Membrane fission during bacterial spore development requires cellular inflation driven by DNA translocation

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SUMMARY

Bacteria require membrane fission for both cell division and endospore formation. In *Bacillus subtilis*, sporulation initiates with an asymmetric division that generates a large mother cell and a smaller forespore that contains only a quarter of its genome. As the mother cell membranes engulf the forespore, a DNA translocase pumps the rest of the chromosome into the small forespore compartment, inflating it due to increased turgor. When the engulfing membrane undergoes fission, the forespore is released into the mother cell cytoplasm. The *B. subtilis* protein FisB catalyzes membrane fission during sporulation, but the molecular basis is unclear. Here we show that forespore inflation and FisB accumulation are both required for efficient membrane fission. Forespore inflation leads to higher membrane tension in the engulfment membrane than in the mother cell membrane, causing membrane to flow through the neck connecting the two membrane compartments. Thus, the mother cell supplies some of the membrane required for the growth of the membranes surrounding the forespore. Oligomerization of FisB at the membrane neck slows equilibration of membrane tension by impeding membrane flow. This leads to a further increase in the tension of the engulfment membrane, promoting its fission through lysis. Collectively our data indicate that DNA-translocation has a previously unappreciated second function in energizing FisB-mediated membrane fission under energy-limited conditions.

INTRODUCTION

When nutrients are scarce, spore-forming bacteria initiate a morphological process called sporulation, resulting in stress-resistant spores that can remain dormant for years until conditions are favorable for germination[1,2]. The first morphological event during sporulation is an asymmetric division that produces a small forespore and a larger mother cell (Figure 1A). The mother cell membranes then engulf the forespore in a process reminiscent of phagocytosis. When the edges of the engulfing membrane reach the cell pole, they undergo membrane fission to release the forespore (Figure 1A inset), now surrounded by two membranes, into the mother-cell cytoplasm. The mother cell then packages the forespore in a protective peptidoglycan cortex and proteinaceous coat while the forespore prepares for dormancy. When the forespore is mature, the mother cell lyses, releasing the dormant spore into the environment. Prior to asymmetric division, the chromosomes are remodeled into an axial filament with the origins of replication at the cell poles and the termini at mid-cell[3,4]. Thus, immediately after asymmetric division, only ~1/4 of the forespore chromosome is trapped in the forespore[5-7]. The rest of the chromosome is pumped into the forespore by an ATPase, SpoIIIE[8,9], consuming one ATP molecule per every two base pairs translocated[10]. As the chromosome is packed into the forespore, the forespore volume doubles from its initial volume of $\sim 0.1\ \mu\mathrm{m}^3$ by the time the engulfment membrane migrates completely around the forespore and continues to grow to $\sim 0.3\ \mu\mathrm{m}^3$ about 3 h after septation[11]. Forespore inflation is driven by osmotic forces which are mainly due to the counterions that neutralize the highly charged DNA[11]. Upon complete DNA packing, the pressure difference between the forespore and mother cell reaches ~60 kPa[11]. It has been suggested that DNA translocation can be seen as an energy transduction process, converting the chemical energy stored in ~1.5 million ATP molecules to mechanical energy, stretching the thin peptidoglycan layer between the forespore and engulfment membranes and smoothing their wrinkles[11]. However, it is not clear if this stored mechanical energy is utilized by the nutrient-deprived, sporulating bacterium, and if so, how.

Here, we investigate the relationship between forespore inflation driven by DNA translocation and the membrane fission event that releases the forespore into the mother cell cytoplasm. We previously showed that this event is catalyzed by the protein FisB that accumulates at the neck of the engulfing membranes during sporulation in *Bacillus subtilis*[12,13]. FisB, a ~250 residue protein that is conserved among endospore-forming bacteria, is produced after asymmetric division in the mother cell[12]. It possesses a short N-terminal cytoplasmic domain, a single-pass transmembrane domain (TMD), and a large extracytoplasmic domain (ECD, Figure 1B)[13]. In cells lacking FisB (*ΔfisB*), engulfment proceeds normally, but the membrane fission step is impaired[12,13]. Membrane fission can be assessed during a sporulation time course by labeling cells with a lipophilic dye that crosses the cell membrane inefficiently (Figure 1C). In cells that have not yet undergone fission, the dye has access to the forespore, engulfing, and mother cell membranes, resulting in a stronger signal where these three membranes are in close proximity. By contrast, in cells that have undergone membrane fission, the dye only weakly labels the internal membranes, allowing the quantification of cells that have undergone fission at a given time point after initiation of sporulation[13] (Figure 1D).

Using this assay, we have previously shown that sporulating cells lacking FisB are impaired in fission. A functional GFP-FisB fusion forms dim mobile clusters (DMC), each containing ~12 GFP-FisB molecules. An intense spot at the engulfment pole (ISEP) containing ~40 copies of GFP-FisB appears around the time of membrane fission (Figure 1E,F and ref. 13). FisB localizes to the membrane neck that connects the engulfment membrane to the rest of the mother cell membrane based only on lipid-binding, self-aggregation, and the unique geometry encountered at the end of engulfment[13]. FisB self-oligomerizes and binds acidic lipids, bridging artificial membranes, but does not appear to have other binding partners. During sporulation, the only location where FisB can bridge membranes is at the membrane neck that eventually undergoes membrane fission (Figure 1F), suggesting homo-oligomerization and trans interactions that bridge membranes are sufficient to explain FisB accumulation in the neck during late stages of engulfment[13]. However, how membrane fission occurs at the last step of engulfment is not known.

Here we show that forespore inflation and FisB accumulation at the membrane neck are both required for efficient membrane fission. Interventions that slowed or prevented forespore area expansion delayed or inhibited membrane fission, even when FisB had accumulated at the membrane neck. Conversely, sporulating cells lacking FisB had impaired membrane fission but their forespores expanded. We find that membrane fission is driven by the combination of high tension in the engulfment membrane due to forespore inflation and FisB oligomerization impeding lipid flux that partially supports forespore and engulfment membrane growth. Our results demonstrate that part of the mechanical energy stored during the process of DNA packaging into the forespore is used for membrane fission.

RESULTS

FisB does not remodel membranes, but forms a stable, extended network on giant unillamellar (GUV) membranes

The simplest mechanism of FisB-catalyzed membrane fission would involve direct membrane remodeling by FisB, similar to eukaryotic membrane fission machineries dynamin[14] or ESCRT-III[15]. To test this idea, we studied interactions of the FisB extracytoplasmic domain (ECD) with model GUV membranes. We fluorescently labeled recombinant purified FisB ECD with iFluor555-maleimide at a cysteine introduced at residue 123 (iFluor-FisB ECD, Figure 2A), a substitution that does not affect FisB's function[13]. GUV membranes were labeled by including a fluorescent lipid in the lipid composition. Membranes and the labeled protein were then visualized using spinning-disc confocal (SDC) microscopy (Figure 2). We first incubated GUVs with various concentrations of iFluor-FisB ECD in a closed chamber for 60 min, then imaged the membranes and FisB ECD (Figure 2B). iFluor-FisB ECD did not bind GUVs lacking acidic lipids, as reported previously[13]. By contrast, iFluor-FisB ECD bound GUVs containing 30 mole % cardiolipin (CL). At 20 nM in solution, FisB binding to GUVs was barely detectable, but at ~75 nM, discrete FisB spots were visible. These spots diffused around the GUV membrane, reminiscent of the DMC in live cells. At 200 nM and above, most GUVs were covered more uniformly and completely by iFluor-FisB ECD.

Consistent with previous work[13], and unlike many proteins implicated in eukaryotic membrane fission[16-18], FisB did not cause any membrane deformations such as tubulation or invaginations. Because such deformations are opposed by membrane tension, we repeated these experiments using deflated GUVs with lower membrane tension. Even at high coverage and when the GUVs were deflated, FisB did not cause any remodeling of GUV membranes (Figure 2B). However, we noticed that deflated GUVs covered with iFluor-FisB ECD did not display shape fluctuations typical of bare deflated GUVs, suggesting FisB may form a fixed, extended network on the membranes stabilizing the membrane shape.

We reasoned that if FisB ECD does indeed form an extended network on membranes, its mobility should be low in such regions. To test this, we performed fluorescence recovery after photobleaching (FRAP) experiments (Figure 2C). We incubated 1 μM iFluor-FisB ECD with GUVs for 2 h. This allowed for a range of iFluor-FisB ECD coverage of GUVs. Some GUVs were uniformly covered by iFluor-FisB ECD whereas some others were only partially covered by iFluor-FisB ECD patches. We bleached the iFluor-FisB ECD fluorescence in a rectangular region of interest (ROI) and monitored the subsequent recovery on a given GUV. Recovery was faster for GUVs that had partial, patchy iFluor-FisB ECD coverage, as new patches diffused into the bleached region (Figure 2C upper row and Figure 2D). By contrast, there was virtually no recovery for GUVs that were uniformly covered by iFluor-FisB ECD (Figure 2C lower row and Figure 2D). To relate FisB ECD membrane coverage to mobility, we plotted the mean iFluor-FisB ECD signal along the contour of the GUV, $F_{\mathrm{mean}}^{\mathrm{ECD}}$, against the fractional fluorescence recovery 45 s after bleaching, as shown in Figure 2E. This analysis showed that increasing FisB ECD coverage led to decreased FisB ECD mobility, consistent with the idea that FisB ECD forms a denser, less dynamic network as coverage increases.

To test how stable the FisB ECD network is, we dissolved the membranes after the network had formed while observing iFluor-FisB ECD signals. Five to ten minutes after addition of Triton X-100 the lipid signal disappeared, suggesting that the membranes were dissolved. Remarkably, iFluor-FisB ECD signals were intact (Figure 2F), indicating that once formed, the three-dimensional FisB ECD network was stable even after the removal of the membrane.

FisB ECD also bridges GUV membranes, accumulating in the membrane adhesion zone (Figure S1 and ref. 13). When membranes from separate GUVs are not available, we speculate that FisB ECD can create folds within the same GUV membrane such that it can still bridge the folded membranes. Such folds cannot be resolved optically, but this idea is supported by the observation that when both the membrane and FisB ECD are labeled, lipid signals are often enhanced at locations where FisB is clustered (Figure 2B and ref. 13). We speculate that similar FisB supramolecular arrangements could be relevant in the FisB clusters (DMC and ISEP) observed in bacteria (Figure S1F).

The self-aggregation, lipid binding, and membrane bridging activities of FisB ECD were independent of the 6-histidine purification tag and occurred in the absence of the last 32 residues that are predicted to be disordered, ruling out potential artifacts due to these features (Figure S1). In addition, using membranes with a more physiological lipid composition containing 5 mole % CL and 50 mole % phosphatidyl glycerol (PG)[19,20]

resulted in similar interactions with FisB ECD (Figure S1 and Figure 6B,C).

Altogether, these data argue that FisB does not remodel membranes but is capable of forming an extended, stable network in which individual FisB molecules are immobile.

Forespore inflation accompanies membrane fission

How can FisB catalyze membrane fission if it cannot remodel membranes by itself? We reasoned that an additional cellular process should be involved, given that direct interactions with other proteins could not be detected[12,13]. A process that could potentially influence membrane fission is the forespore volume increase that occurs after asymmetric division due to translocation of DNA into the forespore by the ATPase SpoIIIE[11] (Figure 3A). However, whether and how this volume increase is related to membrane fission was not known. We began by investigating whether there was a correlation between forespore inflation and membrane fission. We used the lipophilic fluorescent dye 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) that allowed us to detect forespore contours of both cells that had undergone membrane fission and those that had not[13]. We used a semi-automated active-contour fitting algorithm[21] and estimated the membrane areas from the surface of revolution around the long axis of symmetry (Methods). Three hours after initiation of sporulation via nutrient downshift, post-fission forespores were visually larger than their remaining pre-fission counterparts (Figure 3B). The average areas of pre- and post-fission forespores decreased and increased as a function of time after the nutrient downshift, respectively (Figure 3C), suggesting cells with rapidly growing forespores underwent membrane fission, leaving behind smaller pre-fission forespores. Similar trends were obtained using a soluble CFP forespore marker (Figure S2), ruling out potential artifacts due to the use of a lipophilic membrane dye. The close link between forespore inflation and membrane fission was even more evident when we plotted the percentage of cells having undergone membrane fission against the post-fission forespore area, which yielded a nearly linear relationship (Figure 3D).

The forespore area difference between pre- and post-fission cells, ΔA_{FS}, increased during sporulation to reach 1.3 ± 0.1 μm^2 (Figure 3E). Taking into account the 4 membrane leaflets that must grow together and assuming an area per lipid of $\sim 0.7\ \mathrm{nm}^2$, $\sim 7.4 \times 10^6$ new lipids must be added to the engulfment and forespore membranes during sporulation. Given that this growth occurs over ~2 h, lipids must be added at an average rate of ~1,000 lipids/s to the forespore and engulfment membranes. Interestingly, vegetative cell membrane areas grow at a comparable rate ($\dot{A}_{\mathrm{veg}} = 0.020\ \mu\mathrm{m}^2/\mathrm{min}$, or about ~1000 lipids/s, taking into account two leaflets, 95% confidence interval: 0.007-0.023 μm^2/min, n=8 cells), consistent with the finding that phospholipid synthesis during sporulation occurs near the maximal rate associated with vegetative growth[22]. Thus, forespore inflation requires high rates of *de novo* phospholipid synthesis despite energy-limiting conditions, and most of the newly synthesized lipids are utilized for forespore area growth during the late stages of engulfment and after fission.

In summary, there is a clear correlation between membrane fission at the end of engulfment and forespore inflation, suggesting that forespore growth may facilitate

membrane fission.

Hypothesis: increasing membrane tension during forespore inflation drives FisB-dependent membrane fission

How can forespore inflation promote FisB-mediated membrane fission? We hypothesized that increased membrane tension due to forespore inflation, coupled with FisB oligomerization at the membrane neck, could work synergistically to drive membrane fission. Because DNA pumping into the forespore leads to forespore inflation through increased turgor, and smooths membrane wrinkles[11], there must be an associated increase in the tension of the forespore membrane. Furthermore, because the forespore and engulfment membranes are separated through a thin layer of peptidoglycan only ~20 nm wide[23,24], we expect the tension and the area of the engulfment membrane (which eventually undergoes fission) to increase together with that of the forespore membrane. The membrane tension of the forespore and engulfment membranes can be estimated from the excess osmotic pressure in the forespore, ~60 kPa[11]. Assuming the two membranes that surround the forespore balance the excess osmotic pressure, then according to Laplace's Law $\Delta P = 4\gamma/R_{\mathrm{fs}}$, so that a forespore radius of $R_{\mathrm{fs}} = 400$ nm implies a membrane tension $\gamma \sim 6$ pN nm^{-1}. This is a very high value, around values expected for membrane rupture[25-27]. It is thus likely that the peptidoglycan cell wall surrounding the forespore may actually be necessary during forespore inflation to avoid membrane rupture[11].

The hypothesis that increasing membrane tension during forespore inflation drives FisB-dependent membrane fission leads to the following predictions. First, interfering with forespore inflation should hinder membrane fission. Even when forespore inflation is slowed or blocked, FisB should be able to properly localize to the membrane neck (provided engulfment is not compromised), because FisB's localization relies solely on its interactions with itself and with membranes, and the geometry of the neck[13]. Second, in the absence of FisB, forespores should inflate but membrane fission should be impaired.

Blocking lipid synthesis impedes forespore inflation and membrane fission

Lipid synthesis is required during vegetative growth[28,29] and sporulation[22,30] and fatty acid availability determines average cell size[31]. When *B. subtilis* cells are placed in a nutrient poor medium, lipid synthesis is initially downregulated, but with the onset of sporulation, it returns close to the maximal rate[22]. We reasoned that blocking lipid synthesis at different time points after the initiation of sporulation should block forespore area expansion at different stages. Furthermore, if forespore inflation is needed for membrane fission, the percentage of forespores blocked in inflation should correlate with those stalled at the pre-fission stage.

We blocked the synthesis of neutral lipids and phospholipids at different time points t after nutrient downshift using cerulenin, a drug that inhibits *de novo* fatty acid biosynthesis[32] (Figure S3A). We then probed the cells for membrane fission at $t = 3$ h

using TMA-DPH labeling. We found that the earlier lipid synthesis was blocked, the smaller the fraction of cells that had undergone membrane fission at $t = 3$ h (Figure S3B,C). Both pre-and post-fission forespore and mother cell membrane areas decreased with longer cerulenin application (Figure S3D). These results are consistent with previous reports that *de novo* lipid synthesis is needed for engulfment and forespore inflation[11,22]. In addition to perturbing engulfment (Figure S3E,F) FisB accumulation at the fission site was reduced in the presence of cerulenin (Figure S3G), making it difficult to infer the role of forespore inflation in membrane fission. To unmask the contribution of forespore inflation on membrane fission, we focused on cells in which FisB had successfully accumulated at the membrane fission site. We discovered that an increasing fraction of these cells failed to undergo membrane fission with longer cerulenin application (Figure S3H). Thus, although impaired engulfment and FisB accumulation at the fission site partially account for the membrane fission defects observed, blocking lipid synthesis inhibited membrane fission even for cells with proper FisB localization, suggesting forespore inflation facilitates membrane fission.

Slowing DNA translocation slows membrane fission

In addition to lipid synthesis, forespore inflation requires ATP-dependent DNA translocation into the forespore. Accordingly, in a complementary set of experiments we took advantage of previously characterized mutations in the DNA translocase SpoIIIE to investigate whether forespore inflation contributes to membrane fission.

We began by using an ATPase mutant (SpoIIIE36) that is translocation defective[33]. In the absence of DNA pumping, the forespores remained small and engulfment was severely perturbed (Figure S4A), similar to the engulfment phenotype of the Δ*spoIIIE* mutant reported previously[12]. Membrane fission was severely impaired in these cells (Figure S4B) as was forespore area growth, both for pre-fission and the small number of post-fission forespores (Figure S4C). The fission defect can only be partly explained by deficient FisB localization, as 23±4 % of the SpoIIIE36 cells had an ISEP at 3 h after the nutrient downshift, compared to 70±6% of WT cells (Figure S4G), yet only 5% of the SpoIIIE36 cells had undergone membrane fission at this timepoint (Figure S4B). Importantly, among cells with an ISEP at the end of engulfment, only ~16±4% of SpoIIIE36 cells had undergone membrane fission compared to ~91±6% % of WT cells (Figure S4H). This difference was even more pronounced when we used a sensitized background in which FisB was expressed at ~8-fold lower levels than wild-type[12,13]. Under these conditions, there were no post-fission SpoIIIE36 cells at hour 3 of sporulation among the 7% that had an ISEP. By contrast, 95% of the sporulating cells with SpoIIIE^{WT} that had an ISEP had undergone membrane fission in this FisB low-expression background (Figure S4G,H). Altogether, these results suggest that membrane fission requires both FisB accumulation at the neck (ISEP formation) and forespore inflation.

The severe engulfment defects in the SpoIIIE36 mutant have the potential to obscure the contribution of forespore inflation to FisB localization and membrane fission. We therefore took advantage of a second mutant, SpoIIIE(D584A) (SpoIIIE^{SLOW}) that

translocates DNA ~2.5-fold slower than wild-type[34] and does not have engulfment or FisB localization defects (Figure 4G,H). In sporulating cells expressing *spoIIIE*^{SLOW}, we observed slow forespore inflation that correlated with slow membrane fission. Specifically, by hour 3 of sporulation, *spoIIIE*^{SLOW} cells accumulated ~1.4-fold more FisB at the cell pole than did wild-type cells (Figure 4I), yet only ~40% of the *spoIIIE*^{SLOW} cells had undergone membrane fission compared to ~75% of the wild type cells (Figure 4D). Furthermore, forespore inflation was delayed for post-fission cells and the average forespore area of the remaining pre-fission cells increased instead of decreasing as for wild-type cells (Figure 4A-C), suggesting slow forespore inflation slows membrane fission under conditions in which neither engulfment nor FisB localization are impaired. This was more evident when the percentage of cells that had undergone fission was plotted together with the post-fission forespore areas as functions of time (Figure 4D). The percentage of cells that underwent membrane fission increased with the increase in post-fission forespore area (Figure 4E).

Together, these results strongly suggest that FisB accumulation at the membrane neck is *not* a sufficient condition for membrane fission; fission additionally requires forespore enlargement.

In the absence of FisB, forespores inflate but fission is impaired

Our data suggest that FisB accumulation at the fission site and forespore inflation work together to drive membrane fission. To investigate whether these two processes occur independently, we monitored forespore membrane area changes in cells expressing FisB at low levels or altogether lacking it.

We analyzed forespore area at different time points after a nutrient downshift, using TMA-DPH (Figure 5A-D). As anticipated, most sporulating cells lacking FisB (*ΔfisB*) did not undergo membrane fission (Figure 1D) despite completing engulfment (Figure 5A) [12,13]. However, and importantly, the forespore areas of these pre-fission cells, $A_{\mathrm{FS}}^{\mathrm{pre}}$, increased as a function of time after the nutrient downshift, nearly doubling by $t = 3.5$ h, while those of wild-type cells decreased slightly during the same period (Figure 5B). The forespore areas of the small fraction of *ΔfisB* cells that underwent membrane fission (Figure 1), $A_{\mathrm{FS}}^{\mathrm{post}}$, were slightly larger than those of wild-type cells and followed a similar time course, increasing as a function of time (Figure 5C).

Sporulating cells expressing GFP-FisB at ~8-fold lower levels than wild-type undergo membrane fission more slowly (Figure S5). In these cells, post-fission forespore areas, $A_{\mathrm{FS}}^{\mathrm{post}}$, were slightly larger than those expressing GFP-FisB at native levels, and in both cases $A_{\mathrm{FS}}^{\mathrm{post}}$ increased over time (Figure S5F). Similar to the Δ*fisB* mutant, the pre-fission forespore area increased as a function of time when GFP-FisB levels were reduced albeit the increase was more modest (Figure S5E).

Overall, these results indicate that forespore inflation occurs independently of FisB, but that forespore inflation is not sufficient to catalyze efficient membrane fission. When

FisB is present, the fission process is greatly accelerated by forming an aggregate at the membrane neck to be severed, but only if the forespore is inflating (Figure 5D).

Modeling suggests increased engulfment membrane tension is the primary driver of FisB-catalyzed membrane fission

Next, we modeled the effect of increased membrane tension on membrane neck geometry, including both the pulling force on the forespore due to hydrodynamic drag during DNA pumping and the osmotic pressure difference between the cytoplasm and the lumen of the neck. We developed a minimal model based on free energy minimization, considering the free energy F of an axisymmetric and mirror symmetric membrane neck connecting two membrane sheets, corresponding to the local geometry of the neck formed between the forespore and mother cell membranes (see Methods S1 in Supplemental Information for details). The energy functional F consists of a term accounting for membrane bending and tension, F_{m}, another term, F_p, accounting for an osmotic pressure difference $\Delta p = p_{\mathrm{cyto}} - p_{\mathrm{peri}}$ between the cytoplasm and periplasm since the lumen of the neck is continuous with the periplasmic space, and finally a term representing any pulling force f on the forespore, e.g. due to DNA translocation, F_f.

We first explored the role of membrane tension, γ, on neck geometry. As shown previously[13] using a minimal model for the neck as a cylinder of radius r_{cyl} and length L_{cyl}, there are two regimes: (1) For a large neck (radius larger than a critical radius r_{crit}), membrane tension drives the neck to become ever larger. (2) By contrast, for an initial $r_{\mathrm{cyl}} < r_{\mathrm{crit}}$, the neck will relax to a finite equilibrium radius set by the balance between membrane tension and membrane bending stiffness. Intuitively, these two regimes follow because as the radius of the cylindrical neck grows, the area of the cylinder grows $\sim L_{\mathrm{cyl}}\, r_{\mathrm{cyl}}$, but the areas of the associated flat membranes on the mother cell and forespore *shrink* $\sim r_{\mathrm{cyl}}^2$. While the former term can dominate for small r_{cyl} leading to a local minimum, the latter term eventually dominates for large r_{cyl} implying the neck will open. The existence of a local minimum of neck radius is recapitulated by this simplified model in Methods S1 Eqs. 1–5. As seen in Figure 5E (Left) and Fig. S7d, in regime (2) increasing membrane tension γ results in a narrowing of the equilibrium neck radius. While to our knowledge there has been no direct measurement of the membrane tension in *B. subtilis*, we find that reasonable values of $\gamma = 0.1 - 1$ pN nm^{-1} are sufficient to achieve a neck radius < 10 nm[27]. It is likely that cell wall remodeling that drives engulfment[35] brings the neck radius into regime (2), because a neck forms and a small fraction of cells undergo membrane fission even in the absence of FisB (Figure 1 and Figure 5). Moreover, we obtained an estimate of the membrane tension associated to a forespore radius of ~ 300–400 nm for an osmotic pressure difference $\Delta p \sim 60\ \mathrm{kPa}$ and in the absence of a pulling force f, yielding $\gamma \sim 4.5\text{–}6\ \mathrm{pN\ nm^{-1}}$, which according to our model is large enough to produce a narrow neck and thus accumulate FisB in the neck.

To examine the additional role of osmotic pressure difference between the cytoplasm and the lumen of the neck, we also minimized F at finite Δp. While $\Delta p = 0.1$ atm has little effect on the neck radius, $\Delta p = 0.6$ atm, which is realistic for *B. subtilis*[11,27] further

reduces the minimum neck radius, by e.g. ~1 nm (Fig. S7e and S7h,i), for a fixed value of the membrane tension, $\gamma = 0.84$ pN nm^{-1}. This reduction makes sense, as higher osmotic pressure in the mother cell will tend to compress the volume of the periplasmic space contained within the neck.

By contrast, a pulling force on the forespore not only stretches the neck but also *increases* its equilibrium radius (see Figure 5E right, and Fig. S7). A pulling force of $f = 50 - 100$ pN, around the maximum force that can be applied by one or two SpoIIIE motors translocating DNA into the forespore[36,37] (see Methods S1 for more details), increases the minimum neck radius significantly compared to a case where there is no pulling force. In particular, for $f = 100$ pN the membrane neck radius increases by $\sim 4\ \mathrm{nm}$ and the neck length increases by $30\ \mathrm{nm}$, considering $\Delta p = 0, \gamma = 0.84$ pN nm^{-1} (Figure 5E right and S7h). One can understand this effect simply by considering the total force in the neck at the midpoint, which is the product of the circumference of the neck times the membrane tension, $f = 2\pi r_{\mathrm{min}}\gamma$. So at fixed membrane tension γ, an increase of the pulling force f automatically implies an increasing r_{min}. Moreover, an osmotic pressure difference of $\Delta p = 0.6$ atm is able to reduce the minimum neck radius by ~1 nm even if the pulling force f is not zero, as shown in Fig. S7i, for a fixed value of the membrane tension $\gamma = 0.84$ pN nm^{-1}.

It is likely that the membrane tension in the rapidly expanding forespore and engulfment membranes is higher than in the mother cell membrane. We estimated an upper limit for this membrane tension gradient, by estimating the gradient that would be sufficient to drive a flux of lipids in the neck matching the observed average increase in forespore membrane area (see above and Methods S1). Perhaps surprisingly, the resulting difference is only $\sim 10^{-7}$ pN nm^{-1}, which is orders of magnitude less than the expected overall membrane tension. Thus an unimpeded flux of lipids between the mother cell and forespore will have negligible effect on the equilibrium results presented above. However, if this flux is strongly impeded, e.g. by accumulation of FisB at the neck, the tension in the engulfment membrane around the forespore would increase further, possibly contributing to membrane fission (see below).

In summary, modeling suggests that the pulling force on the forespore exerted by DNA translocation has a negligible effect on neck geometry. By contrast, a realistically high membrane tension, possibly augmented by an osmotic pressure difference across the membrane, is sufficient to drive the equilibrium neck radius down to $\leq$ 5-10 nm, with two expected consequences. First, with a small neck radius, FisB can interact in *trans* and accumulate in the neck, facilitating membrane fission[13]. Second, high membrane tension and a small neck radius likely both facilitate membrane fission.

Membrane fission catalyzed by FisB-lipid friction under high membrane tension

Our results so far suggest that increased membrane tension due to forespore inflation drives FisB-catalyzed membrane fission. Increased membrane tension in the engulfment membrane would provide a driving force for membrane flow from the mother cell to the engulfment membrane through the neck connecting the two compartments. One possibility is that by forming an oligomer at the membrane neck, FisB impedes the

movement of lipids that partially support this growth, further increasing the tension in the engulfment membrane, promoting membrane fission. To test this possibility, here we studied lipid flows between membrane compartments in live cells and how lipid mobility is affected by FisB oligomerization.

We first tested if FisB oligomerization on a membrane can impede lipid movement. We incubated FisB ECD with GUVs labeled with fluorescent Cy5-lipids, allowing FisB ECD to form a dense scaffold as in Figure 2. We then probed lipid mobility using FRAP as shown in Figure 6. In the absence of FisB ECD, fluorescence of the bleached region recovered rapidly and nearly completely, indicating unimpeded diffusion, as expected. However, when GUVs were coated with FisB ECD, about half the lipids became immobile (Figure 6C). We conclude that FisB oligomerization severely impedes lipid mobility.

Next, we tested if forespore inflation leads to membrane movement between the mother cell and engulfment membrane compartments. We followed the kinetics of forespore area growth in individual cells and related the kinetics to membrane fission. Using time-lapse microscopy, we imaged individual sporulating cells labeled with the lipophilic dye FM4-64[11] as shown in Figure 6D,E (see Figure S6 for more examples). While TMA-DPH crosses the cell membrane slowly, FM4-64 does not cross it, which allowed us to detect the time of membrane fission in addition to monitoring membrane areas. The non-fluorescent FM4-64 molecules in the aqueous phase exchange with the membrane-inserted fluorescent ones[38]. Membrane fission isolates the small inter-membrane space from the bath, blocking exchange of bleached dyes with unbleached ones in the bath and accelerating decay of the fluorescence signals arising from the forespore and engulfment membranes. A plot of forespore contour fluorescence as a function of time for individual cells shows a stable signal, which starts decreasing upon membrane fission. We cross-correlated these individual fluorescence profiles with a model decay function to determine the timing of membrane fission (Figure 6F). We defined $t = 0$ as the frame just preceding fission (Figure 6E,F) and aligned all measurements with respect to this time before averaging membrane areas as shown in Figure 6G,H. The rate of forespore area increase slowed gradually after membrane fission (Figure 6G). Remarkably, the mother cell area shrank before and grew after membrane fission (Figure 6H).

Until FisB accumulates at the membrane neck and the membrane undergoes fission, there are two sources of lipids to support the growth of the forespore and engulfment membranes: *de novo* lipid synthesis and lipid flux between the mother cell and engulfment membranes (Figure 6I). After membrane fission, substantial expansion of the forespore and engulfment membranes can only be supported by *de novo* lipid synthesis. Thus, analysis of the rates of area changes can be informative about lipid flux through the neck. We focused on the mother cell area changes for this analysis, because lipid synthesis in this cellular compartment is better characterized[22]. The mother cell area changed at rates of $-0.011\ \mu\mathrm{m}^2/\mathrm{min}$ (-262 lipids/s) and $+0.0032\ \mu\mathrm{m}^2/\mathrm{min}$ (+76 lipids/s) before and after fission, respectively. Assuming the rates of insertion into the mother cell of newly synthesized lipids pre- and post-fission do not change appreciably, we estimate ~340 lipids/s move through the neck toward the engulfment membrane before FisB accumulation – a significant fraction of the maximal

cellular lipid synthesis capacity (see above). Membranes lyse when stretched above only ~1 % in area[26]. Hence, assuming that the total area of the engulfment and forespore membranes is ~ 5 μm^2, a deficit of ~300 lipids/s would cause a 1% deficit of the membrane area, i.e. ~ 0.05 μm^2, within ~ 4 min if the area expanded at fixed number of lipids. Thus, it is possible that FisB oligomerization – by interfering with lipid flux through the neck – leads to a further increase in the engulfment membrane tension and thus facilitates membrane fission.

DISCUSSION

Bacteria rely on membrane fission for every division cycle and during endospore formation. FisB is the only molecule described so far with a dedicated role in membrane fission in bacteria[12,13], but how FisB catalyzes membrane fission has remained unclear. Here we report that FisB does not remodel membranes directly but forms a stable network on them. Thus, another cellular process must be involved to achieve membrane fission at the end of engulfment. Surprisingly, we found that translocation of the chromosomal DNA into the forespore by the ATPase SpoIIIE – occurring far from the membrane fission site – is a necessary condition for FisB-dependent membrane fission. DNA translocation leads to forespore inflation through increased turgor, stretching the thin layer of peptidoglycan between the forespore and engulfment membranes and smoothing membrane wrinkles[11]. FisB accumulation at the membrane fission site and forespore inflation are both necessary conditions for efficient membrane fission.

We propose that increased membrane tension in the engulfment membrane due to forespore inflation drives membrane fission (Figure 7). High membrane tension can cause membrane fission through opening of a transient pore[39], as was shown for fission catalyzed by BAR domain proteins scaffolding a membrane tube under extension[40]. The presence of a protein scaffold on a membrane tube has a dual role: slowing equilibration of membrane tension by impeding membrane flow, and acting as a heterogeneous nucleation point for a membrane defect, lowering the energy barrier for membrane poration[40]. Thus, FisB can similarly have a dual role in membrane fission. First, by oligomerizing at the membrane neck, FisB impedes membrane flow from the mother cell membrane to the engulfment membrane where tension is higher, leading to a further increase in tension, making fission more likely. Second, the FisB oligomer-bare membrane interface could provide a nucleation point for a membrane defect at high tension.

Membrane fission requires energy input. All biological membrane fission reactions described to date are both energized and regulated locally. For the best characterized cases, membrane fission proteins dynamin[14] or ESCRT III[15] polymerize at the membrane fission site and use the energy liberated by nucleoside triphosphate hydrolysis to reshape membranes locally. By contrast, here we have found that membrane fission during endospore formation in *B. subtilis* is energized indirectly and non-locally through DNA translocation into the forespore – a process that is required primarily for genetic inheritance and at first sight bears little relation to the membrane fission that occurs at the opposite end of the engulfment membrane. This situation is

analogous to secondary transporters using electrochemical gradients set by primary pumps such as the sodium-potassium ATPase to energize secondary transport processes[41].

Additionally, our results clarify the role of SpoIIIE in membrane fission during endospore formation. It was originally proposed that in addition to DNA translocation, SpoIIIE drives membrane fission because an ATPase deficient mutant resulted in membrane fission defects[42,43]. However, SpoIIIE's role in membrane fission remained unclear because the knock-out or ATPase dead mutants (Figure S4 and ref. 12) cause severe engulfment defects. Since FisB localization and membrane fission occur downstream of engulfment, any engulfment defect would also lead to defects in FisB localization and membrane fission and would not be particularly informative about membrane fission itself. Here we establish that SpoIIIE does indeed play a critical role in membrane fission even in the absence of engulfment defects, but this role is related to forespore inflation.

The membrane fission mechanism we have described here provides an elegant example of how bacteria efficiently use energy under starvation conditions. DNA translocation into the forespore is an energy-consuming process that cannot be bypassed, but the byproduct of this process, namely increased turgor in the forespore, can be harnessed to energize secondary processes. It will be interesting to see if processes other than membrane fission are energized by mechanical energy stored in the inflated forespore.

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AUTHOR CONTRIBUTIONS

EK, MB, AL, and DZR conceived the study. MB carried out most of the experiments with purified proteins and artificial membranes, AL carried out live cell imaging and analysis. AL and CGP performed the lipid mobility measurements. MB, EK wrote matlab scripts

for image analysis, AL, MB analysed images. AMC and NSW developed the mathematical model, DZR, NSW and EK supervised the work. CDAR and TD provided reagents and intellectual input. EK wrote the manuscript, with input from all authors.

DECLARATION OF INTERESTS

The authors declare they have no conflicts of interest.

INCUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

FIGURE LEGENDS

Figure 1. FisB is required for efficient membrane fission during sporulation. **A.** Schematic of sporulation stages. Upon starvation, sporulation is initiated by asymmetric division into a mother cell (MC) and a forespore (FS). The MC then engulf the FS. At the end of engulfment, a thin neck or tube connects the engulfment membrane to the rest of the MC membrane (red box). Fission of the neck releases the FS, now surrounded by two membranes, into the MC cytoplasm. Upon maturation, the FS turns into a spore and the MC lyses to release it. Inset: membrane fission step marking the end of engulfment. **B**. Domain structure of FisB[12,13]. **C**. Membrane fission assay. An aliquot of cells is labeled with the lipophilic dye 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) which fluoresces only once inserted into the membrane. The dye labels internal membranes poorly. If a cell has not yet undergone membrane fission at the time of TMA-DPH labeling, the dye has access to the space between the engulfment and FS membranes, resulting in brighter labeling of the MC, FS, and engulfment membranes when these are adjacent to one another (top row). After fission, the dye labels internal membranes poorly (bottom row). **D**. Time course of membrane fission for wild-type cells, *ΔfisB* cells, or *ΔfisB* cells complemented with mGFP-FisB expressed at native levels (strain BAM003). Some membrane fission occurs even in the absence of FisB (*ΔfisB*, gray markers). Mean$\pm$SEM from 3 independent experiments is shown (>300 cells were analyzed per point). **E.** Wide-field fluorescence microscopy images of cells expressing mGFP-FisB at native levels (strain BAM003). Aliquots were taken from the suspension at indicated times after nutrient downshift, labeled with TMA-DPH, and images of mGFP-FisB and membranes were acquired sequentially. Examples of sporulating cells with mGFP-FisB enriched at the septum (1.5 h), forming a dim mobile cluster (DMC; 2.5 h) and with a discrete mGFP-FisB focus at the cell pole (intense spot at engulfment pole, ISEP, 3 h) are highlighted with arrowheads. Scale bars represent 1µm. **F**. Schematic of FisB dynamics[13]. At the end of engulfment, ~40 FisB molecules accumulate at the neck into an immobile cluster to catalyze membrane fission.

Figure 2. FisB does not remodel membranes. A. The soluble recombinant fragment of FisB comprising its extracytoplasmic domain (ECD) used in the experiments with GUVs. **B**. Interactions of FisB ECD with GUV membranes ("100% PC" and "30% CL" membranes are composed of (all mole %) 99 PC, 1 NBD-PE, and 30 *E. coli* CL, 69 eggPC, 1 NBD-PE, respectively). At low concentrations, FisB ECD forms small mobile clusters. With increasing concentration, labeling becomes more uniform. Even at high coverage or with deflated GUVs (right), no membrane remodeling is evident (deflated GUVs were composed of (all mole %) 25 *E. coli* PE, 5 *E. coli* CL, 50 *E. coli* PG, 19 eggPC and 1 DiD or NBD-PE (BS mix)). **C**. Mobility of FisB ECD decreases with increasing coverage. GUVs composed of BS mix were incubated with 1 µM iFluor555-FisB ECD for 2h. GUVs were covered with protein to varying degrees. Top row shows fluorescence recovery of a GUV with low iFluor555-FisB ECD coverage. Bottom row shows an example of a GUV fully covered with iFluor555-FisB ECD fluorescence that does not recover after photobleaching. Photobleached regions are indicated with boxes. **D**. Normalized fluorescence intensity from the boxed regions in C as a function of time. **E**. Higher protein coverage ($\propto$ mean GUV membrane intensity) leads to lower protein

mobility. The fractional fluorescence recovery 45 s post-bleaching ($(F_{45} - F_0)/(1 - F_0)$, where F_{45} is the fluorescence intensity at 45 s and F_0 is the intensity just after bleaching, both relative to pre-bleach intensity) is plotted as a function of mean GUV membrane intensity. Each dot represents a GUV. **F**. FisB ECD forms a fixed, stable network on the GUV membrane that persists even after the membranes are dissolved with detergent. 1 μM iFluor555-FisB ECD was incubated with deflated GUVs for 2h, then Triton X-100 was added (1.7 mM final concentration, 7-8-fold above the critical micelle concentration[44]), and the sample imaged 5-10 min thereafter. Two different examples are shown in the two columns of images.

Figure 3. Forespore area increase during sporulation correlates with membrane fission activity. A. Schematic of SpoIIIE-mediated pumping of the chromosome into the forespore leading to forespore inflation. **B**. Images show wild-type strain (PY79) at *t*=3 h after nutrient downshift to initiate sporulation. Membranes were visualized using TMA-DPH. Forespores of cells that have undergone membrane fission (red contours on the right panel) are larger than those that have not yet undergone membrane fission (blue contours). Scale bar represents 1 μm. **C**. Quantification of forespore membrane area for cells that have (red, "post") or have not (blue, "pre") undergone membrane fission. **D**. The percentage of cells that have undergone membrane fission as a function of post-fission forespore area. **E**. The difference between post- and pre-fission forespore areas, ΔA_{FS}, grows as a function of time. In C-E, mean±SEM of 3 independent experiments are shown, with 70 cells analyzed per data point.

Figure 4. Slower forespore inflation leads to slower membrane fission with no adverse effect on engulfment or FisB localization. A. Fluorescence microscopy images of TMA-DPH labeled wild-type SpoIIIE strain BAL039 (top row, times after nutrient downshift are indicated). The lower row shows the same images, but with the overlaid forespore contours for cells that have (red) or have not (blue) undergone membrane fission. **B.** As in A, but for the slow DNA-pumping SpoIIIE strain BAL040 (SpoIIIE^{SLOW}). **C**. The average forespore area for pre- (blue) and post-fission (red) cells as a function of time into sporulation for the SpoIIIE^{SLOW} (dashed lines, strain BAL040) or wild-type (solid lines) cells (strain BAL039). (n=25-70 cells per data point). **D**. The percentage of cells that have undergone membrane fission (left axis, black) followed a very similar time course as the forespore area as a function of time (right axis, magenta) for both WT (solid lines) and SpoIIIE^{SLOW} (dashed lines) cells. Both membrane fission and forespore inflation were delayed in SpoIIIE^{SLOW} cells (>300 cells per data point). **E**. The percentage of post-fission cells increased with increasing forespore area for both WT (solid lines) and SpoIIIE^{SLOW} (dashed lines) cells. WT data is copied from Figure 3 for comparison. **F.** Fluorescence microscopy images of WT or SpoIIIE^{SLOW} cells expressing mGFP-FisB at native levels in a *ΔfisB* background, probed 3h after the nutrient downshift. Membranes were labeled with TMA-DPH. In cells expressing WT SpoIIIE (top row) post-fission cells have larger forespores (red contours) and FisB is accumulated at the membrane fission site (red arrowheads). At this stage, most cells expressing SpoIIIE^{SLOW} are still in the pre-fission stage, even if engulfment is visually complete and FisB has accumulated at the membrane fission site. **G.** Percentage of WT or SpoIIIE^{SLOW} cells with engulfment membrane migration completed by t=3 h into sporulation. Engulfment is not perturbed significantly by the slow DNA pumping by

SpoIIIE^{SLOW} (>300 cells per data point). **H**. FisB localization is not perturbed in SpoIIIE^{SLOW} cells compared to WT cells. **I**. Distributions of mGFP-FisB fluorescence intensities for the ISEP in wild-type ("WT") and the slow DNA pumping by SpoIIIE^{SLOW} mutant strain ("slow"). Representative regions of interest (and regions taken for background correction, "Bg") are shown on the example images on the left. The means ($\pm$ SD) are 70 ± 17 a.u. for WT and 99 ± 18 for SpoIIIE^{SLOW} ISEP intensities. Since for WT this intensity corresponds to ~40 copies of FisB[13], we conclude that nearly 60 copies are accumulated in SpoIIIE^{SLOW}. Scale bars represent 1 µm in A, B, and F**.** Means$\pm$SEM for 3 biological replicates are plotted in panels C-E, G,H. See Figure S4 for results with an ATPase dead SpoIIIE mutant.

Figure 5. **In the absence of FisB, forespores inflate but fission is impaired. A**. Representative images of wild-type (PY79) or ***ΔfisB*** cells (BDR1083) at the indicated times after nutrient downshift. Membranes were visualized with TMA-DPH. Lower panels show the FS contours for cells that have (red) or have not (blue) undergone fission. Scale bars represent 1 µm. **B.** The average pre-fission forespore area, $A_{\mathrm{FS}}^{\mathrm{pre}}$, grows during sporulation for ***ΔfisB*** cells, while it decreases for the wild-type strain. **C**. The average post-fission forespore area, $A_{\mathrm{FS}}^{\mathrm{post}}$, increases faster for ***ΔfisB*** cells. **D.** Summary of results. FisB catalyzes membrane fission in rapidly growing forespores (top row, "WT rapid inflation"). Slowly inflating forespores are less likely to undergo membrane fission even in the presence of FisB (middle row, "WT slow inflation"). In the absence of FisB, forespores inflate, but fission is rare. See Figure S5 for results with a strain expressing FisB at reduced levels. In B, C, mean$\pm$SEM of 3 independent measurements are shown (70 cells per data point). **E**. Modeled dimensionless free energy $\mathcal{F}$ of the membrane neck (Methods S1 Eq. 6) as a function of the arc half-length s_{end} for dimensionless pulling force (Left) $f = 0$ and (Right) $f = 1.35$, for boundary condition dimensionless radius $R_{\mathrm{bc}} = 4$ at $s = s_{\mathrm{end}}$. In both panels we neglect any osmotic pressure difference across the membrane, i.e. $\Delta p = 0$. (Left insets) Bottom: sketch of the membrane geometry during the last stage of forespore engulfment, and schematic of the model of the membrane neck showing the parametrization of the surface of revolution, where s is the arc length along the contour and θ the angle with respect to the vertical. Top: shapes of the membrane neck for different values of s_{end} corresponding to the two local minima of the free energy for $f = 0$, as indicated with dots. The values of the parameters used to generate the dimensional axes are $\gamma = 0.84$ pN nm^{-1} and $\kappa = 20\ k_B T$. (Right inset) Shape of the membrane neck for the value of s_{end} corresponding to the minimum value of the free energy for $f = 1.35$, as indicated by the dot. The dimensional axes are scaled with the same values of the parameters as in the left top inset, which corresponds to $f = 100$ pN.

Figure 6. FisB impedes lipid diffusion. A-C. Lipid diffusion is impeded by FisB ECD oligomerization on membranes. **A**. Schematic of the experiment. A region of interest (2 μm by 2 μm by ~1 μm in xyz) is bleached and fluorescence recovery is monitored for bare GUVs or GUVs covered by FisB ECD labeled with iFluor555. The schematic is drawn from a side view. Actual bleaching and imaging are performed through the bottom. **B**. Snapshots from time-lapse movies at the indicated times for a representative protein-free (top) or a FisB ECD covered GUV (bottom). Time zero corresponds to the

frame right after high-intensity bleach. Scale bar is 1 µm. **C**. Quantification of recovery kinetics. To estimate fractional recovery and an approximate recovery time, the corrected and averaged recovery curves were fitted to $f(t) = f_{\infty}(1 - exp(-t/\tau))$ where f_{∞} and τ are the mobile fraction and the recovery timescales, respectively. Best-fit mobile fractions were (with 95% confidence intervals) f_{∞} =0.98 (0.97, 0.99) and 0.48 (0.47, 0.49) for bare and FisB ECD coated GUVs, respectively (R^2=0.98-0.99). In both cases $\tau \approx$ 1.7 s, implying a lipid diffusivity of a few $\mu m^2/s$. **D-I.** Membrane flows between membrane compartments before and after membrane fission. **D**. Schematic showing how the timing of membrane fission is detected in time-lapse movies of individual cells. The dye (depicted as a star) becomes highly fluorescent upon insertion into the membrane. The lipid-inserted and free dyes exchange continuously, minimizing bleaching in pre-fission cells. Upon fission, the free dye has no longer access to the inter-membrane space, leading to the onset of an intensity decay. **E.** An example of a single cell, labeled with FM4-64, followed as a function of time (see Figure S6 for more examples). Top row shows a montage of FM4-64 fluorescence images as a function of time. The middle and bottom rows show the detected forespore and mother cell contours respectively, using an active-contour fitting algorithm. The frame preceding fission is indicated in the image (τ_{fiss}). Bar,1 µm. **F**. Plot of the mean FS contour FM4-64 fluorescence (black) as a function of time for the individual cell in E, displaying a relatively stable signal and a rapid decay upon membrane fission. Cross-correlation of the fluorescence signal with a model decay function (blue) was applied to determine the timing of membrane fission τ_{fiss}. **G**. Average of forespore cell membrane surface area of wild-type sporangia as a function of time (n=25). Time axes for individual cells were aligned such that $\tau_{\text{fiss}} = 0$ min. Error bars represent SEM. The dashed blue and red lines are linear fits to the pre- and fission periods, with slopes 10.6×10^{-3} and $7.9 \times 10^{-3}\ \mu\text{m}^2/\text{min}$. **H**. Similar to G, but for the mother cell areas. The dashed blue and red lines are linear fits to the pre- and fission periods, with slopes -10.5×10^{-3} and $+3.2 \times 10^{-3}\ \mu\text{m}^2/\text{min}$. **I**. Schematic showing sources and sinks of lipids. Before fission, newly synthesized lipids are inserted into the mother cell and engulfment membranes from the mother cell cytoplasm at rates M and S_c, respectively. Lipids synthesized within the forespore are inserted at rate S_f. Lipid flux through the neck (at rate N) is only possible before fission.

Figure 7. Proposed model of how forespore inflation drives FisB-mediated membrane fission. **A**. DNA translocation by the ATPase SpoIIIE inflates the forespore, stretching the thin peptidoglycan layer between two membranes and smoothing membrane wrinkles [11]. Increased membrane tension in the engulfment membrane drives lipid flux through the neck between the engulfment and mother cell membranes, partially supplying lipids needed for forespore inflation. **B**. FisB oligomerizes at the membrane neck, impeding membrane flow and causing the engulfment membrane to stretch further. **C**. Increased membrane tension drives membrane scission.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Erdem Karatekin (erdem.karatekin@yale.edu)

Materials availability

The materials generated in this study are available upon request from the lead contact.

Data and code availability

- ~~This study did not generate any standardized large data sets.~~ Data underlying the results shown have been uploaded to Mendeley (doi: 10.17632/27cdx65wh7.1) and are available as of the date of publication. ~~are provided as supplemental material,~~ They are provided in the form of an excel file or MATLAB .fig files from which numerical values of the plotted data can be extracted.
-
- MATLAB scripts ~~we~~ generated for image analysis are available at Mendeley (doi: 10.17632/27cdx65wh7.1)~~provided as supplemental material~~.
- ~~The supplemental data and scripts can be found at: Karatekin, Erdem; Landajuela, Ane; Braun, Martha; Martinez-Calvo , Alejandro; Rodrigues, Christopher; Gomis Perez, Carolina; Doan, Thierry; Rudner, David; Wingreen, Ned (2022), "Landajuela_CurrentBiology2022", Mendeley Data, V1, doi: 10.17632/27cdx65wh7.1~~
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All *B. subtilis* strains used in this study were derived from the prototrophic strain PY79[45] and were constructed using plasmid or genomic DNA and a 1-step competence method. Briefly, a freshly streaked colony was used to inoculate 1 ml 1XMC (100 mM potassium phosphate pH, 7.03 mM sodium citrate, 2% glucose 22 mg/ml ferric ammonium citrate, 0.1% casein hydrolysate, 0.2 % potassium glutamate, 3 mM MgSO4) and grown at 37°C for 4 h to create competent *B. subtilis* cells. 2 µl and 1:20 and 1:400 dilutions of either plasmid DNA (obtained from *E. coli* using QIAprep Spin Miniprep Kit or *B. subtilis* genomic DNA were added to 20 – 200 µl of competent cells and the transformation was grown for another 2h at 37°C. Finally, the entire transformation was plated on selective LB plates overnight at 37°C.

Synchronous sporulation was induced in liquid medium at 37°C by resuspension in minimal salts according to the method of Sterlini-Mandelstam[46]. Briefly, overnight cultures of *B. subtilis* strains were prepared by inoculating 5 ml of CH medium [per liter: hydrolyzed casein (8.6 g), L-glutamic acid (3.16 g), DL-alanine (2.14 g), L-asparagine

monohydrate (1.2 g), KH_2PO_4 (1.36 g) Na_2SO_4 (0.107 g), $FeCl_3.6H_20$ (0.6 mg), NH_4Cl (0.535 g), NH_4NO_3 (0.096 g), $MgSO_4.7H_20$ (0.0986 g), $CaCl_2.6H_2O$ (0.02 g), $MnSO_4.4H_2O$ (0.022 g), L-tryptophan (0.02 g), pH 7.1] with a freshly streaked colony. 1:5 and 1:25 dilutions were prepared, and all cultures were grown overnight at 22°C. The next morning the OD600 of all cultures was measured and only cultures that were in mid-log phase (OD600 = 0.4 - 1.0) were used in further experiments. Mid-log phase cultures were then diluted in fresh CH medium to OD600 = 0.05 and grown at 37˚C until cultures reached mid-log phase (0.5-0.9). Cells were harvested by centrifugation for 5 min at 5 krpm. To induce sporulation, the cell pellet was resuspended in an equal volume of Resuspension Medium [per liter: $FeCl_2$ (0.046 mg), $MgSO_4$ (4.8 g), $MnCl_2$ (12.6 mg), NH_4Cl (535 mg), Na_2SO_4 (106 mg), KH_2PO_4 (68 mg), NH_4NO_3 (96.5 mg), $CaCl_2$ (219 mg), L-glutamic acid (2 g), L-tryptophan (20 mg). The pH was adjusted to 7.1 with KOH] and transferred back to the original flasks. The cultures were returned to 37˚C at time=0 h ("T0") of sporulation. T1, T2, etc. refer to t=1 h, 2 h, etc. after induction of sporulation. Site directed mutagenesis was performed using Agilent's Quick-change Lightning kit following manufacturer's instructions and mutations were confirmed by sequencing. The list of strains and plasmids can be found in the Star Methods Key Resources Table.

METHOD DETAILS

FLUORESCENCE MICROSCOPY FROM BATCH CULTURES

500 µl samples at indicated times during sporulation were taken and concentrated ~50-fold by centrifugation (3300xg for 30 s). Membranes were stained with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH; Molecular Probes) at a final concentration of 100 µM and the cells were then immobilized on a 2% agarose pad made with sporulation buffer covered with a no.1.5 coverslip, after which the cells were immediately imaged live. In most cases fluorescence microscopy was performed using a Nikon Ti microscope equipped with a ×100 Plan Apo 1.45 NA phase-contrast oil objective, an Orca-Flash4.0 V2 CMOS camera (Hamamatsu Photonics, Shizuoka,Japan) and a Spectra X light engine (Lumencor, Beaverton, OR, USA), all controlled by Nikon Elements software (Nikon Corp. Tokyo, Japan). Excitation of TMA-DPH was achieved using the 395/25 nm band of the SpectraX system and Chroma's ET 49000 single band filter set the DAPI filter set. (λ_{ex}=395/25 nm; λ_{em}=431/28 nm). Excitation light intensity was set to 50% and exposure times were 200 ms.

In experiments shown in Figure 6 and S3, cells were prepared following the same procedure but visualized on a Leica DMi8 Wide-field Inverted Microscope equipped with a HC PL APO 100× DIC objective (NA=1.40), and an Andor iXon Ultra 888 EMCCD camera (Andor Technology Ltd., Belfast, Northern Ireland) and a Spectra X light engine (Lumencor, OR,USA) controlled by the LAS X program (Leica Microsystems, Wetzlar, Germany). Excitation of TMA-DPH was achieved using the 395/25 nm band of the Spectra X system and the Leica DAPI filter set (λ_{ex}=395/25 nm; λ_{em}=460/50 nm). Excitation light intensity was set to 50% and exposure times were 300 ms. .

Images were processed using the ImageJ software. Contours generated using

JFilament were coloured using Photoshop.

TIME-LAPSE FLUORESCENCE MICROSCOPY

Cells were visualized on a Leica DMi8 Wide-field Inverted Microscope equipped with a HC PL APO 100× DIC objective (NA=1.40) and an iXon Ultra 888 EMCCD Camera from Andor Technology. Sporulation was induced at 37°C. To visualize the membranes, 0.5 µg/ml FM4-64 was added to the culture ~1 hours after sporulation induction and incubation continued for another hour. Then a 10 µl sample was taken and transferred to an agarose pad prepared as described[11]. Pictures were taken in an environmental chamber at 37°C every 5 min for at least 2 hours. Excitation of FM4-64 was achieved using the 575/25 nm band of the SpectraX system and a custom FM4-64 filter set (λ_{ex}=395/25 nm; λ_{em}>610nm). Excitation light intensity was set to 5% to minimize phototoxicity and exposure times were 100 ms. For presentation purposes, sporangia were aligned vertically (with forespore on top) by rotating them using ImageJ. Contours generated with JFilament were colored using Photoshop.

EXPRESSION, PURIFICATION, AND LABELING OF RECOMBINANT PROTEINS

His_6-tagged FisB ECD and mutants were purified as previously described[13]. GST-tagged FisB ECD and mutants were expressed in *E. coli* BL21 (DE3) (New England Biolabs, Ipswich, MA, USA). Briefly, protein expression was induced with 1 mM IPTG at OD_{600} = 0.6 overnight at 16°C. Cells were harvested by centrifugation and the pellet was resuspended in Lysis Buffer (phosphate buffered saline supplemented with 1 mg/ml lysozyme (Sigma-Aldrich), 1 mM tris(2-carboxyethyl) phosphine (TCEP, Sigma-Aldrich), bacterial protease inhibitors (Roche), 2.5 µg/ml DNAase (Sigma-Aldrich), 2.5 µg/ml RNAase (Sigma-Aldrich) and 25U/µL benzonase nuclease (Novagen)). Lysates were then subjected to 5 passes through a high-pressure homogenizer (Avestin EmulsiFlex-C3, Ottawa, Canada). The lysate was then spun down at 100,000 g and the soluble fraction was incubated with glutathione Sepharose 4B (GE Healthcare) for 3 h at 4°C. Proteins were eluted by cleavage with PreScission Protease (GE Healthcare) in Precission Buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA) with freshly added 1 mM TCEP, for 4 h at 4°C. Proteins were concentrated up to ~ 2-3 mg/ml using a Vivaspin with a 10 kDa molecular weight cutoff and labeled with iFluor555 maleimide dye (AAT Bioquest, Sunnyvale, CA, USA) by adding a 20x molar excess of dye to the protein and incubating the reaction overnight at 4°C. Free dye was removed using a HiPrep 20/10 (GE, Chicago, IL, USA) column. For SEC analysis, indicated proteins were loaded onto a Superose 6 Increase 10/300 GL column (GE, Chicago, IL, USA) previously equilibrated with RB-EDTA buffer (25 mM HEPES at pH 7.4, 140 mM KCl, 1mM EDTA, 0.2 mM TCEP) running at a flow rate of 0.5 ml/min at 4˚C. We additionally measured the OD260/280 ratio of all our proteins, which were ~0.7-0.8, close to the value expected for a completely pure preparation for a typical protein (~0.6).

LIPOSOME PREPARATION AND EXPERIMENTS

Small unilamellar vesicles (SUVs) were prepared by mixing 1 μmol of total lipids at desired ratios. A thin lipid film was created using a rotary evaporator (Buchi, New Castle, USA). Any remaining organic solvent was removed by placing the lipid film under high vacuum for 2 hours. The lipid film was hydrated with 1 ml of RB-EDTA by shaking using an Eppendorf Thermomix for >30 minutes. The lipid suspension was then frozen and thawed 7 times using liquid nitrogen and a 37˚C water bath and subsequently extruded 21 times through a 50-nm pore size polycarbonate filter using a mini-extruder (Avanti Polar Lipids). SUVs were composed of (all mole %) *E.coli* PE : *E.coli* CL : *E.coli* PG:EggPC : Cy5-PC = 25:5:50:19:1). The absorbance at 350 nm of 50 μM total lipid was measured for 5 minutes, before addition of 1 μM FisB ECD. Absorbance increases with increasing liposome aggregation due to increased scattering[47].

Giant unilamellar vesicles (GUVs) were formed by electroformation[48]. Briefly, chloroform-dissolved lipids were mixed in a glass tube at desired ratios and spotted on two indium tin oxide (ITO) coated glass slides. Organic solvent was removed by placing the lipid films in a vacuum desiccator for at least 2 h. A short strip of copper conductive tape was attached to each ITO slide which are then separated by a PTFE spacer and held together with binder clips. The chamber was filled with 500 μl Swelling Buffer (SweBu, 1 mM HEPES, 0.25 M sucrose, 1 mM DTT) and sealed with Critoseal (McCormick Scientific LLC, Saint Louis, MO, USA). GUVs were formed by applying a sinusoidal voltage of 10 Hz and an amplitude of 1.8 V for at least 2 h at room temperature. GUV membranes were labeled by including 1 mole % 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) or 1,2-dioleoyl-sn-glycero-3-phosphocholine-N-(Cyanine 5) (18:1 Cy5 PC) in the lipid composition. In Figure 2, "100% PC" and "30% CL" membranes are composed of (all mole %) 99% phosphatidylcholine (PC), 1% NBD-PE, and 30% *E. coli* cardiolipin (CL), 69% eggPC, 1% NBD-PE, respectively. Deflated GUVs were composed of (all mole %) 25% *E. coli* phosphatidylethanolamine (PE), 5% *E. coli* CL, 50% *E. coli* phosphatidylglycerol (PG), 19% eggPC and 1% DiD or NBD-PE (BS mix). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL), except for DiD which was from Thermo Fisher (Waltham, MA).

GUVs were imaged using a Nikon Eclipse TE2000-E microscope. Prior to adding the GUVs, the imaging chamber was filled with a 5 mg/ml β-Casein (Sigma, Saint Louis, MI, USA) solution to prevent attachment of the GUVs to the glass coverslip. The β-Casein solution was removed and replaced with RB-EDTA.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

Protein mobility. FRAP measurements were conducted using a Leica SP8 inverted microscope in the same open imaging chamber. As described above, GUVs were diluted in RB-EDTA (25 mM HEPES at pH 7.4, 850140 mM KCl, 1 mM EDTA, 0.2 mM tris(2-carboxyethyl) phosphine) and incubated with 1 μM FisB ECD for 2-3h. A rectangular area was chosen to bleach as indicated in Figure 2C. Five images (laser power 0.7%) were recorded before bleaching, 10 during bleaching (laser power 100%)

and 60 after bleaching (laser power 0.7%).

The mean GUV intensity was determined using ImageJ. A segmented line (10 pixels wide) was drawn to manually follow the GUV membrane. The line was smoothed using 'fit spine' and the mean pixel intensity was calculated. The background was determined as the mean pixel value of a 20 x 20-pixel box (close to but outside the GUVs) and subtracted from the mean pixel intensity of the GUVs.

Lipid mobility. To monitor lipid mobility in the presence or absence of FisB ECD, GUVs (E.ColiPE :E.ColiCL:E.ColiPG:EggPC:Cy5-PC = 25:5:50:19:1) were prepared as described above and deposited on top of a 0.1% poly-lysine treated glass-bottom dish. After incubation for 30 min at room temperature, the poly-lysine solution was removed and replaced with RB-EDTA. For some samples, 1 μM iFluor555-FisB ECD was added and incubated with the GUVs for 1.5 h. Unbound FisB-ECD was washed with RB-EDTA before imaging. Photobleaching was performed using a Leica SP8 inverted confocal microscope by scanning the 633 nm and 649 nm beams operating at 100% laser power over a rectangular region of interest (ROI, 2 μm by 2 μm). The focal plane was set to mid-GUV height. Note that the bleached membrane area is the cross-section of the ROI set in the imaging plane and the optical thickness (~1 μm), i.e. ~ 2 μm^2. Five frames were acquired at low (1%) laser power for normalization of the fluorescence signal before bleaching the ROI for 1.53 s and recovery was monitored for 60 frames every 154 ms at low laser power (1%). Image size was 128 x 128 pixels. The background was determined with the same ROI area outside the vesicle using ImageJ and subtracted from the signal. The mean pixel intensity of the entire GUV was used to correct for photobleaching during low intensity excitation read-out after rescaling to account for polarization effects. Because the unbleached lipid reservoir is finite, the mean pixel intensity of the entire GUV decreases during the high-intensity bleach. The GUV contour intensity just after bleaching was used as the maximum possible recovery value when calculating the fractional recovery of the ROI fluorescence. To estimate fractional recovery and an approximate recovery time, the corrected and averaged recovery curves were fitted to $f(t) = f_\infty(1 - \exp(-t/\tau))$ where f_∞ and τ are the fractional recovery and the recovery timescales, respectively.

THEORETICAL MODELING

In previous work[13] we showed that a neck of radius $\leq 5 - 10$ nm in the region where the engulfment membrane meets the rest of the mother cell membrane [see sketch in Figure S7A] is necessary for FisB to accumulate. This accumulation in turn favors membrane fission, as shown in the current main text. Here, first, we develop a minimal model for the neck based on free-energy minimization. This minimal model assumes a highly simplified geometry of the membrane neck, as depicted in Figure S7B, that accounts for membrane tension, bending, and an osmotic pressure difference, $\Delta\tilde{p} = p_{\text{cyto}} - p_{\text{peri}}$, between the cytoplasm and periplasm since the lumen of the neck is continuous with the periplasmic space. Using this model, we explore if an increase of membrane tension in the neck, e.g., due to forespore inflation, along with the osmotic pressure difference are able to narrow the radius of the neck. Then, we consider a more realistic model by solving the complete Helfrich equation[49] including the possibility of a

pulling force on the neck, e.g. due to DNA translocation through the SpoIIIE motor. For this more realistic model, the shape of the membrane neck is obtained as part of the solution.

Minimal model for the neck: uniform cylinder connecting two planar membranes

We first consider a highly simplified model based on the free energy $\tilde{\mathcal{F}}_0$ of an axisymmetric cylindrical membrane neck of radius $\tilde{R}$ and length $\tilde{L}$ that connects two planar membrane sheets, corresponding to the local geometry depicted in Figure S7B. We employ the classical Helfrich-Canham theory[49-54] for the energy of the membrane, $\tilde{\mathcal{F}}_0$, which reads

$$\tilde{\mathcal{F}}_0 = \int_{\tilde{S}_\mathrm{n}} \mathrm{d}\tilde{S}_\mathrm{n} \left[\frac{\kappa}{2}\left(2\tilde{H}\right)^2 + \gamma\right] + \int_{\tilde{S}_\mathrm{s}} \mathrm{d}\tilde{S}_\mathrm{s}\gamma + \int_{\tilde{V}} \mathrm{d}\tilde{V}\Delta\tilde{p}, \quad (\mathrm{Eq.}\,1)$$

where $\tilde{S}_\mathrm{n}$ and $\tilde{S}_\mathrm{s}$ are the surfaces of the membrane neck and sheets, and $\tilde{V}$ is the volume inside the neck. The tildes denote dimensional variables which will subsequently be non-dimensionalized. Here $\tilde{H}$ is the mean curvature, κ is the bending modulus, γ is the membrane surface tension, and $\Delta\tilde{p} = p_\mathrm{cyto} - p_\mathrm{peri}$ is the osmotic pressure difference. The two surrounding membrane sheets are assumed to be planar since their curvature is much less than that of the neck, thus their only contribution to the energy comes from their area times the membrane tension. In this simplified model, we neglect the bending energy of the junctions where the cylinder meets the membrane sheets. With these simplifications, the free energy $\tilde{\mathcal{F}}_0$ in Equation 1 reads:

$$\frac{\tilde{\mathcal{F}}_0}{2\pi} = \left(\gamma\tilde{R} + \frac{\kappa}{2\tilde{R}}\right)\tilde{L} + \gamma\left(\tilde{R}_\mathrm{bc}^2 - \tilde{R}^2\right) + \frac{\Delta\tilde{p}}{2}\tilde{R}^2\tilde{L}, \qquad (\mathrm{Eq.}\,2)$$

where $\tilde{R}_\mathrm{bc} > \tilde{R}$ is the outer boundary condition depicted in Figure S7B. Minimizing Equation 2 with respect to the radius $\tilde{R}$ yields

$$2\gamma\tilde{R}^2 - \kappa + 2\tilde{R}^3\left(\Delta\tilde{p} - \frac{2\gamma}{\tilde{L}}\right) = 0. \qquad (\mathrm{Eq.}\,3)$$

To non-dimensionalize Equations 2 and 3 we introduce the natural length scale arising from the balance between bending and tension in Equation 1[55] as the characteristic length scale, $R_\mathrm{c} = \sqrt{\kappa/(2\gamma)}$, and define $R = \tilde{R}/R_\mathrm{c}$. The free energy is also made dimensionless as $\mathcal{F}_0 = \tilde{\mathcal{F}}_0/(\gamma R_\mathrm{c}\tilde{L})$. Hence, the dimensionless forms of Equations 2 and 3 read:

$$\frac{\mathcal{F}_0}{2\pi} = R + \frac{1}{R} + \frac{\left(R_\mathrm{bc}^2 - R^2\right)}{\tilde{L}/R_\mathrm{c}} + \frac{\Delta p}{2}R^2, \quad (\mathrm{Eq.}\,4)$$

$$R^2 - 1 + R^3\left(\Delta p - \frac{2}{\tilde{L}/R_\mathrm{c}}\right) = 0, \quad (\mathrm{Eq.}\,5)$$

where $\Delta p = \Delta\tilde{p}R_\mathrm{c}/\gamma$ and $R_\mathrm{bc} = \tilde{R}_\mathrm{bc}/R_\mathrm{c}$.

Zero osmotic pressure difference. The solution of Equations 5 and 6 when $\Delta p = 0$ reveals that for a long enough neck there is a metastable state of the neck at finite radius[56,57] R_min. This local minimum arises from a balance between membrane bending

energy, which always favors larger R, and membrane tension, which contributes a non-monotonic function of R to the membrane energy. The existence of this local minimum can be observed in Figures S7D,E, which shows the dimensionless free energy $\mathcal{F}_0$ as a function of R for different values of $\tilde{L}/R_\mathrm{c}$, along with the location R_min of the local minimum. Intuitively, for R around R_min, increasing the radius R increases the total amount of membrane in the vicinity of the neck and so increasing R is opposed by surface tension. By contrast, for larger values of R, increasing R removes more membrane from the parallel sheets (removed area scaling $\sim\tilde{R}^2$) than is added to the neck (added area scaling $\sim\tilde{L}\tilde{R}$), so further increase of R is energetically favored [specifically when $R > \sqrt{3} + \sqrt{2}\sqrt{\tilde{L}/R_\mathrm{c} - 3\sqrt{3}}/3^{3/4} + O(\tilde{L}/R_\mathrm{c})$]. We find that, for lengths below a critical value $\tilde{L}/R_\mathrm{c} < 3^{3/2}$, the energy functional $\mathcal{F}_0$ does not have a local minimum, so that expanding the radius of the neck always decreases the total energy of the system, implying the neck opens.

Finite osmotic pressure difference. Equation 5 indicates that a positive osmotic pressure difference between the cytoplasm and the periplasm favors the formation of a locally stable, narrow membrane neck. Indeed, the critical neck length below which there is no locally stable narrow neck decreases as Δp increases, $\tilde{L}/R_\mathrm{c} \leq 2 \cdot 3^{3/2}/(2 + 3^{3/2}\Delta p)$. Moreover, the local maxima shown in Figure S7D for $\Delta p = 0$, disappears when $\Delta p > 2/(\tilde{L}/R_\mathrm{c})$, as shown in Figure S7E. This implies that the free energy $\mathcal{F}_0$ increases monotonically beyond the local minimum, i.e. the neck will not open up. Moreover, the minimum radius of the neck R_min always decreases as Δp increases.

Equilibrium shapes of the axisymmetric membrane neck

We now go beyond the minimal model described in the previous section by solving the complete Helfrich equation, where the shape of the neck is obtained as part of the solution. To this end, we describe the equilibrium shape of an axisymmetric and mirror symmetric membrane neck connecting two membrane sheets, corresponding to the local geometry of the neck connecting the forespore engulfing and mother cell membranes [see Figures S7A,C]. The energy functional $\tilde{\mathcal{F}}$ consists of a term accounting for membrane bending and tension, $\tilde{\mathcal{F}}_\mathrm{m}$, another term accounting for the osmotic pressure difference between cytoplasm and periplasm, $\tilde{\mathcal{F}}_\mathrm{p}$, and a term representing any pulling force $\tilde{f}$ on the forespore, $\tilde{\mathcal{F}}_\mathrm{f}$[49-55,58,59]:

$$\tilde{\mathcal{F}} = \tilde{\mathcal{F}}_\mathrm{m} + \tilde{\mathcal{F}}_\mathrm{p} + \tilde{\mathcal{F}}_\mathrm{f} = \int_{\tilde{A}} \mathrm{d}\tilde{A}\left[\frac{\kappa}{2}\left(2\tilde{H}\right)^2 + \gamma\right] + \int_{\tilde{V}} \mathrm{d}\tilde{V}\Delta\tilde{p} - \tilde{f}\tilde{L}, \quad (\mathrm{Eq.}\,6)$$

where $\Delta\tilde{p} = p_\mathrm{cyto} - p_\mathrm{peri}$, $\tilde{A}$ is the area of the membrane neck, and $\tilde{V}$ is the volume inside the neck.

For axisymmetric surfaces, it proves convenient to describe the surface in terms of the angle $\theta(\tilde{s})$ between the contour and the ordinate axis, as parametrized by the arclength $\tilde{s}$ (see sketch in Figure S7C). To non-dimensionalize Equation 6, we again introduce the natural length scale $R_\mathrm{c} = \sqrt{\kappa/(2\gamma)}$ as the characteristic length. The definition of the radial $R(s)$ and axial $Z(s)$ coordinates, and the first variation of Equation 6 yield the

following nonlinear boundary-value problem[58,60,61]

$$R' = \cos\theta, \quad \text{(Eq. 7)}$$

$$Z' = -\sin\theta, \quad \text{(Eq. 8)}$$

$$\theta'' = -\frac{1}{2}(\theta')^2 \tan\theta - \frac{\cos\theta}{R}\theta' + \frac{\cos^2\theta + 1}{2R^2}\tan\theta + \frac{1}{2}\tan\theta + \frac{\Delta p R}{4\cos\theta} - \frac{f}{R\cos\theta}, \quad \text{(Eq. 9)}$$

where derivatives are with respect to contour length s, $\Delta p = \Delta\tilde{p} R_{\mathrm{c}}/\gamma$, and $f = \tilde{f}/(4\pi\gamma R_{\mathrm{c}})$. Concerning the boundary conditions, we impose $R(s = s_{\mathrm{end}}) = R_{\mathrm{bc}}$, $Z(s = s_{\mathrm{end}}) = 0$, $\theta(s = s_{\mathrm{end}}) = 0$, and $\theta(s = 0) = \pi/2$. The dimensionless free energy of the system is computed as

$$\frac{\mathcal{F}}{2\pi} = \int_A \mathrm{d}A[(2H)^2 + 1] + \frac{1}{2}\int_V \mathrm{d}V\Delta p - 2f\mathcal{L}, \quad \text{(Eq. 10)}$$

where $\mathcal{L} = \tilde{L}/R_{\mathrm{c}}$. The system of differential equations, Equations 7-9, is solved using the Matlab built-in boundary-value problem solver bvp4c[62].

Figure S7F shows the dimensionless version of the free energy from Equation 10, as a function of the arc length s_{end} for different values of the radial boundary size R_{bc} indicated in the legend. When $R_{\mathrm{bc}} \gtrsim 3$, the free energy exhibits two local minima. One of them corresponds to a relatively small value of s_{end}, where the minimum neck radius is large, i.e. the neck opens up. The other local minimum occurs at a larger value of s_{end} with a larger associated free energy, corresponding to a narrow neck.

Increasing the value of s_{end} beyond the latter local minimum increases the total free energy drastically due to a sharp increase of the bending energy, as shown for $R_{\mathrm{bc}} = 4$ (zoomed region in Figure S7F). When $R_{\mathrm{bc}} \lesssim 3$, the free energy only displays one local minimum, which corresponds to that of a narrow neck. Figure S7G is obtained using the same values of the parameters as in Figure S7F, but displaying some of the multiple higher-energy branches arising for sufficiently large values of s_{end}.

Additionally, Figures S7H,I show the role of the pulling force f and the osmotic pressure difference Δp on the free energy and the shape of the membrane neck. Panel H depicts the dimensionless free energy $\mathcal{F}$ as a function of s_{end} for $f = 0$ and $f = 1.35$ (see the table below), and $\Delta p = 0$. The insets, showing the shape of the axisymmetric membrane neck at different values of s_{end}, demonstrate that, for the same values of R_{bc} and s_{end}, the pulling force tends to increase both the length of the neck and the minimum neck radius. Panel I displays the free energy with a non-zero value of the pulling force and the osmotic pressure difference, i.e. $f = 1.35$ and $\Delta p = 0.5$. As expected, incorporating a non-zero value of the osmotic pressure difference between the cytoplasm and the periplasm, slightly favors the narrowing of the membrane neck.

We now estimate some of the physical and dimensionless parameters used in the model and show that realistic values of the membrane tension and the osmotic pressure difference are able to sufficiently narrow the neck radius, to allow FisB proteins to interact and accumulate inside the neck. These estimates are shown in the table below. In particular, we consider a pulling force of $\tilde{f} \sim 50 - 100$ pN, which corresponds to the SpoIIIE maximum stall force[36,37], and an osmotic pressure difference of $\Delta\tilde{p} \sim 60$ kPa, as

estimated in 11. Based on previous works[13,63], we assume that FisB proteins are able to undergo homo-oligomerization in trans when the radius of the membrane neck is below ~ 10 nm, which is within the range of the estimated characteristic neck radius shown in the table below, $R_c = \sqrt{\kappa/(2\gamma)} \sim 2 - 20$ nm, where we have considered $\kappa \sim 20\, k_B T$[55], and $\gamma \sim 0.1 - 10$ pN nm^{-1} [25-27] (see last section of this Methods section for an upper estimate of the membrane tension). According to the model, when $\Delta p = f = 0$, for the local minimum of the free energy $\mathcal{F}$ corresponding to the larger value of s_{end}, shown in Figure S7F, the dimensional minimum radius of the neck is $\tilde{R}_{\text{min}} \sim 0.1 - 1$ nm, which is significantly below the minimum radius required for FisB to interact. Moreover, the local free-energy minimum corresponding to a wider neck yields a minimum neck radius $\tilde{R}_{\text{min}} \sim 6.2 - 62$ nm, implying that, if membrane tension increases sufficiently, the neck radius for this local minimum can also be narrow enough for FisB to interact and accumulate inside the neck.

When $f = 1.35$ and $\Delta p = 0$, which corresponds to, for instance, $\gamma \sim 0.84$ pN nm^{-1} and $\tilde{f} \sim 100$ pN, we find $\tilde{R}_{\text{min}} \sim 4.5$ nm, corresponding to the membrane neck shown in the right bottom inset of Figure S7H. Hence, the action of a pulling force increases the radius of the neck, as compared to the case of $f = 0$ shown in Figures S7F,H. If we now consider the case of a non-zero value of the osmotic pressure difference, shown in Figure S7I, where we have used $f = 1.35$ and $\Delta p = 0.5$, corresponding to $\gamma \sim 0.84$ pN nm^{-1}, $\Delta\tilde{p} \sim 60$ kPa, and $\tilde{f} \sim 100$ pN, the minimum radius is $\tilde{R}_{\text{min}} \sim 4.1$ nm, thereby favoring the accumulation of FisB in the neck. Nonetheless, such osmotic pressure difference only reduces the minimum neck radius slightly, as we also observed in our highly simplified model shown in Figure S7E.

Flux of lipids

To estimate the membrane tension difference between the two ends of the neck that would drive a flux of lipids from the mother cell to the forespore of $\sim 10^3$ lipids s^{-1} (see *Forespore inflation accompanies membrane fission* in the main text), we balance the force due to the membrane tension difference $\Delta\gamma$ and the drag force on a cylinder (i.e. the neck in our highly simplified model), translating parallel to its axis in an outer bath of viscosity μ,

$$2\pi\tilde{R}\Delta\gamma = \frac{4\pi\mu U\tilde{L}}{\ln\left(\tilde{L}/\tilde{R}\right) - 1/2}. \qquad \text{(Eq. 11)}$$

Considering that 1 lipid occupies ~ 0.7 nm^2 and assuming a neck radius of $\tilde{R} \sim 5$ nm, the required lipid velocity along the neck is $U \sim 22.3$ nm s^{-1}. Taking $\tilde{L} \sim 40$ nm and $\mu \sim 10^{-3}$ Pa s, the estimated membrane tension difference is $\Delta\gamma \sim 2.3 \times 10^{-7}$ pN nm^{-1}. Hence, a flux of $\sim 10^3$ lipids s^{-1} would require a relatively small excess membrane tension in the forespore engulfing membrane compared to the mother cell membrane, which we assume is of the same order as the estimate in the table below[25-27]. So, in the absence of an accumulation of FisB in the neck that would slow lipid flow, a flux of lipids in the neck could readily provide $\sim 10^3$ lipids s^{-1} to the forespore membranes.

Comparing the stall force of SpoIIIE to the osmotic pressure difference

Here we analyze how the osmotic pressure difference between the mother cell and the

forespore compares to the stall/disassembly force of SpoIIIE[36,37]. According to ref. 11, the excess pressure in the forespore is of the order of 60 kPa, which can be related to a salt concentration difference $\Delta\tilde{\rho}$ between the mother cell and the forespore via $\Delta\tilde{p} = N_{\mathrm{A}} k_{\mathrm{B}} T \Delta\tilde{\rho}$, yielding $\Delta\tilde{\rho} \sim 25$ mM. Taking the salt concentration in the mother cell to be $\Delta\tilde{\rho}_{\mathrm{mc}} \sim 200$ mM [64,65], the salt concentration inside the forespore is $\tilde{\rho}_{\mathrm{fs}} \sim 225$ mM. We can now estimate the energy required to move one salt ion into the forespore as $\tilde{E} = k_{\mathrm{B}} T \log(\tilde{\rho}_{\mathrm{fs}}/\tilde{\rho}_{\mathrm{mc}}) \sim 2.23\, k_{\mathrm{B}} T$. This estimate is of the same order as the energy available from SpoIIIE, which is its stall maximal force, ~ 50 pN [36,37], multiplied by half the base spacing, i.e. ~ 0.17 nm, since each translocated base of DNA brings ~2 counterions, which yields $\tilde{E}_{\mathrm{SpoIIIE}} \sim 2.1\, k_{\mathrm{B}} T$. This near equality of energies suggests that SpoIIIE works near its stall force, by creating the highest pressure difference it can in the forespore.

Moreover, we can estimate the forespore membrane tension that would be required to balance this osmotic pressure difference (for this estimate, we neglect the possible role of the peptidoglycan surrounding the forespore in opposing this osmotic pressure difference). Assuming that the forespore is a sphere surrounded by two membranes, the equilibrium excess pressure is given by the Laplace law, $\Delta\tilde{p} \sim 4\gamma/\tilde{R}_{\mathrm{fs}}$. Taking $\tilde{R}_{\mathrm{fs}} \sim 400$ nm, we obtain $\gamma \sim 6$ pN nm^{-1}. According to refs. 25,27, the rupture tension of a lipid membrane is $\gamma_{\mathrm{rup}} \sim 20$ pN nm^{-1}. Nonetheless, as shown in 26, the membrane rupture tension depends on the application time of the tension, and it can be of the order of $\gamma_{\mathrm{rup}} \sim 2 - 30$ pN nm^{-1} when $\tilde{t}_{\mathrm{app}} \sim 4 - 0.01$ min. Altogether, we argue that forespore inflation can give rise to an increase of membrane tension enough to narrow the neck below 10 nm (see *Equilibrium shapes of the axisymmetric membrane neck*). Moreover, given that the characteristic time scale of forespore inflation is $\tilde{t} \sim 1 - 10$ min, the increased membrane tension due to forespore inflation may contribute directly to membrane fission.

Parameter	**Value**
κ	$20\, k_{\mathrm{B}} T$ [55]
γ	$0.1 - 10$ pN nm^{-1} [25-27]
$\tilde{L}$	40 nm
$\Delta\tilde{p}$	60 kPa [11]
$\tilde{f}$	$50 - 100$ pN [36,37]
$R_{\mathrm{c}} = \sqrt{\kappa/(2\gamma)}$	$2 - 20$ nm
$\tilde{L}/R_{\mathrm{c}}$	$9 - 90$
$\Delta p = \Delta\tilde{p} R_{\mathrm{c}}/\gamma$	$0.012 - 12$
$f = \tilde{f}/(4\pi\gamma R_{\mathrm{c}})$	$0.4 - 4$

QUANTIFICATION AND STATISTICAL ANALYSIS

Determination of forespore and mother membrane surface area. Using the ImageJ plugin Filament 2D[21], "snakes" were fitted to forespores and mother cells (Figure S2A). Snakes were fitted to forespores using either the TMA-DPH membrane stain (using ridges) or a soluble CFP marker (using gradients) expressed in the forespores. Snakes were fitted to mother cells using the corresponding phase contrast image using gradients. In all cases the snakes were fitted as contours. Fitting parameters were as follows: $\alpha = 100$, $\beta = 100$, $\gamma = 800$, weight=0.5, stretch force=100, deform iterations=50, point spacing=0.5, image smoothing=1.01.

To determine the surface area of forespores and mother cells, the snakes were then further analyzed with MATLAB. First, an ellipse was fitted to the snakes to determine the symmetry axis of the forespore or mother cell (Figure S2B). Half of the snake is then rotated around the symmetry axis to create a surface of revolution from which the membrane surface area for the forespore or mother cell was determined (Figure S2C).

For the analyses shown in Figure 5I, FisB foci were semi-automatically selected using SpeckleTrackerJ[66]. For each spot, the sum of pixel values in a 6 pixels × 6 pixels (0.5 µm×0.5 µm) box around the center of the spot were calculated. For each corresponding cell, the same operation was performed at a membrane area where no clusters were present and subtracted from the individual FisB cluster intensity.

Determination of mean fluorescence intensity around the forespore. We used ImageJ plugin Filament 2D to fit snakes around forespores (see above) to define forespore contours. Mean contour intensity was calculated using Matlab, using snake coordinates from Filament 2D dilated to 4 pixels used as a mask.

~~**Supplemental movie and/or data files.** A compressed file contains data underlying all the plots supporting the conclusions of the paper, in the form of Matlab .fig files (from which the data can be extracted), Matlab .mat files, and/or excel files. Matlab code used for quantification can be found at:~~

~~Karatekin, Erdem; Landajuela, Ane; Braun, Martha; Martinez-Calvo , Alejandro; Rodrigues, Christopher; Gomis Perez, Carolina; Doan, Thierry; Rudner, David; Wingreen, Ned (2022), “Landajuela_CurrentBiology2022”, Mendeley Data, V1, doi: 10.17632/27cdx65wh7.1~~

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