FisB mediates membrane fission during sporulation in *Bacillus subtilis*

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How bacteria catalyze membrane fission during growth and differentiation is an outstanding question in prokaryotic cell biology. Here, we describe a protein (FisB, for fission protein B) that mediates membrane fission during the morphological process of spore formation in *Bacillus subtilis*. Sporulating cells divide asymmetrically, generating a large mother cell and smaller forespore. After division, the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. Membrane fission releases the forespore into the mother cell cytoplasm. Cells lacking FisB are severely and specifically impaired in the fission reaction. Moreover, GFP-FisB forms dynamic foci that become immobilized at the site of fission. Purified FisB catalyzes lipid mixing in vitro and is only required in one of the fusing membranes, suggesting that FisB–lipid interactions drive membrane remodeling. Consistent with this idea, the extracytoplasmic domain of FisB binds with remarkable specificity to cardiolipin, a lipid enriched in the engulfing membranes and regions of negative curvature. We propose that membrane topology at the final stage of engulfment and FisB–cardiolipin interactions ensure that the mother cell membranes are severed at the right time and place. The unique properties of FisB set it apart from the known fission machineries in eukaryotes, suggesting that it represents a new class of fission proteins.

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required for these lipid rearrangements. In the case of eukaryotic membrane fusion, SNARE proteins anchored in apposed membranes form trans-complexes that overcome repulsive forces to bring bilayers into intimate contact. In enveloped viruses, membrane fusion proteins that are anchored in the viral membrane insert into host cell membranes and, through a series of conformational changes, bring the lipid bilayers into close proximity, leading to fusion. As for fission, the only factors described to date that participate in it are dynamin [Ferguson and De Camilli 2012] and the endosomal sorting complex required for transport (ESCRT-III complex) [Hurley 2010; Wollert and Hurley 2010; Henne et al. 2011]. Dynamin and dynamin-like proteins are involved in endocytosis, multivesicular membrane budding, cytokinesis in chloroplasts, and mitochondrial genesis. These proteins are thought to function as “pinching machines”: They polymerize around the outside of the neck that connects two parts of a contiguous membrane, constricting the neck to a critical radius. GTP hydrolysis then produces conformational changes that result in fission [Ferguson and De Camilli 2012]. In contrast, the ESCRT-III complex appears to assemble on the inside or at one of the openings of the neck to induce membrane constriction and, ultimately, fission [Hurley 2010; Henne et al. 2011]. The ESCRT-III complex has been implicated in the biogenesis of multivesicular bodies [Gruenberg and Stenmark 2004; Hurley 2008; Wollert et al. 2009; Wollert and Hurley 2010], membrane abscission during cytokinesis [Elia et al. 2011], and budding of some enveloped viruses from the host membrane [Strack et al. 2003; Morita and Sundquist 2004; Bienasz 2006]. Interestingly, a homologous set of proteins has been proposed to function in cytokinesis in the Crenarchaea Sulfolobus [Lindás et al. 2008; Samson et al. 2008].

The molecular mechanism underlying membrane fission during cytokinesis in bacteria remains enigmatic. It has been proposed that protein components of the cell division machinery could catalyze fission [Sharp and Pogliano 1999; Liu et al. 2006; de Boer 2010; Fleming et al. 2010]. Alternatively, it has been suggested that cell wall synthesis on the outside of the cell could force the constricting membranes into close proximity, ultimately leading to fission [Weiss 2004; Judd et al. 2005; Meyer et al. 2010]. To date, no protein has been directly implicated in this fission reaction.

Here, we investigated a specialized membrane fission event that occurs during sporulation in B. subtilis [Fig. 1]. In response to starvation, B. subtilis differentiates into a dormant spore [Stragier and Losick 1996; Errington 2003]. Upon initiation of this developmental process, the cell divides asymmetrically, generating two cells of unequal size and dissimilar fate. The smaller cell is the prospective spore and is referred to as the forespore. The larger cell is called the mother cell. Initially, these two cells lie side by side, separated by a double-membrane septum. However, shortly after polar division, the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. In the last stage of this process, the leading edges of the migrating membranes meet at the cell pole and, upon membrane fission, the forespore is released into the mother cell cytoplasm [Fig. 1]. The forespore is thus surrounded by two membranes: an inner membrane that contains the forespore cytoplasm and an outer membrane derived from the mother cell membrane. The molecular mechanism underlying this membrane remodeling event is the focus of our work.

We describe the identification and characterization of a membrane protein, FisB, that is required for membrane fission at the last stage of engulfment. FisB is a bitopic membrane protein that is produced in the mother cell after polar division. In its absence, the migration of the mother cell membranes around the forespore proceeds normally, but engulfment stalls when the membranes reach the cell pole. In support of the idea that FisB functions in membrane remodeling, a functional GFP-FisB fusion localizes as a focus at the cell pole at the time of membrane fission. Consistent with a direct role in membrane remodeling, we show that recombinant FisB catalyzes lipid mixing in vitro. Interestingly, lipid-mixing activity required FisB in only one of the two interacting membranes, suggesting that FisB–lipid interactions drive membrane remodeling. In support of this idea, we show that the extracellular domain of FisB directly interacts with liposomes. Moreover, FisB–liposome interaction required the presence of the anionic phospholipid cardiolipin (CL). Altogether, our data support the idea that FisB

![Figure 1. Membrane fission during spore development. Schematic representation of the morphological stages of the engulfment process. After polar division, the mother cell membranes migrate around the forespore. When they meet at (or near) the cell pole, membrane fission releases the forespore into the mother cell cytoplasm. Enlarged schematics highlight the engulfing membranes pre- and post-fission. (Middle panel) At the final stage, the membranes form a tube that is severed in the fission reaction. Fluorescent images show sporulating cells (strain BKM015) before and after membrane fission. Prior to fission, the lipophytic dye FM4-64 labels the peripheral membranes of the mother cell and the double membrane surrounding the forespore. After fission, the dye cannot access the membranes surrounding the forespore. The presence of a forespore within the mother cell is assessed by a fluorescent forespore reporter (PspoIIQ-cfp);](Image)
directly catalyzes the membrane fission event that marks the end of engulfment. Given the topology of FisB and that of the membrane tube to be severed, we suggest that FisB promotes fission from within or near one of the openings of the tube by interacting with CL that is enriched in the engulfment membranes and in regions of high negative curvature.

Results

FisB is required for membrane fission at the last stage of engulfment

Previous studies on membrane fission during sporulation implicated the polytopic membrane protein SpoIIIE (Sharp and Pogliano 1999, 2003). SpoIIIE is a DNA transporter that is required to translocate the forespore chromosome into the forespore compartment (Wu and Errington 1994). In the absence of SpoIIIE, sporulating cells are unable to pump the forespore chromosome and fail to make viable spores. Using an innovative membrane fission assay (described below), Sharp and Pogliano (1999) discovered that cells lacking SpoIIIE are also defective in membrane fission at a late stage of engulfment. In support of the idea that SpoIIIE functions directly in catalyzing fission, a SpoIIIE-GFP fusion protein localized to the forespore pole around the time of membrane fission (Sharp and Pogliano 1999). In the course of our studies on chromosome organization and segregation during sporulation (Burton et al. 2007, Marquis et al. 2008, Sullivan et al. 2009), we observed that sporulating cells lacking SpoIIIE exhibited defects in engulfment at stages prior to membrane fission. Under our assay conditions, 80%–90% of the SpoIIIE mutant cells had bulged septal membranes and/or invaginations that appeared to prevent complete membrane migration around the forespore (Supplemental Fig. S1). These observations led us to consider the possibility that SpoIIIE might only be indirectly involved in membrane fission and that other proteins catalyze this reaction.

We began our studies by investigating two candidate factors for a role in catalyzing fission: the B. subtilis dynamin homolog DynA (Bürmann et al. 2011) and the peptidoglycan synthetic machinery (Meyer et al. 2010). Using this assay, we found that engulfment and membrane fission were indistinguishable in wild-type cells and cells lacking DynA (Fig. 2A). Thus, dynamin does not appear to function in membrane fission during sporulation.

Next, we investigated whether cell wall synthesis between the forespore and mother cell membranes is involved in membrane fission. Dworkin and colleagues (Meyer et al. 2010) recently proposed that the action of cell wall synthetic complexes anchored in the mother cell membrane could promote membrane migration during engulfment and membrane fission. Using a membrane fission assay similar to the one described above, these researchers reported that the addition of antibiotics that inhibit cell wall synthesis during the engulfment process resulted in a fourfold reduction in fission. To investigate whether the force generated from cell wall synthesis was the principal driver of membrane fission, we performed similar antibiotic addition experiments (see the Materials and Methods). Under our assay conditions, we observed a defect in membrane fission, although the effect was more modest than observed previously. Membrane fission was reduced from 86% to 51% when the antibiotic fosfomycin was added at hour 1.5 of sporulation (Fig. 2A). The reduction in fission was even less pronounced when the drug was added at later time points (data not shown). These results suggest that peptidoglycan synthesis and the force generated by it could help bring the membranes into close proximity but indicate that the protein (or proteins) that catalyze membrane fission remain to be discovered.

DynA was not required for membrane fission, and we were unable to identify homologs of the ESCRT-III fission machinery in the B. subtilis genome. Accordingly, we turned to the hypothesis that fission is mediated by a fusion-like mechanism. We reasoned that if such a fission protein existed, it would likely be synthesized in the mother cell at an early stage of sporulation prior to the fission reaction and would have at least one transmembrane segment, and cells lacking this protein would have reduced sporulation efficiency. The first mother cell-specific transcription factor that is induced during sporulation is the alternative σ factor σF [Stragier and Losick 1996; Piggot and Losick 2002]. The σF regulon has been defined by transcriptional profiling (Eichenberger et al. 2003; Feucht et al. 2003; Steil et al. 2005), and strains harboring deletions in most of the genes in the regulon have been generated (Eichenberger et al. 2003). Using the criteria described above, we examined a subset of this ordered library of mutants using the fluorescent membrane fission assay of Sharp and Pogliano (1999). Our analysis identified one mutant (in the yunB gene) that was severely impaired in membrane fission (Fig. 2A). Based on its mutant phenotype and the characterization
of the yunB gene product described below, we renamed this gene fisB for fission protein B.

To quantitatively assess the requirement for FisB in membrane fission, we compared wild type and the FisB mutant in a sporulation time course (Fig. 2B; Supplemental Fig. S2). Two hours after the initiation of sporulation, 13% of wild-type cells \((n > 1000)\) had completed membrane fission, releasing the forespore into the mother cell cytoplasm. At this time point, there was no detectable fission in the mutant. Thirty minutes later, at hour 2.5, 51% of wild-type sporulating cells \((n > 1000)\) had undergone membrane fission compared with <1% in the FisB mutant \((n > 1000)\). By hour 3, 79% of wild-type sporulating cells had completed membrane fission, but only 2% had completed membrane fission in the mutant. Importantly, in cells lacking FisB, the migration of the mother cell membranes around the forespore appeared indistinguishable from wild type (Fig. 2B; Supplemental Fig. S2). Moreover, similar to wild type, membrane migration appeared complete in most cells lacking FisB by hour 2.5. These results indicate that the absence of FisB does not impact the early steps of engulfment and thus suggest that FisB plays a specific role in the membrane fission process.

Interestingly, membrane fission was not completely abolished in cells lacking FisB. By hour 4.5, 
\(~21\%\) of the FisB mutant sporulating cells appeared to have undergone fission. This percentage is likely to be an overestimate because sporulating cells lacking FisB begin to lyse at this time point, reducing the total number of sporulating cells in the culture (Supplemental Fig. S2). FisB mutants have a sporulation efficiency of 12%–15%, suggesting that most of the cells that successfully undergo membrane fission are able to produce mature spores.

Altogether, these results suggest that FisB plays a specific role in membrane fission. In its absence, membrane migration around the forespore proceeds normally, but most sporulating cells fail to undergo membrane remodeling and release of the forespore into the mother cell cytoplasm. Cells that stall at the fission step ultimately lyse. Those that do complete fission do so after an 
\(~2\)-h delay.

FisB forms dynamic foci that localize to the pole at the time of membrane fission

FisB is predicted to be a bitopic membrane protein with a small N-terminal domain that resides in the mother cell cytoplasm and a larger (23-kDa) extracellular domain. To determine the subcellular localization of FisB, we generated an N-terminal monomeric GFP fusion to FisB expressed under its native promoter- and ribosome-binding site. The fusion protein complemented the FisB-null mutant for membrane fission and sporulation (data not shown). We visualized the GFP fusion by epifluorescence microscopy in a sporulation time course. For this experiment, we visualized engulfment and membrane fission using the membrane dye TMA-DPH. TMA-DPH inefficiently crosses the lipid bilayer and thus weakly labels the mother cell membrane that surrounds the forespore after fission is complete. Accordingly, using TMA-DPH, it is possible to distinguish pre- and post-fission stages without a forespore reporter. Supplemental Figure S3 shows a direct comparison of the two membrane dyes FM4-64 and TMA-DPH.
During engulfment at hour 2.5, GFP-FisB localized as discrete foci in both the peripheral membranes as well as the mother cell membranes that were engulfing the forespore (Fig. 3A). We were unable to quantify the number of FisB proteins in the foci, but the fluorescence intensity suggests that each focus is not a single protein and likely represents an oligomeric complex. Consistent with this idea, we found that both full-length FisB and the extracellular domain of FisB when purified from Escherichia coli can form oligomeric complexes in vitro (Supplemental Fig. S4). Time-lapse imaging revealed that the GFP-FisB foci were highly mobile in the membrane (Supplemental Movie M1). Importantly, at the time of membrane fission or just after it, a GFP-FisB focus was present near the cell pole adjacent to the forespore in a significant proportion of the cells. Strikingly, time-lapse imaging indicates that these polar foci remained immobile (Supplemental Movie M2). Figure 3B shows representative stills from a typical movie and an image in which each pixel is averaged over the entire time lapse. Dynamic foci are lost upon averaging, while immobile ones at the cell pole are retained.

In some cases, lowering the level of a fluorescent fusion will reveal or highlight specific subcellular localization, perhaps by reducing nonspecific or low-affinity binding (Rudner et al. 2002; Gregory et al. 2008). Accordingly, we constructed a YFP-FisB fusion expressed at levels approximately fivefold lower than the native protein (see the Materials and Methods; data not shown). The fusion protein complemented the fisB-null, restoring sporulation efficiency to wild-type levels. Moreover, YFP-FisB supported efficient membrane fission, albeit with a 20- to 30-min delay compared with wild type (Fig. 3C; data not shown).

During early engulfment stages, YFP-FisB localized in foci in the mother cell membranes, as observed with the GFP-FisB fusion expressed at wild-type levels. These foci were more often found in the mother cell membranes that surrounded the forespore (Fig. 3C; Supplemental Fig. S5). Importantly, at hour 3, when sporulating cells were about to undergo fission or had just completed it, ~65% of the sporulating cells (n > 1000) exhibited a YFP-FisB focus at or near the cell pole adjacent to the forespore (Fig. 3C, yellow arrowheads; Supplemental Fig. S5, yellow arrowheads). In most cases, the polar focus was brighter than the foci in the peripheral and spore membranes, consistent with the idea that FisB assembles into a larger oligomeric complex at the pole. Because of the lower expression levels, we were unable to assess FisB dynamics due to rapid photobleaching of the fusion protein.

These results suggest that FisB forms mobile oligomeric complexes in the membranes of the mother cell. When the engulfing membranes reach the cell pole, a subset of these complexes localize to this site, where they participate in the fission reaction.

**FisB mediates membrane remodeling in vitro**

Altogether, our results show that FisB localizes to the site of fission and is required for membrane remodeling in vivo. To investigate whether FisB plays a direct role in the fission reaction and gain insight into the mechanism, we turned to in vitro experiments. Based on tomographic cryoelectron microscopy of Caulobacter crescentus cells undergoing cytokinesis (Judd et al. 2005), the connection between daughter cells prior to fission can be viewed as a tube 20–30 nm in length. We imagine a similarly sized membrane tube exists between the peripheral membrane of the mother cell and that surrounding the forespore. Since most of the FisB protein [amino acids 39–254] is present on the extracytoplasmic side of the membrane, if
FisB indeed mediates membrane fission, it is unlikely to do so via a dynamin-like constriction from outside of the tube. Instead, FisB must do work on the membrane tube from within or at one of its openings, interacting with proteins and/or lipids across the tube. This mechanism is reminiscent of those employed by eukaryotic SNAREs and viral fusion proteins to force apposing bilayers into close proximity. We therefore reasoned that if FisB catalyzes fission in vivo, purified full-length FisB inserted into artificial liposomes should be able to induce lipid mixing and membrane remodeling in vitro, as has been shown for SNAREs (Weber et al. 1998). We used a standard fluoroescence dequenching assay for lipid mixing (Struck et al. 1981) in which a population of liposomes bearing the fluorescent lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[7-nitro-2,1,3-benzoxadiazol-4-yl] [NBD-PE] and its lipid-linked quencher, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] [LR-PE], are mixed with unlabeled liposomes. Fusion of labeled and unlabeled liposomes increases the mean distance between the NBD-PE fluorophores and their quenchers, resulting in an increase in fluorescence (Fig. 4).

Full-length his-tagged FisB was expressed in E. coli and purified from detergent-solubilized membranes using the lipid-like detergent Fos-choline-12 [see the Materials and Methods]. The purified protein was then reconstituted into labeled or unlabeled liposomes composed of a mixture of phospholipids (phosphatidylycholine [PC], phosphatidylglycerol [PG], phosphatidylethanolamine [PE], and CL) found in sporulating B. subtilis cells (Griffiths and Setlow 2009) or a mixture of the minimal lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine [POPC] and dioleyl phosphatidyl serine [DOPS]) commonly used for SNARE-mediated fusion (Weber et al. 1998; Karatekin et al. 2010). To control for contaminating membrane proteins in the His-FisB-purified fraction, we performed a mock purification from E. coli cells harboring the empty expression plasmid (Supplemental Fig. S6). Labeled liposomes reconstituted with FisB or with mock-purified proteins (mock) or that were protein-free were mixed with unlabeled protein-free, FisB, or mock liposomes, and lipid mixing was monitored as an increase in NBD fluorescence as a function of time.

Using liposomes composed of lipids found in sporulating B. subtilis, efficient lipid mixing was observed when FisB liposomes were mixed with either FisB or protein-free liposomes (Fig. 4). Labeled FisB liposomes and unlabeled FisB liposomes resulted in slightly less efficient fusion than between FisB and protein-free liposomes, perhaps due to surface coverage by FisB reducing the available area of target membranes to interact with FisB in trans. Importantly, all combinations involving protein-free liposomes and liposomes containing mock-purified proteins resulted in negligible lipid mixing (Fig. 4, data not shown). Interestingly, using liposomes composed of POPC/DOPS (the minimal SNARE lipid mixture), no fusion was observed between FisB–FisB or FisB–protein-free liposomes [data not shown]. In contrast, neuronal SNAREs incorporated into liposomes containing POPC/DOPS resulted in efficient lipid mixing comparable with previous reports (Supplemental Fig. S7). These results show that FisB possesses an intrinsic capacity to remodel lipid membranes in vitro and strongly suggest it can also do so in vivo. The data further imply that the lipid remodeling activity of FisB critically relies on specific protein–lipid interactions.

**FisB binds to CL**

The results of the membrane fusion assay with the two different lipid mixtures suggest that an interaction between a particular lipid in the B. subtilis lipid composition was essential for FisB’s membrane remodeling activity. The simplest model is that this interaction occurs in trans and involves the extracellular domain of FisB (ECD). To test this idea and identify the lipids to which FisB binds, we used a coflotation assay (Fig. 5A; Bigay et al. 2005). The extracellular domain of FisB lacking its transmembrane segment was fused to a His tag, and the fusion protein was expressed in E. coli and purified from the soluble fraction [see the Materials and Methods]. The soluble protein was then mixed with protein-free liposomes consisting of either the B. subtilis lipid mix [PG/PE/CL/PC] or the standard minimal SNARE composition.
The mixture was loaded at the bottom of a density step gradient, and the free liposomes and liposomes with bound protein were separated from unbound protein by ultracentrifugation (see the Materials and Methods). Consistent with the membrane fusion results, the His-FisB extracellular domain bound to and cofloated with the liposomes composed of the *B. subtilis* lipid mixture but not of the SNARE lipid mix (Fig. 5B).

To identify the lipids that FisB binds, we repeated the coflotation assay with liposomes in which we varied the lipid compositions (Fig. 5C, D). These experiments revealed that the extracellular domain of FisB binds CL but not to any appreciable degree to PC, PS, PE, or PG. The binding of FisB to CL was confirmed using both CL extracted from *E. coli* and synthetic tetra-oleoyl CL [data not shown]. These results rule out the possibility that FisB binding was due to an impurity in the CL from *E. coli* and further suggest that FisB interacts with the head group of CL.

**Discussion**

The release of the engulfed forespore into the mother cell cytoplasm through membrane fission is a key step in the developmental process of sporulation. Here, we showed that the mother cell membrane protein FisB plays a central role in this membrane fission reaction. Collectively, our data support a model in which FisB is inserted into the mother cell membrane during the early stages of engulfment, where it forms mobile complexes. As engulfment nears completion, FisB oligomers assemble at the site of membrane closure that connects the peripheral and spore membranes. FisB then severs this membrane tube, releasing the spore into the mother cell cytoplasm (Fig. 6).

Virtually all endospore-forming bacteria in the low G+C Gram-positive branch possess a FisB homolog, while the protein appears to be absent in all other bacteria. Thus, FisB is likely to function exclusively in the fission reaction that occurs at the final stage of engulfment during sporulation, taking advantage of the unusual topology of the membranes at the site of fission.

Given enough time, a subset of sporulating cells lacking FisB successfully complete engulfment and membrane fission. This implies that an additional mechanism promotes fission during sporulation. Possible candidates include peptidoglycan synthesis as proposed by Dworkin and colleagues [Meyer et al. 2010] or peptidoglycan hydrolysis carried out by the membrane-anchored peptidoglycan hydrolase complex composed of SpoIID, SpoIIP, and SpoIIIM [Abanes-De Mello et al. 2002; Aung et al. 2007; Morlot et al. 2010]. Either of these machines could bring the engulfing membranes into close proximity.
and promote fission (Judd et al. 2005), although at a slower rate than FisB. Alternatively, SpoIIIE or an as yet unidentified factor could function in an independent fission pathway.

**SpoIIIE and membrane fission**

Our analysis of engulfment in cells lacking the SpoIIIE DNA translocase suggests that its activity is critical for membrane migration around the forespore prior to fission. These results do not exclude the possibility that it could also function in the fission reaction. However, our analysis of the FisB protein in SpoIIIE mutants provides a simpler explanation for the original observations of Sharp and Pogliano [1999]. Immunoblot analysis of sporulating cells revealed that the levels of FisB were significantly reduced in the SpoIIIE-null mutant compared with wild type [Supplemental Fig. S8A]. Moreover, in a SpoIIIE point mutant [SpoIIIE36] that binds DNA but is impaired in DNA transport [Wu and Errington 1994; Wu et al. 1995], FisB protein levels were unaffected, but YFP-FisB localization was disrupted [Supplemental Fig. S8B]. In accordance with this finding, Sharp and Pogliano (1999) reported that the SpoIIIE36 mutant was impaired in membrane fission but had a less severe defect than the null. Based on these observations, we favor the idea that SpoIIIE is only indirectly involved in the fission reaction, impacting membrane migration and FisB abundance and/or localization. Interestingly, it has also been proposed that SpoIIIE participates in fission during asymmetric division in sporulating cells (Liu et al. 2006; Fleming et al. 2010). This membrane remodeling event, wherein cytosol fills the gap inside the closing membrane annulus, has the opposite topology to the fission at the end of engulfment, where the extracellular medium fills the gap to be closed by the engulfing membrane (Supplemental Fig. S9). Intriguingly, SpoIIIE’s architecture [large cytoplasmic domain anchored to the membrane] is opposite to that of FisB [membrane-anchored extracytoplasmic domain]. Establishing a direct role for SpoIIIE in membrane remodeling awaits in vitro reconstitution.

**Interactions between the extracellular domain of FisB and CL**

We found that the extracellular domain of FisB interacts with CL, and our data suggest that this interaction is important for membrane remodeling in vitro. The specificity of this interaction is remarkable for a soluble domain. Although CL has high affinity for proteins with transmembrane segments involved in oxidative phosphorylation, such as the F_{0}F_{1} ATPase (Haines 2009), many soluble proteins that bind CL, such as cytochrome c [Kagan et al. 2009] and tBid [Epand et al. 2002] in eukaryotes and MinD [Mileykovskaya et al. 2003] in bacteria, also interact with other, negatively charged lipids such as PS and PG to varying extents. In contrast, we could not detect significant interaction between the extracellular domain of FisB and the acidic lipids PG and PS or with the zwitterionic lipids PE and PC [or with digalactosyldiacylglycerol] [data not shown]. Thus, we expect the dynamics of FisB and CL to be intimately coupled [see below]. In this regard, it is noteworthy that during sporulation, the levels of CL increase, and CL is enriched in the engulfing membranes [Kawai et al. 2004]. *B. subtilis* possesses three genes that encode proteins homologous to CL synthases: *clsA* (ywnE), which encodes the major CL synthase; *clsB* (ywjE); and *ywiE* [Kunst et al. 1997; Kawai et al. 2004]. The expression of both *clsA* and *clsB* increases during sporulation, consistent with the idea that CL plays an important role during this process. Cells lacking all three genes are viable and have undetectable levels of CL during vegetative growth [Kawai et al. 2004]. However, CL is readily detectable in the triple mutant during the early stages of sporulation, indicating that an additional sporulation-specific CL synthase exists or that CL is produced as a side reaction by a lipid synthesis/modification enzyme [Kawai et al. 2004, 2006; Tan et al. 2012]. In accordance with the presence of CL during engulfment in cells lacking ClsA, ClsB, and YwiE, we found that the triple mutant was able to undergo fission at a frequency comparable with wild type [data not shown]. This result suggests that if FisB requires CL to catalyze fission in vivo, then the reduced amount of CL made in the absence of the known synthases must be sufficient. Alternatively, FisB could use another phospholipid in addition to CL in vivo.
Late forespore gene expression does not require the completion of engulfment

Previous work has shown that the developmental programs of gene expression in the mother cell and forespore are not free-running clocks but are linked to each other by cell–cell signaling pathways [Stragier and Losick 1996; Rudner and Losick 2001; Rudner and Doan 2008]. Furthermore, it has been hypothesized that these cell type-specific transcriptional programs are coupled to morphogenesis. Specifically, it was proposed that the activation of the forespore transcription factor $\sigma^c$ is coupled to the completion of engulfment and membrane fission [Stragier and Losick 1996; Rudner and Losick 2001; Rudner and Doan 2008; Regan et al. 2012]. One compelling model was that the isolation of the forespore from the external environment as a result of fission served as the trigger for $\sigma^c$. Recently, we reported evidence that argued against the coupling of $\sigma^c$ activation to membrane fission [Doan et al. 2009]. Specifically, we found that a subset (>13%) of sporulating cells that was blocked at early stages of engulfment successfully activated $\sigma^c$ in the forespore. However, the severe defect in morphogenesis in these mutants and the small number of cells that turned on $\sigma^c$ raised concern that the activation might not be biologically relevant. Furthermore, recent genetic experiments using large insertions into the B. subtilis chromosome that delay DNA transport into the forespore suggest that $\sigma^c$ activation could indeed be coupled to completion of engulfment [Regan et al. 2012]. The FisB mutant allowed us to revisit the link between $\sigma^c$ activity and morphogenesis in a strain in which engulfment proceeds normally and only fission is impaired.

Analysis of $\sigma^c$ activity in the FisB mutant revealed that 47% ($n > 1000$) of the sporulating cells that were stalled at the membrane fission step successfully activated $\sigma^c$ in the forespore compartment [Supplemental Fig. S10]. This observation lends further support to the idea that $\sigma^c$ activation and the fission reaction are not coupled events. Intriguingly, we also found that sporulating FisB mutants lyse at an elevated frequency, and this lysis was suppressed in cells lacking $\sigma^c$ [Supplemental Fig. S11]. This observation suggests that activation of $\sigma^c$ prior to the completion of membrane fission is deleterious to the sporulating cell and leads to death. Thus, although a mechanism that holds $\sigma^c$ inactive until fission is complete would appear to be beneficial, our data suggest that B. subtilis does not employ one. We suspect that under normal circumstances [in wild-type cells], $\sigma^c$ activation and fission are temporally distinct enough that a coupling device is not required.

How might FisB mediate membrane scission?

Although membrane fission is a fundamental and ubiquitous biological process used by species ranging from the simplest bacteria and enveloped viruses to the most sophisticated neuronal cells, only two general fission machineries have been described. The first and best understood involves constriction of a membrane neck or tube from the outside by dynamin and its homologs [Ferguson and De Camilli 2012]. This constriction process relies on oligomerization of dynamin around the membrane neck and GTP hydrolysis to sever it. The second fission machinery is the ESCRT-III system [Hurley 2010; Henne et al. 2011], in which all components are soluble proteins that are recruited to the membrane via protein–protein and protein–lipid interactions. The only ESCRT-III component that seems to be absolutely required for fission is Snf7, whose oligomerization initiates the fission process, at least in vitro [Wollert and Hurley 2010]. Other subunits likely terminate Snf7 filament assembly and recruit Vps4, an ATPase that disassembles the ESCRT-III complex and perhaps contributes to the fission reaction. The mechanism by which ESCRT-III mediates fission is still intensely debated [Hurley 2010; Henne et al. 2011]; however, it is clear that the machinery works at the opening of and/or inside the membrane neck to achieve fission, rather than using constriction from outside the tube. Based on the topology of FisB with its large extracytoplasmic domain and very short [15-amino-acid] cytoplasmic domain, we suspect that FisB also catalyzes fission by doing work at one or both of the openings of the tube or from within it.

The activity of eukaryotic fusion and fission machines such as SNAREs and dynamin are exquisitely regulated in time and space by other proteins and intracellular signals. Potential regulators of FisB other than CL have not been identified. However, given the topology of the membranes during engulfment of the forespore, it is possible, in principle, that the dynamics of CL during sporulation, its propensity to associate with negatively curved membranes, FisB binding to CL, and homo-oligomerization of FisB could provide sufficient control over when and where fission occurs.

At the final stages of engulfment, the tube connecting the membrane engulfing the forespore to the rest of the mother cell membrane must shrink in diameter (Fig. 6). This will increase the negative curvature of the tube’s inner leaflet, making it increasingly favorable for CL localization. We hypothesize that FisB becomes immobilized at the fission site as a result of its interaction with CL within the membrane tube. We found that FisB forms oligomers in vitro, and one possibility is that oligomerization leads to a filling of the tube with FisB and overflow to the tube’s openings. The positive curvature of these openings would be unfavorable for CL localization. If FisB recruits CL to these positively curved regions, this could lead to the destabilization and membrane scission. In a variation of this model, FisB would localize to the highly positively curved regions at the openings of the tube by virtue of its own curvature preference and, by recruiting CL to such regions, would accumulate stresses, which could be relieved by severing the tube.

Other alternatives exist that are not mutually exclusive. For example, FisB oligomerization with concomitant conformational changes could be coupled to the destabilization of the tube, perhaps via twisting or compression. Alternatively, FisB could induce lateral segregation of CL (de Kruijff and Cullis 1980), with the resulting line tension and/or the discontinuity in elastic...
properties between the CL-rich and CL-poor phases leading spontaneously to fission. Such fission occurs at domain boundaries between cholesterol-rich and cholesterol-poor tubules under certain conditions (Allain et al. 2004; Roux et al. 2005). Finally, FisB might act through a mechanism reminiscent of viral fusion proteins. Although fusion and fission are topologically opposite processes, microscopically, both require cutting, bending, and rejoining of membranes. From this point of view, it is perhaps not surprising that some homologs of dynamin-1 have been described to mediate fusion, not fission (Kozlov et al. 2010; Ferguson and De Camilli 2012). In this model, FisB anchored inside the membrane tube would interact with CL across the tube via its extracryotyplasmic domain. CL binding could trigger a conformational change in FisB, as in the case of cytochrome c (Kagan et al. 2009), ultimately leading to membrane fission.

Further biochemical analysis of FisB in vitro, along with the identification of other proteins that function with or regulate FisB and the missing CL synthase, will help distinguish among these models for FisB function. However, our characterization of FisB raises the intriguing possibility that it is part of a novel fission mechanism.

Materials and methods

General methods

All _B. subtilis_ strains were derived from the prototrophic strain PY79 (Youngman et al. 1983). Sporulation was induced by resuspension at 37°C according to the method of Sterlini-Mandelstam (Harwood and Cutting 1990) or by exhaustion in supplemented DS medium (Schaeffer et al. 1965). Sporulation efficiency was determined in 36-h cultures as the total number of heat-resistant (20 min at 80°C) colony-forming units (CFUs) compared with wild-type heat-resistant CFUs. Analysis of membrane fission after inhibition of cell wall synthesis was performed as described previously (Meyer et al. 2010) except cells were sporulated at 37°C and fosfomycin (5 mM final) was added at hour 1.5. Membrane fission was assessed at hours 2, 2.5, and 3. Insertion-deletion mutations were generated by isothermal assembly (Gibson et al. 2009) of PCR products followed by direct transformation into _B. subtilis_. Tables of strains, plasmids, and oligonucleotide primers and a description of plasmid construction can be found online in the Supplemental Material (Supplemental Tables S1–S3; Supplemental Material).

Protein purification and antibody production

His6-FisBFL and His6-FisBECD fusion proteins were expressed in _E. coli_ BL21 DE3 pLysS at 30°C and 16°C, respectively, and purified by affinity chromatography on Ni²⁺-NTA agarose (Qiagen). MBP-FisBEC fusion protein was expressed in _E. coli_ NB42 and purified by affinity chromatography on amylose resin (New England Biolabs). A complete description of the purifications can be found in the Supplemental Material. MBP-FisBEC peak fractions were pooled and used to generate rabbit polyclonal antibodies (Covance). Crude serum was affinity-purified as described (Campo and Rudner 2006) using His6-FisBEC.

Immunoblot analysis

Whole-cell lysates from sporulating cells induced by resuspension were prepared as described (Doan and Rudner 2007). Samples were heated for 10 min at 50°C prior to loading. Equivalent loading was based on OD600 at the time of harvest. Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, electroblotted onto Immobilon-P membranes (Millipore), and blocked in 5% nonfat milk in phosphate-buffered saline (PBS) and 0.5% Tween-20. The blocked membranes were probed with affinity-purified anti-His (1:1000; Santa Cruz Biotechnology), anti-FisB (1:10,000), and anti-α-L (15,000) (Carniol et al. 2004) diluted into 3% BSA in 1× PBS-0.05% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit G (Bio-Rad) and the Western Lightning reagent kit as described by the manufacturer (PerkinElmer).

**In vitro lipid mixing assay**

The protocol described in Weber et al. (1998) was followed with minor modifications. All lipids were purchased from Avanti Polar Lipids. For fluorescent donor liposomes, 100 μL of 3 mM lipids [L-α-PC (E. coli; sodium salt), CL (E. coli; sodium salt), L-α-phosphatidylethanolamine (E. coli; PE), L-α-PC (egg, chicken), NBD-PE (ammonium salt; 18:1), LR-PE (ammonium salt; 18:1)] in a molar ratio of 30:45:13:10:1:1 dissolved in chloroform were dried under nitrogen gas, placed under a vacuum dessicator for 1 h, and then dissolved in reconstitution buffer (RB; 25 mM Heps at pH 7.4, 100 mM KCl, 10% glycerol, 1 mM DTT) supplemented with 1% (w/v) octyl-β-D-glucopyranoside (OG) and either FisB protein, the mock purification, or nothing to obtain protein-free liposomes. Unlabeled acceptor liposomes were prepared using 100 μL of 15 mM lipids as above in a molar ratio of 30:45:15:10. FisB protein was reconstituted into the lipids at a 1:1000 [protein:lipid] molar ratio, and then the OG was removed by overnight dialysis (6000- to 8000-Da molecular weight cutoff). Unincorporated protein was removed by flotation of the liposomes through a Nycodenz (Accurate Chemicals) step gradient. Labeled liposomes were recovered in a 150-μL volume (2 mM lipid). Unlabeled liposomes were collected in 400 μL (3.75 mM lipid).

The lipid-mixing assay was performed in white 96-well Maxisorp plates (Nunc). Liposomes (10 μL of labeled and 45 μL of unlabeled) were mixed on ice and then placed in a room temperature SpectraMax M5 Microplate Reader [Molecular Devices], and NBD fluorescence was recorded with filters set at 460 nm (excitation) and 538 nm (emission) at 30-sec intervals for 45 min. To determine the maximum NBD fluorescence, 10 μL of 2.5% (w/v) n-dodecyl-β-D-maltoside (DM) [Thermo Scientific] was added at the end of the experiment, and fluorescence were monitored for 15 min.

**Flotation assay**

Natural [PG, PE, and CL, all from _E. coli_, or egg PC] lipids or synthetic lipids [POPC], 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt), 1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycero-3-phospho[1'-rac-glyceryl] [sodium salt] from Avanti Polar Lipids were used. All liposomes included 0.2 mole percent NBD-PE (ammonium salt; 18:1) as a fluorescent marker. The lipids were dissolved in a 2:1 (v/v) mixture of chloroform and methanol. Lipids (1 μmol total) were mixed at desired ratios, and the mixtures were dried under nitrogen flow. Residual organic solvent was removed under vacuum for at least 2 h in a vacuum dessicator. Lipids were hydrated in 1 mL of RB-EDTA buffer [25 mM Heps at pH 7.4, 140 mM KCl, 1 mM EDTA, 0.2 mM tris(2-carboxyethyl)phosphate] by shaking for >30 min. The lipid suspension was then immersed in liquid nitrogen and then allowed to thaw in a water bath at 37°C. This freeze–thaw cycle
was repeated five additional times to form large unilamellar vesicles (LUVs) [Piek 1981]. The LUVs were either flash-frozen in liquid nitrogen and stored at −80°C or used fresh for extraction. The LUV stock was extruded 19 times through 50-nm pore size polycarbonate filters (Avanti) at 4°C using a minicentrifuge.

The extruded liposomes (40 nmol total lipid) were incubated with 40 pmol of FisB extracytoplasmic domain in a total volume of 100 µL for 1 h at room temperature. Two-hundred milliliters of 60% iodoxanol density gradient medium (Optiprep, Sigma-Aldrich) was added to make a 40% iodoxanol solution (density, 1.215 g/mL) that was layered at the bottom of a 5-mm × 41-mm Beckman Ultra-Clear ultracentrifuge tube (Beckman Coulter, Inc.). This was overlaid with 200 µL of 20% iodoxanol solution (diluted in RB-EDTA; density, 1.110 g/mL), followed by a top layer of 200 µL of RB-EDTA. To assess liposome redistribution after floatation, NBD-PE fluorescence was measured before and after centrifugation as follows: Step gradients were prepared in a centrifuge device (ImageQuant LAS 4000, GE Healthcare). A mirror was parallel, and the tubes were placed in a rack in a gel-imaging instrument (ImageQuant LAS 4000, GE Healthcare). After centrifugation (8000 rpm for 30 sec) prior to visualization. The LUV stock was extruded 19 times through 50-nm pore size polycarbonate filters (Avanti) at 4°C using a minicentrifuge.

References


Fluorescence microscopy

Fluorescence microscopy was performed on an Olympus BX61 microscope as previously described [Doan et al. 2005]. Fluorescent signals were visualized with a phase-contrast objective (UplanF1 100×) and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software (Molecular Device). Exposure times were typically 500 msec for CFP and GFP and 2000 msec for YFP-FisB. Membranes were stained with either TMA-DPH or FM4-64 (Molecular Probes) at a final concentration of 0.01 mM and 3 µg/mL, respectively. Exposure times were typically 200 msec. Images were analyzed, adjusted, and cropped using Metamorph software. Cells were concentrated by centrifugation (8000 rpm for 30 sec) prior to visualization. This step had no impact on the localization of the fusion proteins.

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