the wild type to 63% in Δ*minCD* at t_4 (Figure 4C, D and F). These results suggest that the additional SpoIIIE–GFP foci assembled in Δ*minCD* forespores are active and capable of promoting reverse chromosome translocation.



F

	i	ii
P_{spoIIE}spoIIIE-gfp	100	0
P_{spoIIE}spoIIIE-gfp, Δ*minCD*	100	0
P_{spoIID}spoIIIE-gfp	96	4
P_{spoIID}spoIIIE-gfp, Δ*minCD*	97	3
P_{spoIIQ}spoIIIE-gfp	8	92
P_{spoIIQ}spoIIIE-gfp, Δ*minCD*	43	57
P_{spoIIR}spoIIIE-gfp	18	82
P_{spoIIR}spoIIIE-gfp, Δ*minCD*	56	44

Fig. 2. Effect of *minCD* deletion on SpoIIIE–GFP localization. Cells were harvested at $t_{1.5}$ and stained as described in Materials and methods. Images show Mitotracker red-stained membranes (red), DAPI-stained DNA (blue) and SpoIIIE–GFP (green). (**A**) SpoIIIE–GFP expressed from its native promoter efficiently assembles into foci at the septal midpoint (arrowhead; strain KP629). Scale bar = 1 μm. (**B** and **C**) SpoIIIE–GFP expressed at high levels in the forespore (P_{spoIIQ}-*spoIIIE-gfp*) in (B) a wild-type (KP630) or (C) a Δ*minCD* strain (KP657), the latter of which allows additional foci to assemble. Arrowheads indicate foci of SpoIIIE–GFP at the septal midpoint, while arrows indicate lines of SpoIIIE–GFP along the septum. (**D** and **E**) Localization of SpoIIIE–GFP expressed at low levels in the forespore (from P_{spoIIR}-*spoIIIE-gfp*) in (D) a wild-type (KP632) or (E) a Δ*minCD* strain (KP659). Again, more foci assemble in the Δ*minCD* strain. (**F**) Percentage of sporangia with the fusions indicated showing SpoIIIE–GFP localization as either (i) a focus or (ii) a line along a septum that appeared complete. Only sporangia with flat polar septa were scored, to ensure that only the early stages of SpoIIIE–GFP localization and production were scored. A total of 120–200 sporangia were scored for each strain.

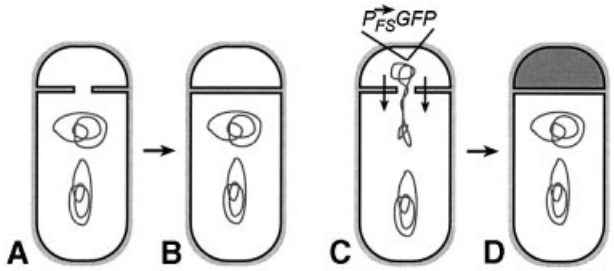


Fig. 3. Method to discriminate anucleate forespores resulting from reverse chromosome translocation from minicells. (**A** and **B**) During growth, *minCD* mutants produce small anucleate cells resulting from sporulation-independent polar division. These anucleate cells can be discriminated from anucleate forespores resulting from reverse chromosome translocation by using a forespore-expressed *gfp* fusion gene located in the origin-proximal chromosome domain. Forespores that never contained a chromosome, such as those which result from defective polar anchoring of the chromosome (Mulder *et al*., 1990; Äkerlund *et al*., 1992; Thomaides *et al*., 2001), will appear identical to minicells in this assay. (**C** and **D**) Anucleate forespores containing GFP fluorescence (indicated by shading in D) must have once contained a chromosome, activated forespore-specific gene expression and subsequently translocated the forespore chromosome into the mother cell.

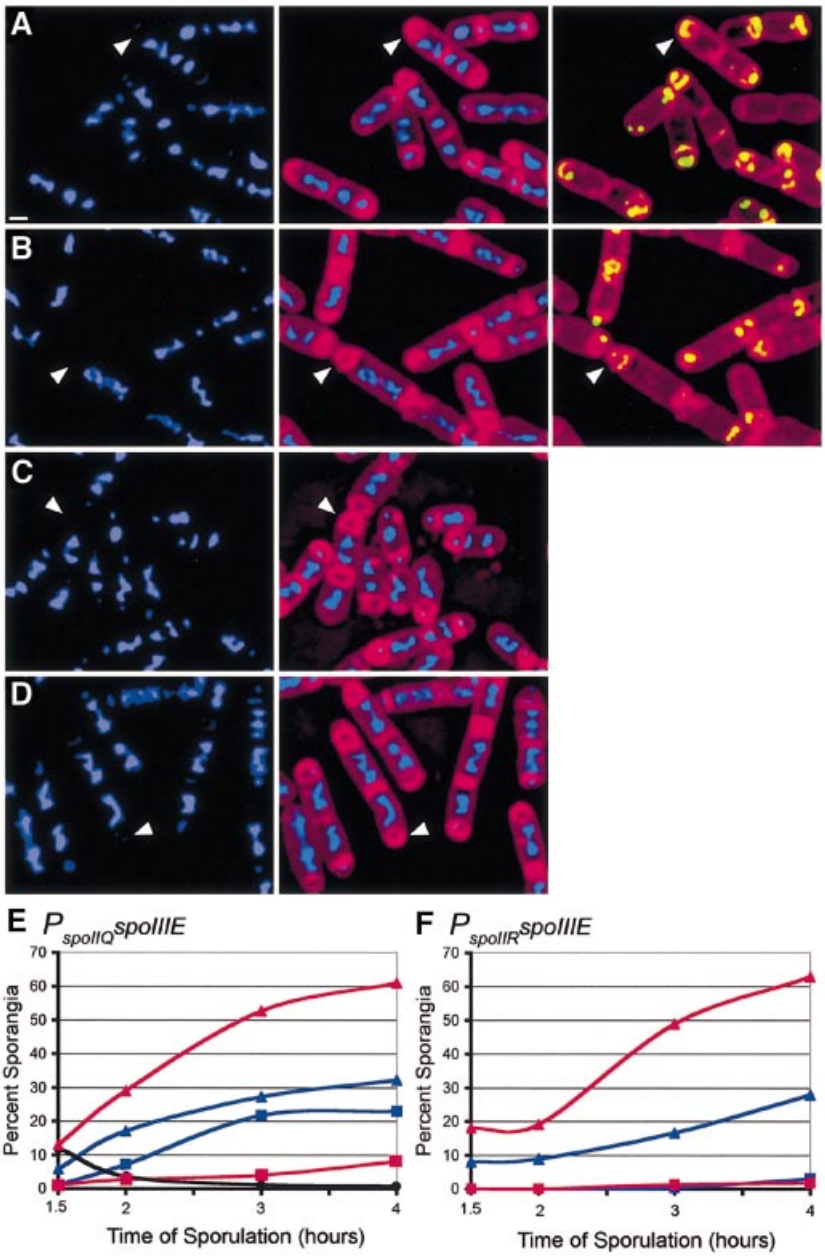


Fig. 4. Effect of the *minCD* deletion on chromosome translocation mediated by forespore-expressed SpoIIIE. (**A**–**D**) Samples from t_3 were fixed and lysozyme treated to ensure uniform permeability to fluorescent stains, then the membranes were stained with FM 4-64 (red) and the DNA stained with DAPI (blue) as described (Materials and methods). Arrowheads indicate sporangia that have undergone reverse translocation. Sporangia expressing *spoIIIE* at high levels in the forespore (P_{spoIIQ}-*spoIIIE-gfp*) in (A) a wild-type (KP630) or (B) a Δ*minCD* mutant background (KP657). SpoIIIE–GFP (green) fluorescence is also shown for this strain, to distinguish between minicells and anucleate forespores. Sporangia expressing *spoIIIE* at low levels in the forespore (P_{spoIIR}-*spoIIIE-gfp*) in (C) wild-type (KP632) or (D) Δ*minCD* mutant background (KP659). GFP fluorescence is not shown, since it was not possible to detect SpoIIIE–GFP fluorescence reproducibly after fixation, due to the low levels of expression from the *spoIIR* promoter. The scale bar in (A) = 1 μm. (**E** and **F**) Chromosome translocation kinetics when *spoIIIE* was expressed at (E) high (P_{spoIIQ}-*spoIIIE-gfp*) or (F) low (P_{spoIIR}-*spoIIIE-gfp*) levels in the forespore. Chromosome translocation was scored and is presented as the percentage of sporangia showing forward (squares) and reverse (triangles) translocation in Δ*minCD* mutant (red) or wild-type (blue) backgrounds. The black line in (E) indicates minicell frequency in Δ*minCD* P_{spoIIQ}-*spoIIIE-gfp*. At least 200 sporangia were scored for each strain.

When SpoIIIE is expressed in the forespore at high levels (from P_{spoIIQ}-*spoIIIE*), some forward DNA translocation is observed, an effect likely to be due to the escape of SpoIIIE into the mother cell, where it assembles into a functional DNA channel more rapidly than in the forespore (Sharp and Pogliano, 2002b). Deletion of *minCD* significantly reduced this forward chromosome translocation (from 23% to 8%; Figure 4E), suggesting that the Δ*minCD* mutation reduces the kinetic disadvantage of SpoIIIE assembly in the forespore, allowing forespore-expressed SpoIIIE to assemble into a functional channel before it can escape into the mother cell.

Normally, production of SpoIIIE in the mother cell closely mimics the wild-type situation, supporting efficient forward translocation and no reverse translocation. However, the deletion of *minCD* allowed mother cell-expressed SpoIIIE to support some reverse translocation (4% of 292 sporangia scored at t_4), which was never

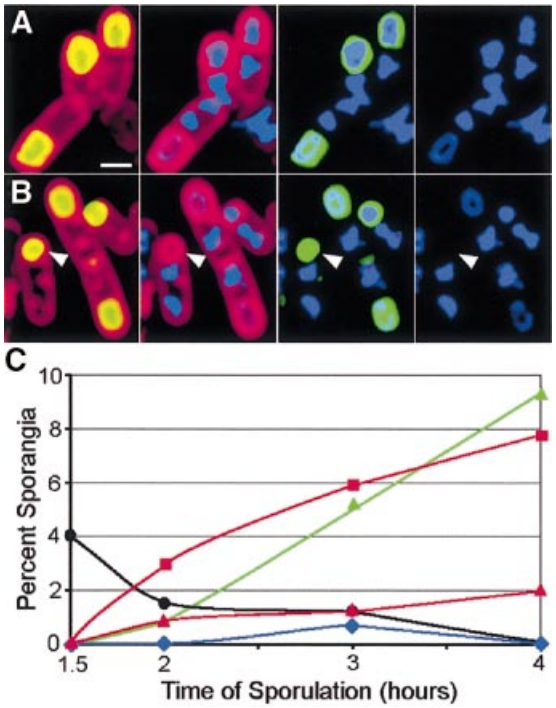


Fig. 5. Anucleate forespores are produced by the Δ*minCD* mutant. Micrographs of (**A**) wild-type (KP646) and (**B**) Δ*minCD* sporangia (KP651) at t_3 fixed and stained (as described in Materials and methods), showing FM 4-64-stained membranes (red), DAPI-stained DNA (blue) and forespore-expressed GFP (green; P_{spoIIQ}-*gfp*). The arrowhead in (B) indicates an anucleate forespore. The scale bar in (A) = 1 μm. (**C**) Scoring of minicell (black circles) and anucleate forespore production. In the Δ*minCD* mutant, the number of minicells declined during sporulation (black circles), whereas the frequency of anucleate forespores increased (red squares). When *minCD* was expressed in the mother cell of the Δ*minCD* mutant (strain KP653), the frequency of anucleate forespores was unchanged (green triangles). Expression of *minCD* in the forespore of the Δ*minCD* mutant reduced anucleate forespore production (red triangles; strain KP652), whereas introduction of a *spoIIIE* mutation abolished anucleate forespore production in the Δ*minCD* mutant (blue diamonds; strain KP655). At least 250 sporangia were scored for each strain.

observed in cells with wild-type *minCD* (Sharp and Pogliano, 2002b). This suggests that in the absence of MinCD, mother cell-expressed SpoIIIE was able to enter the forespore and assemble into an active DNA transporter, which then exported the forespore chromosome. Together, these results suggest that the Δ*minCD* mutation eliminates a forespore-specific inhibitor of reverse chromosome translocation.

MinCD regulates the polarity of SpoIIIE produced prior to division

SpoIIIE normally is produced prior to septation, rather than after division as in the preceding experiments. If MinCD normally regulate the polarity of SpoIIIE DNA translocase assembly, then a Δ*minCD* strain with a wild-type copy of *spoIIIE* should occasionally produce anucleate forespores resulting from the reversal of SpoIIIE polarity. Again, we used forespore-expressed GFP to distinguish minicells from anucleate forespores produced by reverse chromosome translocation (Figure 3), and observed neither anucleate forespores nor minicells in wild type (>2000 sporangia examined). However, Δ*minCD* sporangia produced 8% anucleate forespores (Figure 5B, arrowhead) and 0.5% minicells by t_4 (Figure 5B and C). A *spoIIIE minCD* double mutant produced the same frequency of minicells as the Δ*minCD* single mutant, but no anucleate forespores (Figure 5C), confirming that anucleate forespores result from a SpoIIIE-mediated DNA translocation event. Thus, in an otherwise wild-type strain, the absence of MinCD can lead to a reversal in the direction that SpoIIIE translocates DNA.

To confirm that MinCD acts specifically in the forespore to prevent reverse chromosome segregation, we tested the effect of mother cell- and forespore-produced MinCD on the polarity of chromosome translocation in the Δ*minCD* mutant. When *minCD* was expressed in the mother cell of a Δ*minCD* mutant, there was no effect on the frequency of reverse chromosome translocation (Figure 5C). However, when *minCD* was expressed in the forespore of the Δ*minCD* mutant, the frequency of reverse chromosome translocation dropped from 8% to <2% at t_4 (Figure 5C). Thus, MinCD is required specifically in the forespore to prevent reverse chromosome translocation. The failure to completely abolish reverse chromosome translocation when *minCD* was expressed in the forespore could result from the delay in *minCD* expression relative to *spoIIIE* expression and localization: in this experiment, SpoIIIE assembles during polar septation, while MinCD is produced after polar septation. Therefore, concentrations of MinCD might be limiting in some forespores, allowing some reverse translocation. However, because forespore-expressed MinCD inhibits reverse translocation to a significant extent, it is possible that MinCD can disassemble pre-existing SpoIIIE complexes, similar to its proposed role in disassembling FtsZ polymers (Hu *et al*., 1999).

Discussion

During *B.subtilis* sporulation, the SpoIIIE DNA translocase must act in a vectoral manner across the sporulation septum specifically to move the trapped forespore chromosome into, rather than out of, the forespore. Previously, we provided evidence that SpoIIIE acts as a DNA exporter whose vectoral activity is conferred by its differential assembly in the two cells of the sporangium (Sharp and Pogliano, 2002b). Specifically, we found that mother cell-expressed, but not forespore-expressed, SpoIIIE localizes to the septal midpoint and efficiently catalyzes chromosome translocation into the forespore. Because SpoIIIE is synthesized prior to polar septation and is present in both cells of the sporangium, these results suggest the existence of a mechanism to regulate the assembly of a functional SpoIIIE DNA translocase differentially on each side of the sporulation septum.

Here we demonstrate that the absence of MinCD allows forespore-expressed SpoIIIE to assemble a focus at the septal midpoint and catalyze reverse chromosome translocation with higher efficiency. MinCD are well-conserved proteins responsible for inhibiting cell division at polar sites in *E.coli* and *B.subtilis*, thereby restricting cell division to the cell midpoint (reviewed by Margolin, 2001; Rothfield *et al*., 2001). Our results suggest that MinCD play an unanticipated second role during sporulation, repressing SpoIIIE assembly in the forespore to ensure that

the DNA translocase assembles with the appropriate polarity in the septum. In keeping with this hypothesis, inactivation of *minCD* in an otherwise wild-type background leads to the production of anucleate forespores in a SpoIIIE-dependent manner.

It therefore appears that the ability of MinCD to sense positional information is utilized during sporulation to specifically inhibit assembly of the SpoIIIE DNA translocase on the forespore face of the sporulation septum. MinC has been shown to disassemble FtsZ filaments *in vitro* (Hu *et al*., 1999), which, together with its MinD-dependent localization to the cell poles (Hu and Lutkenhaus, 1999; Marston and Errington, 1999; Raskin and de Boer, 1999a), suggests that MinCD restrict vegetative division to midcell by disassembling FtsZ rings at the cell poles. MinCD might play a similar role in regulating SpoIIIE assembly, acting in the forespore to disassemble SpoIIIE foci. Alternatively, they might interact with another factor, which itself modulates SpoIIIE assembly; further experiments are required to resolve this question. However, our results clearly demonstrate that MinCD affect SpoIIIE localization, and suggest that these proteins are able to contribute to the polarity with which an individual protein assembles within the septum.

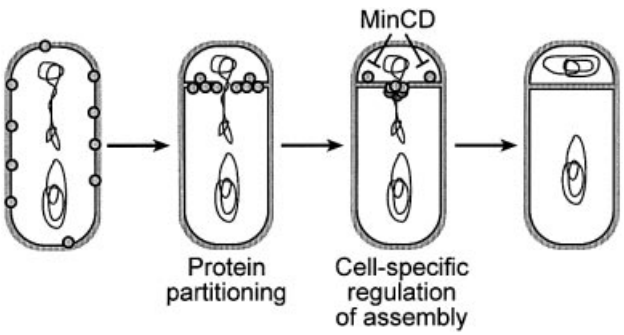


Fig. 6. Model for the role of MinCD in cell-specific SpoIIIE assembly. We propose that the polarity of SpoIIIE assembly is controlled by two events: (i) the asymmetric partitioning of SpoIIIE by polar septation, which results in the mother cell containing ~8-fold more SpoIIIE than the forespore (Sharp and Pogliano, 2002b); and (ii) the MinCD-dependent inhibition of SpoIIIE assembly in the forespore. Together, these two pathways serve to ensure that SpoIIIE assembles specifically on the forespore face of the septum, thereby ensuring the correct polarity of DNA translocation.

We propose that two processes act in concert to ensure the mother cell-specific assembly of the SpoIIIE DNA translocase (Figure 6). The first mechanism involves differences in abundance of SpoIIIE in the two cells of the sporangium (Sharp and Pogliano, 2002b). Prior to polar septation, SpoIIIE is distributed uniformly in the cell membrane; however, as a consequence of asymmetric septation, more SpoIIIE molecules are partitioned into the mother cell than the forespore. Indeed, after division, the mother cell contains ~8-fold more SpoIIIE than the forespore (Sharp and Pogliano, 2002b). SpoIIIE is subsequently recruited to the nascent septum from this pool of free protein, forming a ring at the site of membrane invagination (Sharp and Pogliano, 2002a). We propose that the larger pool size of SpoIIIE in the mother cell leads to a higher concentration of SpoIIIE on the mother cell face of the growing sporulation septum, thereby providing a kinetic advantage to complex assembly in the mother cell. The contribution of protein partitioning to the polarity of SpoIIIE assembly can be overcome by producing SpoIIIE only after polar septation, in which case forespore-expressed SpoIIIE is able to support reverse chromosome translocation, although at a reduced efficiency relative to forward chromosome translocation.

Secondly, we propose that MinCD-dependent repression of SpoIIIE assembly in the forespore serves to ensure that any SpoIIIE molecules partitioned into the forespore during polar division are unable to assemble into an active DNA translocase. Our observation that inactivation of *minCD* in an otherwise wild-type strain allows just 8% reverse chromosome translocation suggests that in most sporangia the increased SpoIIIE pool size in the mother cell achieved by protein partitioning suffices to ensure the correct polarity of SpoIIIE assembly. However, eliminating MinCD-dependent repression of translocase assembly more than doubles the level of reverse chromosome translocation observed when we eliminate the advantage of pool size by expressing SpoIIIE only in the forespore. Under these circumstances, 60% of sporangia show

Table I. *Bacillus subtilis* strains used in this study

Strain	Genotype	Reference
PY79	Wild type	Youngman *et al*. (1984)
KP92	*spoIIIE36*	Wu and Errington (1994)
KP141	Δ*spoIIIE::spc*	Pogliano *et al*. (1997)
KP629	Δ*spoIIIE::spc, amyE::*$P_{spoIIIE}$*spoIIIE-gfp*Ω*cat*	Sharp and Pogliano (2002b)
KP630	Δ*spoIIIE::spc, amyE::*P_{spoIIQ}*spoIIIE-gfp*Ω*cat*	Sharp and Pogliano (2002b)
KP631	Δ*spoIIIE::spc, amyE::*P_{spoIID}*spoIIIE-gfp*Ω*cat*	Sharp and Pogliano (2002b)
KP632	Δ*spoIIIE::spc, amyE::*P_{spoIIR}*spoIIIE-gfp*Ω*cat*	Sharp and Pogliano (2002b)
KP646	*amyE::*P_{spoIIQ}*-gfp*Ω*cat*	This study
KP651	*amyE::*P_{spoIIQ}*-gfp*Ω*tet*, Δ*minCD::erm*	This study
KP652	*amyE::*P_{spoIIQ}*-gfp*Ω*tet*, Δ*minCD::erm, sacA::*P_{spoIIQ}*minCD*Ω*cat*	This study
KP653	*amyE::*P_{spoIIQ}*-gfp*Ω*tet*, Δ*minCD::erm, sacA::*P_{spoIID}*minCD*Ω*cat*	This study
KP654	Δ*spoIIIE::spc, amyE::*P_{spoIIQ}*-gfp*Ω*tet*, Δ*minCD::erm*	This study
KP655	*spoIIIE36, amyE::*P_{spoIIQ}*-gfp*Ω*tet*, Δ*minCD::erm*	This study
KP656	Δ*spoIIIE::spc, amyE::*$P_{spoIIIE}$*spoIIIE-gfp*Ω*cat*, Δ*minCD::erm*	This study
KP657	Δ*spoIIIE::spc, amyE::*P_{spoIIQ}*spoIIIE-gfp*Ω*cat*, Δ*minCD::erm*	This study
KP658	Δ*spoIIIE::spc, amyE::*P_{spoIID}*spoIIIE-gfp*Ω*cat*, Δ*minCD::erm*	This study
KP659	Δ*spoIIIE::spc, amyE::*P_{spoIIR}*spoIIIE-gfp*Ω*cat*, Δ*minCD::erm*	This study

reverse chromosome translocation, while most of the remaining 40% show untranslocated chromosomes.

The fact that we have not yet been able to produce 100% anucleate forespores suggests the existence of additional regulatory mechanisms to ensure accurate chromosome partitioning during sporulation. One candidate for such a factor is DivIVA, which interacts with MinCD to regulate cell division during vegetative growth (Edwards and Errington, 1997; Marston *et al*., 1998). Interestingly, DivIVA has also been proposed to anchor the forespore chromosome to the cell pole prior to asymmetric division, since *divIVA minD* double mutants produce more anucleate cells than *minD* mutants during sporulation (Thomaides *et al*., 2001). However, during cell division, DivIVA is thought to regulate polar division by recruiting the MinCD division inhibitors to the cell poles, where they inhibit FtsZ assembly (Marston *et al*., 1998; Marston and Errington, 1999). It therefore seems likely that if DivIVA affects SpoIIIE assembly, it would do so by controlling the localization and function of MinCD.

Another regulatory mechanism that might contribute to accurate chromosome partitioning is the inherent polarity of the DNA substrate, which might also favor forward over reverse chromosome translocation. Recently, evidence has been provided suggesting that the direction in which the *E.coli* homolog of SpoIIIE, FtsK, moves DNA is determined by the polarity of the DNA substrate itself (Corre and Louarn, 2002). Similarly, it is possible that specific asymmetric sequence motifs are located on the region of the chromosome trapped in the invaginating septum, and that these contribute to the polarity of DNA transfer. A second mechanism by which chromosomal polarity might contribute to the direction of DNA transfer is provided by the observation that most genes in the bacterial chromosome are arranged so that transcription proceeds away from the origin of replication, an arrangement that probably reduces collisions between RNA and DNA polymerase. Similarly, during forward chromosome translocation, most RNA polymerase molecules move along DNA in the same direction as SpoIIIE, whereas during reverse chromosome translocation, RNA polymerase and SpoIIIE would move in opposite directions, perhaps resulting in collisions between RNA polymerase and SpoIIIE that might slow the rate of DNA translocation.

At each key step of *B.subtilis* sporulation, overlapping regulatory mechanisms act in concert to ensure the appropriate outcome. This is true of the switch from medial to asymmetric cell division, which requires the production of the SpoIIE phosphatase and increased levels of FtsZ (Ben-Yahuda and Losick, 2002). It is also true of activation of the first cell-specific transcription factor, σ^F, which requires both specific protein localization (Arigoni *et al*., 1995; Duncan *et al*., 1995) and transient genetic asymmetry, which allows the specific depletion of the anti-σ^F factor from the forespore (Dworkin and Losick, 2001). Here we present evidence that the polarity of DNA transfer is controlled similarly by multiple mechanisms including protein partitioning, cell-specific regulation of protein assembly and an as yet unidentified third mechanism. It seems likely that these overlapping regulatory mechanisms serve to convey robustness to sporulation, ensuring that a viable spore is produced even in a changing and uncertain environment.

Materials and methods

Strains, growth conditions and genetic manipulation

Strains were induced to sporulate by the resuspension method (Sterlini and Mandelstam, 1969). All strains (Table I) are derivatives of the wild-type strain PY79 (Youngman *et al*., 1984) and were produced using standard methods (Hoch, 1991). Disruptions of *amyE* were achieved by integrating the specified plasmid construct by a double recombination event. All plasmids used for *amyE* integration were derivatives of pDG1662 (Guerout-Fleury *et al*., 1996) with a variety of modifications as described in Sharp and Pogliano (2002b), and those summarized below. All PCR amplification was done using the Roche Expand High Fidelity PCR Kit. Restriction digests were performed with the specified restriction enzymes (New England Biolabs), ligations used T4 DNA ligase (New England Biolabs) and all cloning was performed in *E.coli* DH5α.

The first modification to pDG1662 involved inserting a promoterless super bright variant of *gfp* to yield pMDS12 (Cormack *et al*., 1996). The *gfp* gene was amplified using the primers GACTGAGAATTCGGATCCAAGCTTACTAGT**AGTAAAGGAGAAGAACTTTTCACTG** and CTGACTAGATCT**CTATTACGGCCGTTTGTATAGTTCATCCATGCCATGTG** (template homology shown in bold), digested with *Eco*RI and *Bgl*II and ligated into pDG1662 digested with *Eco*RI and *Bam*HI. We next constructed pMDS13, pMDS14, pMDS16 and pMDS78, all derivatives of pMDS12, with the *spoIIQ*, *spoIID*, *spoIIIE* and *spoIIR* promoters and translational start sites, respectively, cloned upstream of *gfp*. The *spoIIQ* promoter and translational start site was amplified using the primers GACTGAAGATCTGCTAGC**GCCATAAGTGAGCGGATGCCAAG** and CTGACTAAGCTTGGATCC**GTTTTCTTCCTCTCTCATTGTTTCATC**, the *spoIID* promoter and translational start site with GACTGAAGATCTGCTAGC**GTTGATTTAGCAAACTATATCAACGG** and CTGATCACTAGTGGATCC**TGCGAATTGTTTCATATTCAGCTGC**, the *spoIIIE* promoter and translational start site with GACTGAAGATCTGCTAGC**AACGTAAACCGATGATCATCC** and CTGACTAAGCTTGGATCC**TTTCTTTGCCACACTCATCACCTTAC**, and the *spoIIR* promoter and translational start site with GACTAGATCT**GCTTTCTTTGTTGCGGCCATACC** and CTGAAAGCTTGGATCC**TACTGTTTTTTTCATCGGTCCCCAC**. Each promoter fragment was digested with *Bgl*II and *Hin*dIII and ligated into pMDS12 digested with *Bam*HI and *Hin*dIII. Finally, the *spoIIIE* genes were inserted between the promoters and *gfp*. The full-length *spoIIIE* gene was amplified using the primers CTGACTGGATCC**CGAAAATCAAGAAAAAAACAGGCGAAA** and GACTGAACTAGT**AGAAGAGACCTCATCATATTTCTCTT**, digested with *Spe*I and *Bam*HI and ligated into pMDS13, pMDS14, pMDS16 and pMDS78 digested with the same enzymes to yield pMDS24, pMDS27, pMDS33 and pMDS80, respectively.

Cell-specific expression of *minCD* was accomplished by fusing the entire coding sequence of *minCD* to either the *spoIID* or *spoIIQ* promoters and translational start sites and integrating the hybrid genes at the *sacA* locus using derivatives of pRM52 (a gift from R.Middleton and A.Hofmeister). The *sacA* gene is located within the chromosomal domain trapped in the forespore by polar septation. The coding sequence of *minCD* was amplified using the primers CAGTGGATCC**AAAAAGCAATATG** and GTCACGGCCG**TTAAGTCTTACTCCGAAA**AATG.

Microscopy

Cells were grown to the desired stage of sporulation, harvested and processed in two different ways. When examining GFP localization, 0.5 ml of live cells were mixed with 5 μl of Mitotracker Red at 10 μg/ml and 10 μl of DAPI at 5 μg/ml (Molecular Probes), and concentrated 10-fold as described in Sharp and Pogliano (2002b). After staining, 3 μl of cells were applied to a slide, immobilized with poly-L-lysine-treated coverslips and visualized with an Applied Precision optical sectioning microscope. To evaluate chromosome translocation efficiency, cells were fixed (Pogliano *et al*., 1995), permeabilized with 0.4 mg/ml lysozyme and stained with 0.2 μg/ml DAPI and 2 μg/ml FM 4-64 (Molecular Probes). Images were acquired and deconvolved using DeltaVision v2.10 software (Applied Precision); typically, three optical sections were taken for each sample (Sharp and Pogliano, 2002b). Figures were assembled using Photoshop v5.0 software (Adobe).

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