FtsK and SpoIIIE, coordinators of chromosome segregation and envelope remodeling in bacteria

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Glossary

Divisome: group of proteins that contribute to cytokinesis in bacteria and includes tubulin and actin-like cytoskeletal elements, cell wall remodeling enzymes and their regulators.

Sporulation: genetic and morphological process that results in the formation of dormant and stress-resistant cells called spores.

Chromosome dimer resolution: molecular process that leads to the resolution of chromosome dimers after DNA replication by site-specific recombination.

RecA family of ATPases: family of proteins with a characteristic fold, a series of beta sheets sandwiched between alpha helices, that enable the protein to bind and hydrolyse ATP.

LysM domain: widely distributed protein domain that binds to N-acetyl-glucosamine in bacterial peptidoglycan or eukaryotic chitin.

Transposon-sequencing: genetic approach involving DNA-sequencing of a transposon insertion mutant library, which then allows quantification of the fitness contribution of each gene, to any given *in vivo* or *in vitro* condition.

Peptidoglycan: mesh-like material composed of sugars and peptides, that provides bacterial cells with their shape, rigidity and ability to cope with osmotic tension.

Sigma factor (σ factor): protein that facilitates binding of RNA polymerase to promoters of specific genes and the initiation of RNA polymerization.

Compartmentalization (during sporulation): separation of the mother cell and forespore cytoplasm during development.

Abstract

The translocation of DNA during bacterial cytokinesis is mediated by the SpoIIIE/FtsK family of proteins. These proteins ensure efficient chromosome segregation into sister cells by ATP-driven translocation of DNA and control chromosome dimer resolution. How FtsK/SpoIIIE mediate chromosome translocation during cytokinesis in Gram-positive and Gram-negative organisms has been the subject of debate. Studies on FtsK in *Escherichia coli* and recent work on SpoIIIE in *Bacillus subtilis*, have identified interactions between each translocase and the division machinery, supporting the idea that SpoIIIE and FtsK coordinate the final steps of cytokinesis with completion of chromosome segregation. Here we summarize and discuss the view that SpoIIIE and FtsK play similar roles in coordinating cytokinesis with chromosome segregation, during growth and differentiation.

Bacterial Cytokinesis and DNA translocases

Successful growth, division and differentiation in all organisms, including bacteria, involves precise coordination of multiple processes, often taking place simultaneously. The final step of cell division, called cytokinesis, involves fission of invaginating membranes to separate the dividing cell into two individual sister cells. In bacteria, cytokinesis also involves remodeling of the cell wall **peptidoglycan** (PG) concurrent with segregation of sister chromosomes into each daughter cell, as well as resolution of chromosome dimers that might arise during replication. This coordination is carried out by proteins of the cell division machinery, collectively known as the **divisome**. The divisome is assembled around the septal membrane after being recruited by the ring-forming, tubulin-like protein, FtsZ [1, 2]. The divisome comprises cell wall synthases and hydrolases that remodel the septal cell wall as the septum closes [3]. Importantly, since cytokinesis occurs concurrently with chromosome segregation, the divisome harbours a DNA translocase of the widely conserved FtsK/SpoIIIE family that actively pumps chromosomal DNA across the division septum.

The FtsK DNA translocase has been well studied in *Escherichia coli*, where it is an essential gene [4]. The role of FtsK is to coordinate chromosome segregation with septum closure, by clearing the septum of chromosomal DNA to ensure that the chromosome does not become bisected by the closing septum [5, 6] (Figure 1C). During **sporulation,** bacteria face a similar but more complex problem when translocating a copy of the chromosome across the asymmetric septum from the mother cell to the forespore **(Box 1)**. In *Bacillus subtilis*, the most well-studied spore-forming bacterium, approximately 25% of the chromosome is trapped in the forespore by the asymmetric septum, while the remaining 75% is translocated across the septum from the mother cell into the forespore by the DNA transporter, SpoIIIE (Figure 1D) [7, 8].

Both FtsK and SpoIIIE are membrane-anchored proteins that share similar domain structures: four transmembrane helices in the N-terminal domain, a linker region and a C-terminal translocase motor (Figure 1A) [9]. The FtsK/SpoIIIE motor domain contains three subdomains (α, β, and γ) and is classified as a member of the **RecA family of ATPases** [10]. The $\alpha\beta$ subdomain assembles into a hexameric ring containing the ATPase machinery and a central channel through which double-stranded DNA is threaded [11, 12] (Figure 1B). The γ domain ensures DNA is translocated directionally by recognizing chromosomal DNA sequence motifs **(Box 2).** The linker region of *E. coli* FtsK is very long compared to SpoIIIE, and indeed compared to other FtsKs (Figure 1A): across bacteria, the FtsK linker length is variable and the sequence poorly conserved [10].

Although functioning during different cellular events, there is emerging evidence to suggest that FtsK and SpoIIIE share similar functions, not only in DNA translocation and dimer resolution (for detailed reviews see [13, 14]), but also in regulating division proteins at the septum, such as the PG synthases and hydrolases as well as site-specific DNA recombinases, to ensure coordination between chromosome translocation and timely septal closure. This review aims to highlight recent advances in our understanding of the roles of FtsK and SpoIIIE during cytokinesis, with a focus on their interactions at the septum.

SpoIIIE, a DNA translocase in Gram-positive bacteria

SpoIIIE is a DNA translocase that functions during both *B. subtilis* vegetative growth and sporulation. During vegetative growth, chromosome translocation across the division septum is primarily carried out by a soluble DNA translocase, SftA **(Box 3)**. However, SpoIIIE is required for efficient dimer resolution and for clearing the septum of entrapped chromosomes [15-18]. During *B. subtilis* sporulation, SpoIIIE complexes assemble at the asymmetric septum to translocate a chromosome from the mother cell into the developing forespore [19]. Here, SpoIIIE has been recently shown to interact with other septal proteins and together they contribute to maintaining cytoplasmic **compartmentalization** of the mother cell and forespore, and to coordinating chromosome translocation with PG remodeling at the asymmetric septum [20].

Conflicting models for SpoIIIE-mediated chromosome translocation during sporulation - a historical perspective

Historically, studies of the mechanism of chromosome translocation by SpoIIIE during sporulation arose from the characterization of SpoIIIE mutants that, in addition to their defects in chromosome partitioning, were categorized into two mutant classes based on their ability to constrain σ^F activity to the forespore compartment: class I mutants exhibit compartmentalized σ^F activity, whereas class II mutants, which include the *spoIIIE* null mutant (Figure 2A), exhibit miscompartmentalized σ^F activity [21, 22]. Based on miscompartmentalization of the SpoIIIE class II mutants, in addition to its role in chromosome translocation, it became obvious that another role of SpoIIIE is to prevent cytoplasmic transfer between the mother cell and forespore, enabling compartment specific activity of σ^F (forespore) and σ^E (mother cell). Exactly how SpoIIIE functions in chromosome translocation,

while at the same time maintaining compartmentalization during sporulation has been the subject of a debate spanning two decades.

Early studies examining SpoIIIE class II mutants found that the mutant proteins were generally unstable and failed to localize efficiently to the septal membrane [23]. This observation suggested that SpoIIIE stability is critical to maintaining compartmentalization. Based on this observation, two possible functions for SpoIIIE had been put forward: 1) SpoIIIE acts a plug or diffusion barrier, preventing molecules, including the sporulation sigma factors, from passing through a tight septal pore [23] or 2) SpoIIIE is directly required for septal membrane fission and in its absence the septal membranes remain unfused, resulting in an aberrant pore in the septum, allowing cytoplasmic mixing and miscompartmentalization [24, 25]. Based on these possible functions for SpoIIIE, two main models, the Aqueous Pore Model (Figure 2Aii) and the Membrane Channel Model (Figure 2Ai), evolved and dominated the debate surrounding the mechanism of chromosome translocation [23, 24, 26-28]. The debate was fueled by advances in microscopy and was centered on whether the septal membranes are fused or unfused during chromosome translocation in sporulation, whether SpoIIIE plays a direct role in membrane fission and finally whether SpoIIIE exists on both sides of the sporulation septum.

Following from their earlier work on the characterization of SpoIIIE mutants [21, 22] , in 1997, using biochemical assays and immunofluorescence, the Errington laboratory proposed that SpoIIIE localizes in the septal membrane and translocates the chromosome through a “septal annulus” (i.e. septal aqueous pore) (Figure 2Aii) [23]. According to this model, the septal membranes are unfused during chromosome translocation to allow for passage of the DNA, and SpoIIIE localizes to the septal pore through its transmembrane segment [23].

Around the same time, the Pogliano laboratory proposed the idea that SpoIIIE could function in septal membrane fission. In one study, they identified an additional phenotype for a SpoIIIE mutant, *spoIIIE36* [29]. This mutant, which had been previously shown to block chromosome translocation, was found to block the engulfment membrane fission event that releases the forespore into the mother cell cytoplasm (Figure 1D) [29]. The authors also found that wild-type SpoIIIE relocalizes to the forespore pole, where engulfment membrane fission takes place, suggesting a direct role in membrane fission (Figure 1D). In later work, Pogliano and co-workers identified mutants in SpoIIIE that are miscompartmentalized (septal membrane fission is defective) but capable of engulfment membrane fission [25]. These studies led to the idea that SpoIIIE may play a general role in membrane fission. Based on this, they further proposed that SpoIIIE-mediated chromosome translocation occurs across a fused septal membrane, with two distinct lipid bilayers. The idea that SpoIIIE is directly involved in

engulfment membrane fission has since been challenged by the identification of FisB, a protein which mediates engulfment membrane fission [30, 31] and requires SpoIIIE for its stability [30].

Interestingly, in 2004 the Piggot laboratory demonstrated that the cytoplasmic miscompartmentalization defect of cells lacking *spoIIIE* is suppressed in cells lacking the mother cell engulfment PG hydrolases, SpoIID and SpoIIP [32]. This observation led Piggot and colleagues to propose that there is a septal pore that would typically be too small to allow miscompartmentalization of molecules like GFP (green fluorescent protein), but upon septal PG hydrolysis in cells lacking SpoIIIE, the pore enlarges, allowing GFP, and likely σ^F, to pass through, generating miscompartmentalization [32]. Although the exact significance of this observation remained elusive for many years to come, it hinted at a more direct relationship between SpoIIIE and PG remodeling during engulfment and that maintaining compartmentalization depends on the amount of PG present within the asymmetric septum.

To explain the translocation of DNA, a hydrophilic molecule, across the hydrophobic septal membranes, Pogliano and co-workers suggested that the transmembrane domains of SpoIIIE assemble a channel across closed septal membranes [25]. A similar model was put forth by the Rudner laboratory, except it was suggested that SpoIIIE forms two transmembrane channels, with each channel translocating an arm of the circular chromosome (Figure 2Bi), but with SpoIIIE not being required for septal membrane fission [27]. This model was based on various approaches in genetics, cell biology and biochemistry. In elegant cell biology experiments, they demonstrated that the left and right arm of the chromosome are translocated into the forespore at a similar translocation rate, suggesting coordination between two transmembrane SpoIIIE channels [27]. Furthermore, isolation of SpoIIIE complexes from membranes of sporulating cells, suggested the existence of large SpoIIIE complexes that could harbour 12 SpoIIIE molecules (2 SpoIIIE hexamers - representing one SpoIIIE transmembrane channel complex) and in theory would transverse the septal membranes [27]. Consistent with the idea that SpoIIIE could form channels transversing a fused septum, electron microscopy combined with immunogold labelling suggested that SpoIIIE-GFP is present on both sides of a continuous septal membrane.

The transmembrane channel element of the Pogliano and Rudner models led to the idea that SpoIIIE must exist on both the mother cell and forespore side of the sporulation septum. However, a complex problem originating from the idea of SpoIIIE transmembrane channels is how the final loop of chromosomal DNA is translocated across the fused septal double membrane. Rudner and Pogliano proposed similar solutions to this problem, that as the final loop of DNA reaches the SpoIIIE channels on the mother cell side, the two channels would

merge to form a larger channel which allows the DNA loop to move across the septum [24, 27]. Another possibility involving cleavage of the final loop of DNA, with subsequent ligation of DNA in the forespore, was also put forward [27]. Exactly when the final portion of the chromosome would enter completely in the forespore remained an open question. It seemed possible that the final steps of chromosome translocation could occur well after the majority of the chromosome is translocated into the forespore (occurring over a period of 20 min after asymmetric division) [27], particularly since SpoIIIE had been shown to remain as a focus well throughout engulfment [29] and SpoIIIE focus formation has been shown to depend on DNA being present in the septal membranes (Figure 1D).

Later, in 2013, propelled by advances in high-resolution microscopy, the Nöllmann laboratory utilized PALM (photoactivated localization microscopy) to study SpoIIIE localization in great detail [26]. Their work suggested that the C-terminus of SpoIIIE localizes mainly to the mother cell side of the septum, thereby translocating the chromosome only in one direction, from the mother cell into the forespore through an aqueous pore [26]. Furthermore, based on the research developments on the mechanism of chromosome translocation by FtsK, the Nöllmann laboratory proposed that SpoIIIE acts as a checkpoint to prevent completion of cytokinesis until the chromosome is fully translocated into the forespore. They proposed that this checkpoint is maintained through possible interactions with proteins involved in septal PG remodeling and completion of cytokinesis.

Finally, in 2015, building upon their earlier work with PALM [24], the Pogliano laboratory demonstrated that under certain conditions (in sporulating cells where engulfment is blocked) it is possible to observe SpoIIIE complexes on both sides of the sporulation septum in approximately 1/3 of the population, although in most cells SpoIIIE complexes are present only on the mother cell side of the septum [28]. Experiments using a targeted degradation approach [33], involving a degradable allele of *spoIIIE* (*spoIIIE-gfp-ssrA**), suggested that approximately half of the SpoIIIE molecules in the septal focus are in the mother cell and the other half in the forespore. Based on these observations, the Pogliano laboratory proposed that SpoIIIE exists on both sides of the sporulation septum, forming two coaxially paired channels (four SpoIIIE complexes). Intriguingly, using the *spoIIIE* degradable allele, they also found that SpoIIIE degradation in the mother cell, or in the forespore, resulted in chromosome translocation defects [28]. These observations led to the hypothesis that SpoIIIE may function as a bidirectional motor: the mother cell SpoIIIE complexes are essential for chromosome translocation into the forespore and in their absence, the forespore SpoIIIE complexes can function as a DNA exporter, translocating DNA out of the forespore. How SpoIIIE complexes

in the mother cell could contribute to preventing the DNA exporter activity of forespore SpoIIIE complexes is not clear. One possibility is that additional players are involved.

SpoIIIE coordinates chromosome segregation with PG remodeling at a highly-stabilized pore – a new perspective

Until recently, a lack of genetic evidence in support of either the Aqueous Pore or Channel model allowed the debate surrounding these models to remain open for over 20 years. Recent work from the Rodrigues laboratory has provided strong genetic evidence that the forespore chromosome is translocated across a septal pore, and not a closed septal membrane [20]. Importantly, Rodrigues and co-workers also provided evidence that SpoIIIE not only functions in chromosome translocation but also functions to maintain the size and integrity of the septal pore by interacting with two proteins: PbpG, a forespore PG synthase, and SpoIIIM, a mother cell **LysM domain**-containing protein (Figure 2Biii). Collectively this work has led to a comprehensive model (Highly Stabilized Septal Pore Model) for chromosome translocation during sporulation, one that integrates PG remodeling and biophysical processes occurring at the asymmetric septum at the onset of engulfment.

Using a genetic approach called **transposon-sequencing** [34] to identify proteins that function with PbpG in PG remodeling during engulfment, the authors identified SpoIIIM. Using fluorescence microscopy, sporulating cells lacking PbpG and SpoIIIM were found to have miscompartmentalized σ^F activity, to a similar degree as cells lacking SpoIIIE (Figure 2A). To understand why cells lacking PbpG and SpoIIIM exhibit severe miscompartmentalization, the authors investigated the possibility that these proteins contribute to SpoIIIE stability. Interestingly they found that SpoIIIE is stable and localizes as a discrete focus in the absence of PbpG and SpoIIIM, like that observed in WT cells. However, unlike WT cells, in cells lacking PbpG and SpoIIIM, the SpoIIIE focus fails to disassemble. Using chromosome translocation assays based on the *lacO*-LacI system developed for *B. subtilis* [7], the authors found that SpoIIIE is active in cells lacking PbpG and SpoIIIM and capable of translocating the chromosome into the forespore. However, here the authors made yet another interesting observation: although cells lacking PbpG and SpoIIIM could translocate the chromosome into the forespore, the chromosome failed to remain there. Instead the chromosome was lost back to the mother cell, in a process designated as chromosome efflux, that is likely passive in nature based on the retention pattern of fluorescently labelled *oriC/ter* markers in the forespore [20]. Thus, the absence of PbpG and SpoIIIM allowed SpoIIIE activity but led to a leaky pore through which the chromosome could diffuse back out of the forespore. Future experiments examining the chromosome translocation rate of SpoIIIE in the absence of PbpG and SpoIIIM may reveal if these proteins play a more direct role in chromosome translocation.

Importantly, blocking engulfment PG hydrolysis suppressed the miscompartmentalization and chromosome efflux phenotypes of cells lacking PbpG and SpoIIIM [20]. Thus, SpoIIIM and PbpG appear to function in constraining the septal pore, by counterbalancing the activity of the PG hydrolases during engulfment. Consistent with this idea, the catalytic activity of PbpG was shown to be required for compartmentalization and chromosome retention in the forespore. The role of SpoIIIM and PbpG in maintaining the balance between PG synthesis and hydrolysis was also shown to contribute to sustaining the increased turgor pressure on the septal PG that results from the chromosome being translocated into the forespore compartment, a biophysical effect characterized by Pogliano and co-workers [35]. Consistent with this idea, the authors found that miscompartmentalization in the absence of SpoIIIM and PbpG can be partially suppressed by abolishing chromosome translocation [20].

Despite recent studies examining sporulating cells using cryo-focused ion beam milling coupled with cyro-electron miscroscopy (Cryo-FIB-ET) [36], visual evidence of the septal pore containing SpoIIIE is lacking. It is formerly possible that the septal pore is too small and thus can be easily missed, despite the thin sections generated using Cryo-FIB-ET. Interestingly, in some tomograms it is possible to visualize a “constriction” in the asymmetric septa of sporulating cells [36]. This constriction can be viewed as evidence that the septum is not closed and harbors a pore during chromosome translocation. Alternatively, as hypothesized by Pogliano and co-workers, this constriction may represent the site of SpoIIIE channels traversing the closed septum [36]. Assuming a total of four SpoIIIE hexamers (24 SpoIIIE molecules, ~2.1 MDa) needed to establish two coaxially-paired SpoIIIE channels across the septal membranes, it is noteworthy that direct evidence of SpoIIIE transmembrane channels remains elusive. Nonetheless, one of the most remarkable observations supporting the idea of a highly stabilized septal pore, and argues against the idea that the chromosome is translocated through a closed septum [27, 28], is the complete retraction of the septal membranes that delineate the pore during chromosome translocation in certain genetic conditions. Rodrigues and co-workers found that cells lacking SpoIIIE, PbpG or SpoIIIM are susceptible to septal retraction when the highly conserved SpoIIIAH-SpoIIQ interaction is abolished (Figure 2Biii). SpoIIIAH-SpoIIQ forms a complex bridging the forespore membrane and the mother cell membrane and this interaction holds the two sets of membranes in close proximity and might help prevent retraction of the membranes [37]. Cells lacking these proteins initiate asymmetric division and activate forespore transcription, but due to the activity of the PG hydrolases required for engulfment, the asymmetric septum retracts, abolishing compartmentalization and spore development. This dramatic phenotype suggests that the septal pore is not only stabilized by newly synthesized PG within the septum but also by protein-protein interactions across the septum. In other experiments, using bacterial two-

hybrid assays and fluorescence microscopy, the authors demonstrated that PbpG and SpoIIIM interact with SpoIIIE, thus revealing a likely direct coordination between chromosome translocation and septal PG remodeling during engulfment. Altogether, these data support the idea that the septal pore is stabilized by multiple molecular mechanisms to ensure genetic and cytoplasmic compartmentalization during development.

Finally, several questions remain answered regarding the molecular relationships between SpoIIIE, PbpG and SpoIIIM, as well as the exact organization of these proteins at the septal pore (**Outstanding Questions Box**). For instance, it remains unclear what role SpoIIIM plays in maintaining the septal pore and compartmentalization. One possibility is that SpoIIIM binds to PG through its LysM domain and bridges SpoIIIE to the septal PG, thereby stabilizing the pore through protein-PG interactions during chromosome translocation. In this capacity, SpoIIIM may also function to inhibit a putative DNA exporter function of SpoIIIE [28], although this hypothesis seems unlikely based on the pattern of chromosome loss to the forespore in cells lacking SpoIIIM and PbpG [20]. Alternatively, SpoIIIM may function to activate PG synthesis from the mother cell side of the septal pore, by interacting with a yet-to-be-defined PG synthase [20]. The answer to these questions may reveal the exact biochemical mechanisms underlying septal pore stabilization during chromosome translocation and its subsequent closure after completion of chromosome translocation.

FtsK, a DNA translocase in Gram-negative bacteria

FtsK is a multidomain, multifunctional protein, with high homology to SpoIIIE in the C-terminal motor domain. It is essential in *E. coli* as it forms part of the divisome, but also functions in chromosome segregation and dimer resolution, and manages to co-ordinate these functions with cell division.

Interactions between FtsK and other divisome proteins

FtsK is a key part of the divisome and interacts with several cell division proteins mainly through its N-terminal and linker domains (Figure 2C). FtsK is recruited to the septum via interaction with components of the FtsZ ring; two-hybrid screens by several groups have shown that multiple regions of FtsK interact with FtsZ [38-40]. Two-hybrid interaction has also been shown between FtsK and ZapA in both *Streptococcus pneumoniae* and *E. coli*, while a FtsK interaction with FtsA has been shown in *S. pneumoniae* but was not detected in *E. coli* [41, 42]. Once localized to the site of septation, FtsK interacts with and helps recruit several proteins involved in PG synthesis – FtsW/Q/L/I [38-40]. Constriction of the septum is then

dependent upon the activity of the FtsQ/L/B complex and the FtsW/I transglycosylase/transpeptidase complex [47]. These interactions have also been demonstrated by two-hybrid screens as well as co-immunoprecipitation in the case of FtsK-FtsQ [45], and FtsK-FtsW in *S. pneumoniae* [41], and are consistent with the failure to localize these divisome components in a FtsK mutant. Furthermore, FtsK has been identified as a member of the ~1MDa divisome complex in *E. coli* [46]. Despite the wealth of data suggesting these multiple interactions, definitive delineation of the FtsK-divisome interaction network is still lacking. Interestingly, evidence suggests that FtsK may be involved in the switch from lateral peptidoglycan synthesis for cell growth, to peptidoglycan synthesis for septum closure [5, 43], and that these two states are mutually exclusive [44]. For example, it was shown that under certain conditions (large chromosome inversion), FtsK activity seems to delay cell division, and concurrently cell elongation is stopped [5].

Recently it has been shown that FtsK interacts with an outer-membrane protein RlpA and recruits it to the division septum, potentially forming a link between inner membrane invagination, PG synthesis and the outer membrane [48]. The precise role of RlpA is unknown but several lines of evidence suggest the FtsK-RlpA interaction may be involved in the switch between cell elongation and cell division [48]. In the same study, a number of other potential FtsK interactions with periplasmic/outer membrane proteins were identified by protein-crosslinking and mass spectrometry, including many involved in cell envelope remodeling [48], but direct interactions between FtsK and these proteins awaits confirmation.

Evidence that FtsK coordinates chromosome segregation with cell division

Current evidence suggests that FtsK influences cell division via its many interactions with key divisome proteins. However, the converse is also true: the translocase activity of FtsK appears to be dependent upon the initiation of cell division; FtsK is active as a translocase only once septation has begun [5, 6, 49, 50]. Addition of cephalexin, which inhibits FtsI, also prevents FtsK activity [6, 50], but this may be due to a failure to localize FtsK in the presence of cephalexin. The dependence of FtsK activity upon later stages of cell division was elegantly shown by the Barre laboratory [6].

The N-terminus of FtsK forms hexamers, independently of the C-terminus [51], which did raise the possibility of the N-terminus forming a septal channel through which DNA could be pumped across a fused septum similar to the early models for SpoIIIE that proposed a septal channel with fused membranes [24, 27, 52]. However, there is no evidence that this is the case for FtsK, and to the contrary, there are several lines of evidence suggesting that FtsK acts before

membrane fission and that FtsK can even delay cell division while it is actively segregating chromosomes [5, 6].

FtsK was shown to be active once septation was under way, but before it had completed [5, 6]. In cells that had a large chromosomal inversion, with roughly 2/3 of the right replichore being inverted, FtsK's translocase activity became essential for viability [5]. In this strain, one replication fork copies the majority of the chromosome, leading to longer replication times and alterations in chromosome segregation. This leads to a greater requirement for FtsK to translocate DNA to achieve proper partitioning of the chromosomes before division, and is reminiscent of other mutants that cause defects in chromosome segregation, such as the *mukB* mutant, where FtsK activity is also essential [53]. In the inversion strain, cells were seen with invaginated septa but had delayed cell division, presumably because division was delayed whilst FtsK was still actively translocating misaligned chromosomes. Further, when FtsK was inactivated, these cells often displayed aberrant morphologies characteristic of mutants deficient in PG synthesis [5]. These data strongly support a model where FtsK acts to segregate chromosomes at an invaginating septum; PG synthesis by FtsI is required to begin the process, but FtsK then somehow delays cell division while it is active, until all DNA has been cleared from the septum [5, 6]. The exact nature of how this signaling might occur is not currently known but has been suggested to be linked to a structural/allosteric change in the FtsK linker region when the motor is active [54]. If the active motor stretches the linker region behind it, then this could disrupt contacts between the linker, the PG remodeling enzymes and possibly FtsZ, turning off cell constriction activity. This model still awaits rigorous testing.

Another, seemingly conclusive, line of evidence against a model where the N-terminus of FtsK forms a septal pore is the finding that the N-terminal domain is dispensable for FtsK translocation and activation of recombination [40]. In a *ftsA** hyperactive background, $FtsK_N$ is not required for divisome assembly. In these cells a fusion of the linker and C-terminal domains of FtsK to an integral membrane protein of the late divisome (like FtsW, L or Q) was sufficient to support chromosome segregation and recombination. FtsK can thus act as a translocase and activate dimer resolution without needing specialized pore or channel to bridge fused septal membranes.

Roles of FtsK in dimer resolution

During DNA repair by homologous recombination, as often occurs during replication, a crossover can occur between the two nascent chromosomes. In a cell with a circular genome, any odd number of crossovers will result in the two daughter chromosomes becoming joined

in a chromosome dimer. **Chromosome dimer resolution** is an essential process: unresolved dimers lead to cell death. Bacteria and archaea overcome this impediment by introducing another crossover to resolve the chromosome dimer into monomers. This is a site-specific recombination event catalyzed by the Xer recombinase proteins [55]. In *E. coli,* and many other bacteria, there are two related tyrosine recombinases, XerC and XerD, that act at the 28bp *dif* site to carry out this reaction. The *dif* site is at the centre of the terminus region of the chromosome, between the inner replication fork trap structures (*Ter* sites in *E. coli*). The recombination catalyzed by XerCD is dependent upon FtsK: the very C-terminus of FtsK (the γ domain) is necessary and sufficient to promote the first catalytic step of the reaction mediated by XerD [56, 57]. As FtsK is anchored at the septum, it requires its directional translocation to be able to segregate chromosomes and to localize the *dif* sites. Recombination will then be activated. Remarkably, many of the same amino acids within the FtsKγ winged-helix fold recognize the KOPS sequence (**Box 2**) and interact with XerD to activate recombination [56, 58].

Concluding remarks

Despite years of controversy, emerging evidence suggests SpoIIIE and FtsK function in a similar way and play a critical role in coordinating cell envelope remodeling and the final steps of chromosome segregation during cytokinesis (Figure 3, Key Figure). They do so by governing the localization and activity of a variety of proteins that are directly connected to cell envelope layers. While defining these interactions has been key to elucidating the multifunctional nature of SpoIIIE and FtsK, and bridging the differences between them, deciphering the exact biochemical and structural relationships they establish with their interacting partners could reveal the finer details of how a single multidomain protein connects molecular events in the cell envelope to the DNA (see Outstanding Questions).

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Box 1 - Stages of Bacterial Sporulation

Some bacteria in the phylum Firmicutes enter a developmental pathway called sporulation. Sporulation is induced by starvation and results in dormant, stress-resistant spores. Spores resist various stresses, including desiccation, UV radiation, high-temperatures, digestion by protozoans, detergents and acid [59]. Spores underlie the epidemiology of spore-forming pathogens, including for example, *Bacillus anthracis* (Anthrax disease*)*, *Bacillus cereus* (food poisoning), *Clostridioides difficile* (infectious diarrhoea), *Clostridioides botulinum* (botulism) and *Paenibacillus larvae* (honey-bee pathogen) [60].

The first distinctive step in sporulation is asymmetric division (Figure 1D). Asymmetric division generates two cells of different size and developmental fates. The smaller cell (i.e. forespore) develops into a dormant spore. The larger cell (called mother cell) contributes to forespore development but then dies. Asymmetric division precedes chromosome segregation and traps approximately 25% of the chromosome in the forespore, while the remaining 75% is translocated into the forespore by SpoIIIE (Figure 1D) [7].

The morphogenetic events during forespore development are controlled by cell-specific **sigma factors**, that activate cell-type-specific gene expression in the mother cell or forespore (Figure 1D) [61]. Upon asymmetric division, σ^F is activated in the forespore, which then signals activation of σ^E in the mother cell. As the spore develops, activation of σ^G in the forespore signals σ^K activation in the mother cell [61].

Concurrent with chromosome translocation into the forespore, and as gene expression occurs in the forespore and mother cell, the asymmetric septum that compartmentalizes the forespore and mother cell undergoes remodeling (Figure 1D). σ^E directs expression of PG hydrolases that assemble into the DMP complex (composed of SpoIID, SpoIIM and SpoIIP), which thins the septal PG and facilitates migration of the mother cell membranes around the developing forespore in a phagocytic-like process called engulfment (Figure 1D) [62, 63]. In addition to PG degradation, engulfment is thought to involve PG synthesis by biosynthetic complexes [64]. During engulfment, the conserved SpoIIIAH-SpoIIQ protein-protein interaction holds the mother cell and forespore membranes together, promoting forward migration of the engulfing membranes, functioning like a ratchet [37]. Upon engulfment completion, the engulfing membranes undergo fission and the forespore is released into the mother cell as a double-membrane protoplast, with an inner membrane from the forespore and an outer membrane from the mother cell [65] (Figure 1D). Inside the mother cell, the forespore matures through the deposition of cortex PG and protective coat layers around it [66]. Upon spore maturation, the mother cell lyses, releasing the spore into the environment, where it remains dormant until nutrients become available.

Box 2 - Mechanism of sequence-directed DNA translocation by SpoIIIE and FtsK

The comparable functions and high sequence similarity of FtsK and SpoIIIE suggest a similar mechanism of DNA translocation. Underlying the mechanism of DNA translocation is the ability of FtsK and SpoIIIE to translocate the chromosome unidirectionally.

Directional DNA transport by FtsK: FtsK recognizes an 8 bp sequence, called KOPS (FtsK orienting polarized sequences) that has the consensus 5´ GGGNAGGG 3´ [67] [68]. These sequences are over-represented in the terminus of the chromosome and are highly polarized so that they point toward *dif* on each chromosome arm. Three γ domains of FtsK bind to one KOPS site and it is thought that this helps to nucleate motor hexamer formation on the DNA, in a loading reaction [58]. The loading is such that the motor will subsequently translocate towards *dif* on each chromosome arm.

Directional DNA transport by SpoIIIE: The chromosome of *B. subtilis* also has similar polarized sequences that give directionality to SpoIIIE: the SRS sequences (5´ GAGAAGGG 3´), which are similar to the *E. coli* KOPS [69]. As with FtsK-KOPS, these sequences are recognized by the γ domain of SpoIIIE. However, the prevailing model for SpoIIIE-SRS interaction is that SpoIIIE binds DNA as a hexamer in a random orientation and slides upon DNA until it encounters a SRS sequence in the proper direction, whereupon its ATPase motor is activated and chromosome translocation begins [70].

Box 3 - Roles of SftA and SpoIIIE during vegetative growth

SpoIIIE is essential for chromosome translocation during *B. subtilis* sporulation, however, it is not essential for chromosome segregation during vegetative growth in normal conditions [15, 22]. Instead, *B. subtilis* encodes a second DNA translocase, called SftA, that contains a C-terminal ATP-dependent DNA translocase domain that is homologous to the C-terminal DNA translocase domains of FtsK and SpoIIIE [18].

During vegetative growth, *B. subtilis* SpoIIIE and SftA function independently, and synergistically, in chromosome segregation [18]. SpoIIIE, which is membrane-bound via its N-terminal transmembrane segments, has a punctate distribution throughout the cell membrane [18, 71]. SftA, however, is a soluble, hexameric protein that localises to the division septum in an FtsZ- and FtsA-dependent manner [18, 71]. The exact mechanism of SftA localization to the division septum remains unclear, however, existing localization data suggest that it is dependent on the cytosolic N-terminal domain of SftA [18, 71]. Here, SftA translocates

unsegregated chromosomes to clear the midcell of DNA prior to septum closure [18], in a manner presumably analogous to KOPS- or SRS-mediated directional DNA translocation by *E. coli* FtsK or *B. subtilis* SpoIIIE, respectively [67, 68, 72]. Meanwhile, in vegetative cells, SpoIIIE is almost exclusively recruited to the division septum to rescue entrapped DNA under conditions of DNA damage or when chromosome segregation and cell division become uncoupled [15, 18, 73].

The *E. coli* DNA translocase, FtsK, also functions in chromosome dimer resolution by activating the XerCD recombinases [74, 75]. Similarly, in addition to mediating chromosome translocation, *B. subtilis* SftA, synergistically with SpoIIIE, facilitates the resolution of chromosome dimers by bringing the *dif* sites into close proximity to allow the site-specific DNA recombinases, RipX and CodV, to catalyse DNA strand exchange and dimer resolution [17, 76]. Thus, *B. subtilis* has mechanisms to resolve chromosome dimers before and after septation, via SftA and SpoIIIE, respectively.

Homologues of SftA are present in the *Firmicutes*, including in *S. aureus* [18], which encodes a *B. subtilis* SftA and *E. coli* FtsK homologue, FtsK, and a second DNA translocase, SpoIIIE, that is homologous to *B. subtilis* SpoIIIE [24, 77]. These two DNA translocases appear to have partially-redundant roles, since *S. aureus* cells lacking both *ftsK* and *spoIIIE* have more deleterious chromosome segregation and morphological defects compared to cells lacking either gene alone [77]. Thus, the presence of two DNA translocases appears to be widely adopted in bacteria to clear the closing septum of DNA, including the rescue of septum-entrapped DNA, and, at least in *B. subtilis*, to facilitate dimer resolution.

Figure Legends

Figure 1. The FtsK/SpoIIIE family translocases share similar protein domains and functions. (A) Graphical representation of protein domains of the DNA translocases SpoIIIE and FtsK, illustrating the transmembrane, linker, motor and DNA-interacting domains. (**B)** Crystal structure of the FtsK motor domain of *Pseudomonas aeruginosa* (PDB 2IUU), modelled with double-stranded DNA shown in purple [75]. The hexameric motor domain is coloured in green. **(C)** Chromosome translocation by SpoIIIE during *B. subtilis* cell division (top) and by FtsK during *E. coli* cell division (bottom). SpoIIIE and FtsK complexes assemble at midcell and translocate replicated chromosomes and resolve chromosome dimers to clear the septum of DNA prior to septum closure. (**D)** Chromosome translocation by SpoIIIE during *B. subtilis* sporulation. SpoIIIE complexes assemble at the asymmetric septum during

sporulation to translocate a chromosome from the mother cell (mc) into the forespore (fs). In addition to DNA translocation, SpoIIIE is required to maintain cytoplasmic (white and cyan) and genetic (eg. sporulation-specific sigma factors: σ^F, σ^E, σ^G, σ^K) compartmentalization of the mother cell and forespore. After chromosome translocation is complete, SpoIIIE disperses around the forespore membrane. SpoIIIE dispersal is thought to represent detachment from DNA, since SpoIIIE focus formation has been shown to depend on DNA trapping in the asymmetric septum [78]. SpoIIIE and FtsK complexes are shown in green, chromosomes as black squiggles, origin of replication (*oriC*) as blue circles, *ter* sites as red circles and PG in grey.

Figure 2. Models of DNA translocation by SpoIIIE and FtsK highlighting their interacting partners. (A) Schematic illustrating miscompartmentalization of mother cell and forespore cytoplasmic contents in the absence of *spoIIIE* (Δ*spoIIIE*) and in the absence of *pbpG* and *spoIIIM* (Δ*pbpG* Δ*spoIIIM*). Miscompartmentalization is represented by leakiness of forespore contents (cyan) into the mother cell. SpoIIIE complexes are shown as green circles, chromosomes as black squiggles, origin of replication (*oriC*) as blue circles, *ter* sites as red circles and PG in grey. (**B).** Close-up of dotted area in A, showing models of DNA translocation by SpoIIIE during *B. subtilis* sporulation. **(i)** Membrane Channel Model. SpoIIIE complexes (green) form channels through a fused septal membrane via their transmembrane domains, with each channel translocating an arm of the chromosome. **(ii)** Aqueous Pore Model. Septal membranes are unfused and SpoIIIE assembles at an aqueous pore via its transmembrane segments, with its C-terminal domain predominantly on the mother cell side. **(iii)** Highly Stabilised Septal Pore Model. An aqueous septal pore is stabilised by protein-protein interactions involving SpoIIIE, PbpG (orange) and SpoIIIM (light blue). These interactions are required for coordinating chromosome translocation with PG remodeling during engulfment, while maintaining cytoplasmic and genetic compartmentalization. The septal pore is further stabilised by interactions between SpoIIIAH (dark blue) and SpoIIQ (magenta) across the forespore membranes. PG is shown as grey dots and lines. fs: forespore; mc: mother cell. **(C)** Model of DNA fssetranslocation by FtsK during *E. coli* cell division**.** Left: FtsK complexes (green) assemble at midcell as part of the divisome. Chromosomes are shown as black squiggles and PG is shown in grey. Right: Close-up of dotted area on the left. FtsK complexes assemble on either side of the division septum to translocate both chromosomes and clear the septum of DNA before septal closure. DNA translocation is coordinated with PG synthesis and septal closure by interactions between FtsK and the divisome components FtsQLB (orange), FtsWI (blue) and FtsN (pink). Further interactions are made between FtsK and cell envelope proteins including RlpA (magenta). PG is shown as grey dots and lines.

Figure 3 (Key Figure). 3-D illustration of SpoIIIE and FtsK at the division site during *B. subtilis* sporulation and *E. coli* vegetative growth. SpoIIIE and FtsK (shown in pastel green) assemble at the division site and coordinate chromosome segregation and cell envelope remodeling during cytokinesis. The PG is shown as light grey cables and the DNA as white helical cables. Proteins involved in cell envelope remodeling are shown in different colours (for more details on these proteins refer to Figure 2). The rendering effect that is apparent on the proteins is inspired on structural data but does not contain structural information. For simplicity, only a limited number of SpoIIIE and FtsK molecules are shown to assemble into DNA-bound complexes. Existing data suggest that an average of 34 to 47 SpoIIIE molecules [26, 28] and an average of 25 FtsK molecules [51] may exist at the division site. While these molecules likely delineate the closing septum during cytokinesis, how all these molecules are organized there is unknown (see Outstanding Questions Box).

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Figure 1

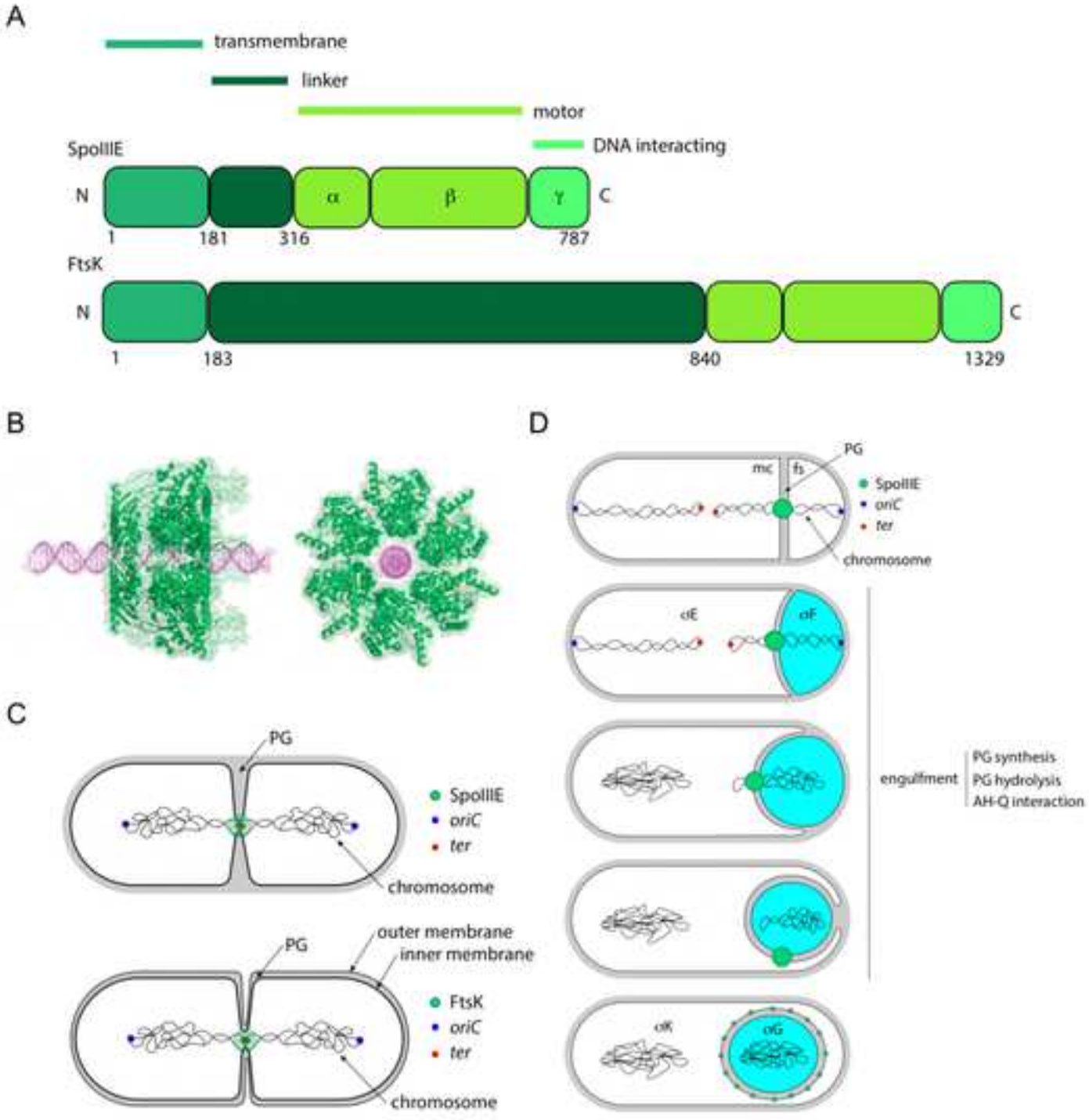

Figure 2

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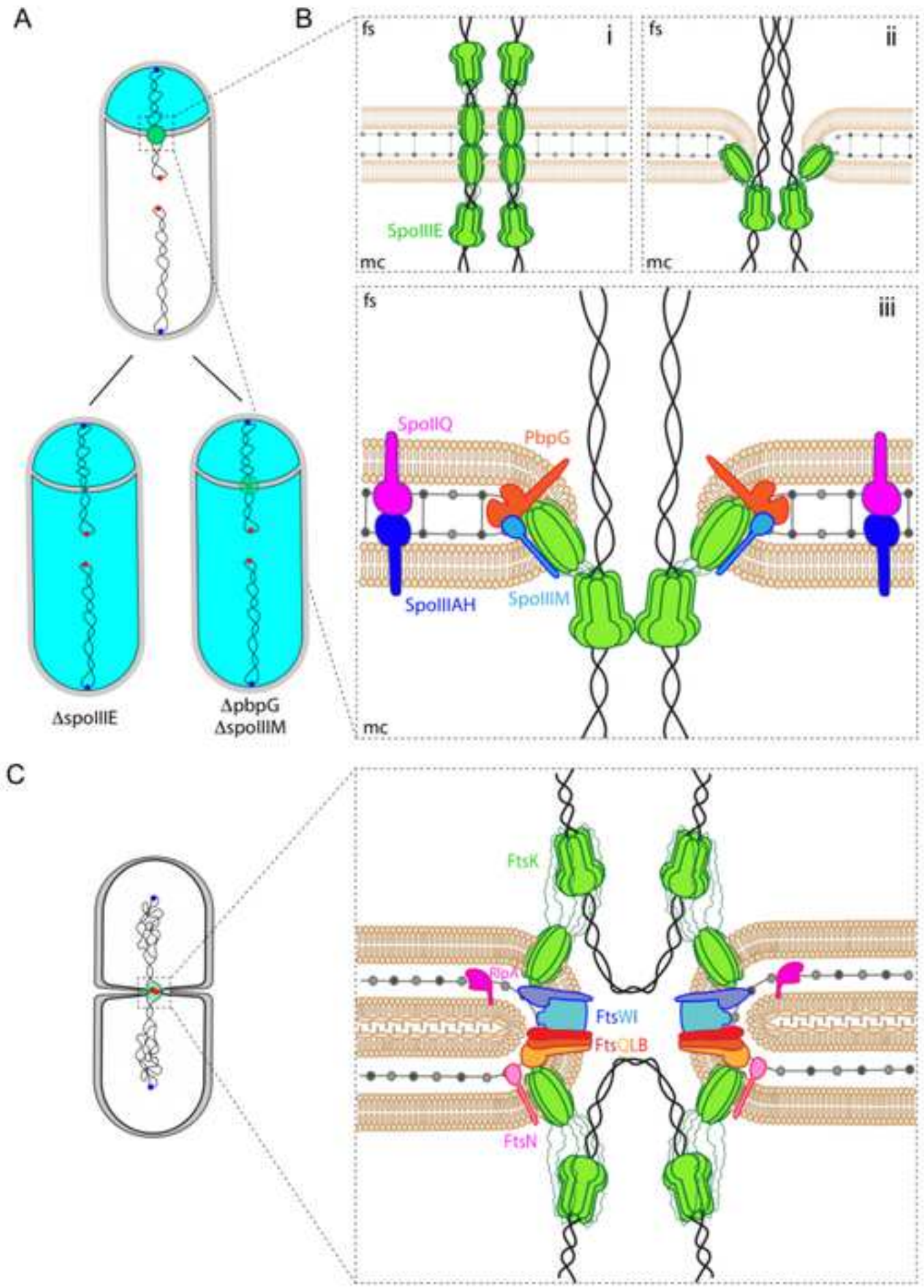

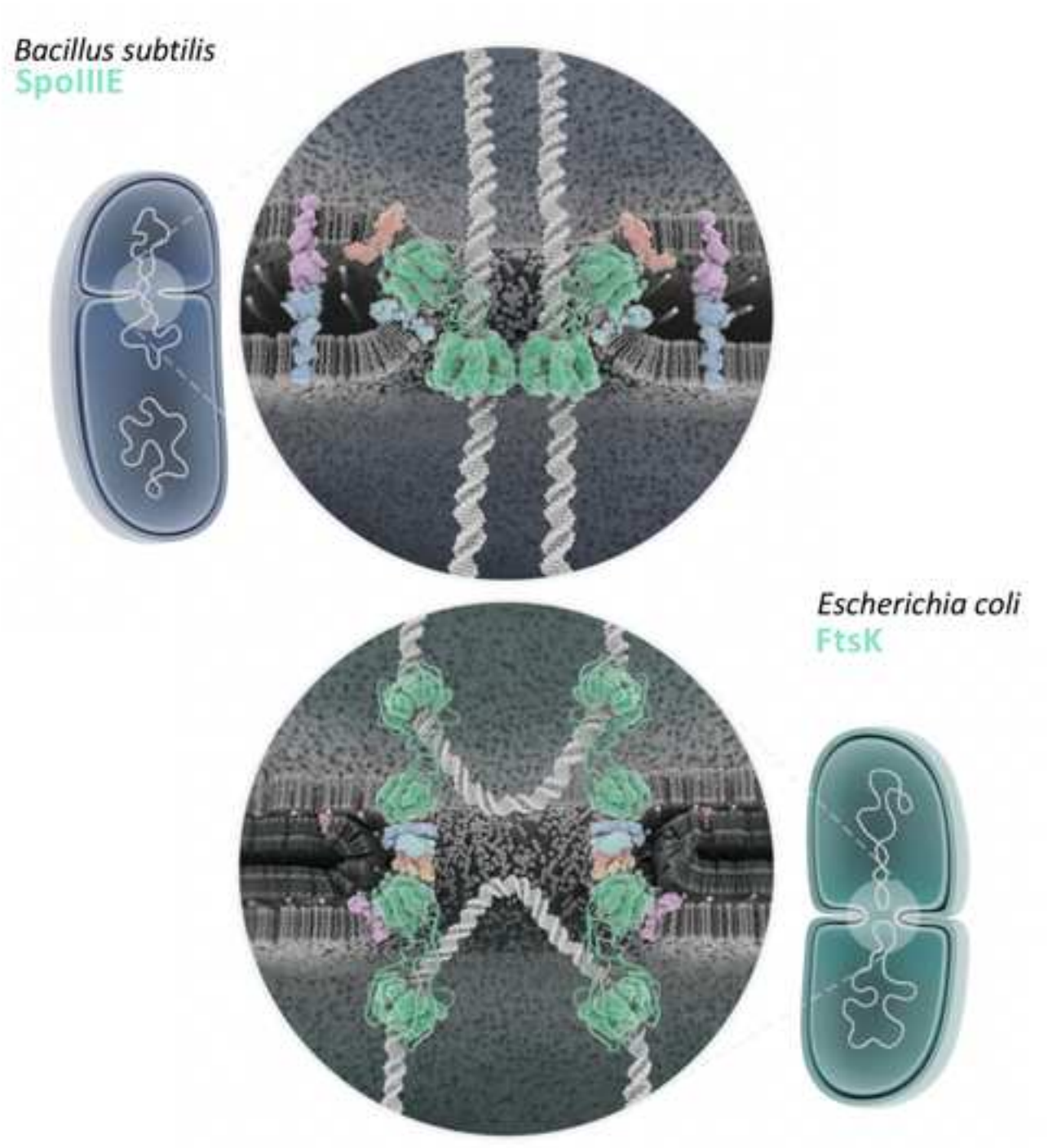

Outstanding Questions Box

- **How are SpoIIIE and FtsK organized in the septum?**

Is it possible to reveal the configuration of SpoIIIE and FtsK within the septum using cryo-electron and correlative light cryo-electron microscopy?

- **How do SpoIIIE and FtsK regulate cell envelope remodeling?**

Can *in vitro* experimental approaches using purified components, reveal direct biochemical links between cell envelope remodeling and chromosome translocation?

- **How conserved is the role of SpoIIIE in other spore-formers?**

Does SpoIIIE function in a similar capacity in other spore-formers or play additional roles in development?

- **How does SftA mediate DNA translocation?**

Is this mechanism different from that of membrane-bound DNA translocases such as FtsK and SpoIIIE?

Can *in vitro* experimental approaches reveal the biochemical mechanism by which SftA translocates DNA?

Highlights

- A recent study shows that SpoIIIE interacts with proteins that are connected to the cell envelope during spore development
- FtsK appears to interact with proteins within all layers of the Gram-negative cell envelope
- SpoIIIE and FtsK are both multifunctional and coordinate cell envelope remodelling with chromosome segregation
- Both SpoIIIE and FtsK likely regulate peptidoglycan remodelling at the septum
- Advances in cryo-electron microscopy may reveal how FtsK and SpoIIIE complexes are organized during cytokinesis