

Cell wall synthesis is necessary for membrane dynamics during sporulation of *Bacillus subtilis*

Pablo Meyer,¹ Jennifer Gutierrez,² Kit Pogliano² and Jonathan Dworkin^{1*}

¹Department of Microbiology and Immunology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA.

²Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093, USA.

Summary

During *Bacillus subtilis* sporulation, an endocytic-like process called engulfment results in one cell being entirely encased in the cytoplasm of another cell. The driving force underlying this process of membrane movement has remained unclear, although components of the machinery have been characterized. Here we provide evidence that synthesis of peptidoglycan, the rigid, strength bearing extracellular polymer of bacteria, is a key part of the missing force-generating mechanism for engulfment. We observed that sites of peptidoglycan synthesis initially coincide with the engulfing membrane and later with the site of engulfment membrane fission. Furthermore, compounds that block muropeptide synthesis or polymerization prevented membrane migration in cells lacking a component of the engulfment machinery (SpolIQ), and blocked the membrane fission event at the completion of engulfment in all cells. In addition, these compounds inhibited bulge and vesicle formation that occur in *spolID* mutant cells unable to initiate engulfment, as did genetic ablation of a protein that polymerizes muropeptides. This is the first report to our knowledge that peptidoglycan synthesis is necessary for membrane movements in bacterial cells and has implications for the mechanism of force generation during cytokinesis.

Introduction

A central question in cell biology is the nature of the forces driving membrane movement. During endocytosis, the cellular membrane invaginates and ultimately forms a

lipid-bounded compartment that is distinct from the membrane. This event rarely occurs spontaneously and is dependent upon enzymes that facilitate this process. Proteins that polymerize into filaments, such as actin and tubulin, convert the chemical energy of polymerization into mechanical work (Theriot, 2000; Phillips *et al.*, 2009). For example, actin polymerization mediates contractile ring formation during cytokinesis in *Schizosaccharomyces pombe* (Pelham and Chang, 2002) and facilitates deformations of the plasma membrane seen during endocytosis (Qualmann *et al.*, 2000) or in protruding lamellipodia (Mitchison and Cramer, 1996). Microtubule polymerization drives the movement of organelles and chromosomes (Inoue and Salmon, 1995). However, while the polymerization of actin (Miyata *et al.*, 1999) or tubulin (Elbaum *et al.*, 1996) can generate a mechanical force that distorts membranes *in vitro*, it remains unclear whether polymerization is sufficient to explain *in vivo* membrane dynamics.

A process of membrane fission is seen in *Bacillus subtilis* that are responding to nutritional limitation. As part of this response, called sporulation, *B. subtilis* divides asymmetrically and undergoes engulfment, an endocytic-like process of membrane fission that results in one cell, the forespore, being entirely encased in the cytoplasm of the larger cell, the mother cell (Fig. 1A, top). Initially, an asymmetric division septum forms generating the two differently sized cells. The subsequent increasing curvature of this septum causes the forespore to assume a rounded shape comprised of a double membrane separated by a thin layer of peptidoglycan. SpolID and SpolIP are autolysins that hydrolyse cell wall and they have been proposed to drive this membrane movement by generating a force through a ratchet-like mechanism driven by the hydrolysis of peptidoglycan (Abanes-De Mello *et al.*, 2002; Broder and Pogliano, 2006). Engulfment is also facilitated by the formation of the SpolIQ–SpolIIAH protein–protein zipper between the forespore and mother cell, which is required for membrane migration when the activity or levels of SpolID and SpolIP are reduced (Broder and Pogliano, 2006). During this movement, the forespore remains attached to the mother cell but in the final step, the forespore pinches off from the mother cell. However, the source of the force responsible for this final step remains unknown.

Accepted 24 March, 2010. *For correspondence. E-mail jonathan.dworkin@columbia.edu; Tel. (+1) 212 342 3731; Fax (+1) 212 305 1468.

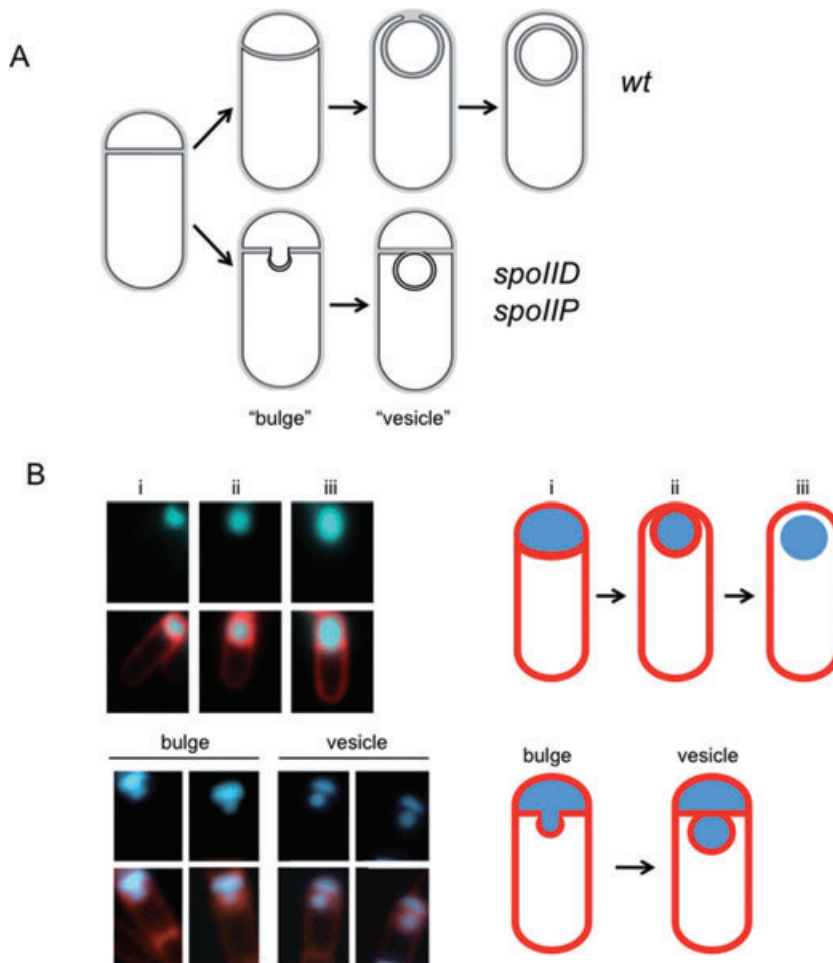


Fig. 1. Membrane bulge and vesicle formation in engulfment mutants.

A. Sporulating wild-type cells undergo engulfment where the smaller forespore becomes a free cell within the larger mother cell (top). Strains carrying *spoIID* or *spoIIP* mutations are blocked at the stage of asymmetric septation and undergo membrane bulging, eventually leading to the formation of membrane bound vesicles (bottom).

B. Wild-type (AES574) and *spoIID* strains (JDB2494) expressing CFP (blue) under control of a forespore-specific promoter (P_{spoIIQ}) as a forespore marker were imaged at T4 in resuspension medium and stained with FM4-64 (red) to visualize membranes. Top, CFP is expressed in: (i) the forespore of wild-type cells undergoing asymmetric septation stained with FM4-64, (ii) engulfing cells stained with FM4-64 and (iii) engulfed cells not stained with FM4-64. Bottom, *spoIID* cells contained either a continuous CFP signal distribution between the forespore and the membrane protrusion ('bulge'), or two distinguishable CFP signals separated by a membrane ('vesicle').

Membrane-bound compartments in other bacteria are thought to result from involutions of the cell membrane. For example, magnetosomes are membrane-associated organelles observed in magnetotactic bacteria composed of crystals of magnetite that are surrounded by a lipid bilayer (Komeili *et al.*, 2006; Scheffel *et al.*, 2006) and *Escherichia coli* carrying mutations in a gene necessary for proper cell shape produce membrane invaginations and cytoplasmic vesicles (Bendezu and de Boer, 2008). Sporulating *B. subtilis* strains carrying *spoIID* or *spoIIP* null mutations form asymmetric septa that fail to proceed with engulfment. Instead, membrane bulges appear gradually at the septa (Fig. 1A, bottom). Electron microscopy (Lopez-Diaz *et al.*, 1986; Illing and Errington, 1991; Smith and Youngman, 1993; Bylund *et al.*, 1994; Frandsen and Stragier, 1995) and staining with the membrane impermeable stain FM4-64 (Abanes-De Mello *et al.*, 2002) indicate that the bulges in *spoIIP* and *spoIID* mutants are continuous with the forespore compartment. The origin of the forces which drive bulge formation are unknown, although by analogy with the roles of actin and tubulin polymerization in

eukaryotic membrane fusion and fission, a protein or peptide capable of polymerizing could serve a similar role. Bulge formation in these mutants therefore offers a useful system to examine candidate force-generating mechanisms responsible for membrane movements in bacteria.

Most bacteria contain peptidoglycan, a rigid polymer built from disaccharide peptide monomers that can be up to 5000 units in length (Hayhurst *et al.*, 2008). Peptidoglycan monomers, also known as muropeptides, are synthesized in the bacterial cytoplasm by a series of essential and highly conserved enzymes that convert the sugar UDP-GlcNAc to the lipid-linked UDP-disaccharide pentapeptide (van Heijenoort, 2001) which is flipped across the membrane to the outside of the cell where cross-linking transpeptidation and polymerizing transglycosylation reactions link these muropeptides to mature peptidoglycan (Sauvage *et al.*, 2008). While the glycan polymers of peptidoglycan are generated by transglycosylases, inhibition of the transpeptidation reaction by vancomycin also results in a loss of this polymerization activity in *Staphylococcus aureus* (Kim *et al.*, 2008).

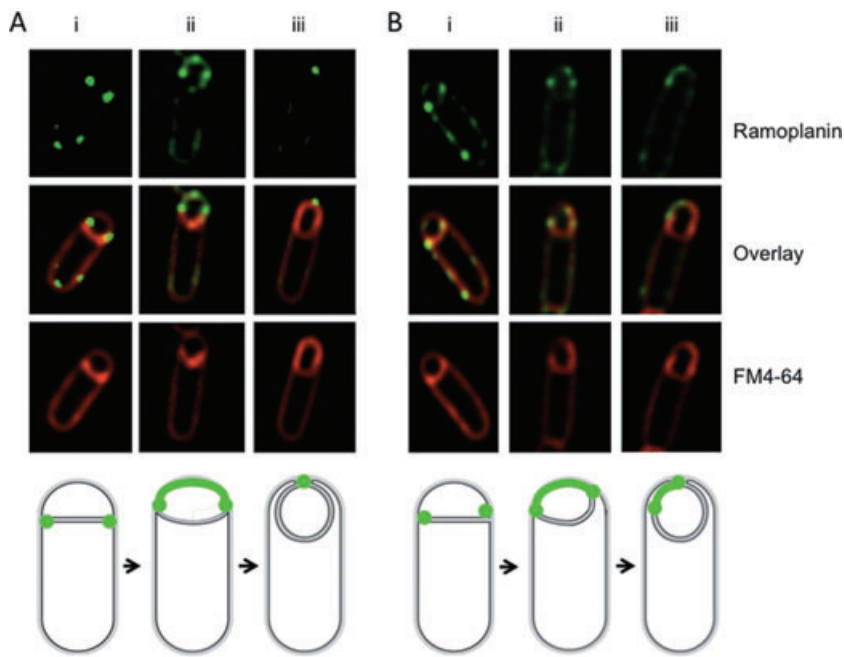


Fig. 2. Peptidoglycan synthesis during engulfment. Engulfing cells contain sites of active peptidoglycan synthesis. Ramoplanin-FL (top) and FM4-64 (bottom) staining of (A) wild-type (PY79) cells and (B) *spoIID(D210A)* cells; centre panel are the overlay of the two images. (i) Ramoplanin-FL staining at septal tips, (ii) ramoplanin-FL staining around the forespore, (iii) ramoplanin-FL staining restricted to the point of contact between the advancing arms. Diagrams shown represent ramoplanin-FL (green) signals at different stages of engulfment corresponding to wild-type (left) and *spoIID(D210A)* (right) images.

Finally, since cell wall peptidoglycan has a Young's modulus of 1.7–25 MPa (Yao *et al.*, 1999; Francius *et al.*, 2008), it is up to 10^4 times stiffer than actin, so formation of mature peptidoglycan would produce mechanical work.

The requirement for peptidoglycan synthesis during bacterial growth complicates assessment of the hypothesis that it provides a driving force for membrane movement. We have therefore examined the role of peptidoglycan synthesis in three different membrane movements that occur during the non-essential process of *B. subtilis* sporulation. First, during the initial stages of engulfment, the membranes surrounding the forespore undergo a process of migration that results in the formation of curved membranes. We find that inhibition of peptidoglycan synthesis blocks this migration in a strain missing a secondary membrane migration system requiring SpoIIQ and we find that peptidoglycan biosynthesis is localized to the leading edge of the engulfing membrane in wild-type cells and in a mutant in which membrane migration occurs asymmetrically. Second, during the last stage of engulfment, peptidoglycan synthesis is localized to the last site of attachment between the two cells and antibiotics that inhibit peptidoglycan synthesis prevent separation of the two cells and completion of engulfment. Third, peptidoglycan synthesis occurs at sites of bulge formation in *spoIID* and *spoIIP* mutants and compounds that inhibit the synthesis of mucopeptides block the initiation of these bulges and their ultimate formation as membrane-bounded, 'vesicle'-like compartments. Consistent with the action of these compounds, a null mutation in a gene encoding a protein

necessary for the synthesis of mature cross-linked peptidoglycan suppresses bulge formation. Together these results suggest that during engulfment, membrane migration and membrane fission require peptidoglycan biosynthesis.

Results

Peptidoglycan synthesis during engulfment

During the process of engulfment, the asymmetric septum becomes rounded and eventually pinches off from the mother cell, resulting in the formation of a membrane-bounded compartment (Fig. 1A, top). Sites of active peptidoglycan synthesis in engulfing cells were identified using a fluorescent ramoplanin that binds the reducing end of nascent glycan chains found at the initiation sites of peptidoglycan synthesis (Tianont *et al.*, 2006). Sporulating cells showed a clear strong fluorescent signal at the sporulation septum during engulfment (Fig. 2A, i). This signal was due to active peptidoglycan synthesis because treatment of sporulating cells before polar septation with fosfomycin, an inhibitor of peptidoglycan synthesis, showed no ramoplanin labelling (Fig. S1, middle) and a derivative of ramoplanin that does not label lipid II (Tianont *et al.*, 2006) did not produce similar patterns of staining (Fig. S1).

Ramoplanin-FL fluorescence was enriched at the edges of the septal disk at early (Fig. 2A, i) and intermediate stages of engulfment (Fig. 2A, ii and Fig. S2A) and eventually localized to the area of membrane fission (Fig. 2A, iii and Fig. S2B). The reduced signal in other

regions of the forespore was likely not the result of reduced accessibility to the space between the two forespore membranes because forespore staining was similar in a *spoIIQ* mutant where the forespore membranes are less tightly associated (Broder and Pogliano, 2006) as compared with the wild type (Fig. S2). Once engulfment completed and the forespore had separated from the mother cell, the ramoplanin signal in the forespore disappeared (data not shown) likely because it is unable to cross the lipid bilayer (Hamburger *et al.*, 2009). Next, we took advantage of a *spoIID(D210A)* mutant (KP1102; Gutierrez *et al.*, 2010) where cells stained with FM4-64 show a clear asymmetry in membrane migration (80% of cells, 53 out of 66 cells) as exemplified by a single cell where one of the tips of the migrating membranes advances further than the other tip (Fig. 2B, cells ii and iii). Sites of peptidoglycan synthesis showed a similar asymmetrical staining pattern (Fig. 2B, top and middle), indicating that the pattern of peptidoglycan synthesis during engulfment reflects the asymmetrical migration of the membrane.

Inhibition of peptidoglycan synthesis blocks membrane migration

During engulfment, the septal membranes migrate around the forespore compartment ultimately resulting in the production of a cell within a cell. The observation that ramoplanin stained the leading edges of the engulfing forespore (Fig. 2A, ii) suggested that active peptidoglycan synthesis was involved in this process. We examined this possibility by performing time-lapse microscopy to follow single sporulating cells with FM4-64-stained membranes (Becker and Pogliano, 2007) and determining the effect of fosfomycin, an antibiotic that specifically inhibits MurAA, the first enzyme in the peptidoglycan biosynthetic pathway (Walsh, 2003). When 5 mM fosfomycin was added to wild-type cells at T1.5 after initiation of sporulation and cells were imaged starting at T2, no significant disruption could be detected in membrane migration (Fig. 3A and B and Fig. S3). However, membrane migration during engulfment is dependent on two partially redundant mechanisms, peptidoglycan hydrolysis by SpoIID and SpoIIP and the zipper-like interaction of SpoIIQ with SpoIIIAH. In mutants with reduced hydrolase activity, the SpoIIQ/SpoIIIAH system becomes essential (Broder and Pogliano, 2006), so a requirement for muropeptide synthesis for membrane migration could be similarly masked. Consistent with this hypothesis, time-lapse movies revealed that fosfomycin reduced membrane migration in *spoIIQ* mutant cells (Fig. 3C–E and Fig. S3A–C), as 54 out of 72 cells (75%) showed membrane migration in the *spoIIQ* mutant, but only 17 out of 55 (31%) in the *spoIIQ* mutant with fosfomycin. To ensure

that fosfomycin did not have pleiotropic effects during sporulation of *spoIIQ* cells, we confirmed that a fluorescent marker under σ^F control was activated in *spoIIQ* cells that showed no membrane migration in the presence of fosfomycin (Fig. S3E).

A fluorescence-based assay to monitor completion of engulfment

The presence of a strong ramoplanin signal at the point of contact between the two engulfing membrane arms (Fig. 2A, iii) suggested that peptidoglycan synthesis was occurring at an appropriate time and place to play a role in the separation of the outer forespore membrane from the mother cell membrane that marks completion of membrane fission during engulfment. We examined this possibility using a strain that expresses CFP only in the forespore along with visualization of membranes by the lipophilic fluorescent dye FM4-64 that was added just prior to microscopy. We expressed CFP under control of a forespore specific promoter (P_{spoIIQ}), which is active only upon completion of the septum and thus CFP-positive cells must have progressed past this point in sporulation. When asymmetric septation was completed in this strain, the forespore contained a CFP signal that was surrounded by an FM4-64 signal (Fig. 4A, panels 'T3.5', 'T4'; numbers indicate the time in hours after the initiation of sporulation). Once engulfment ends, the forespore has detached from the mother cell and since FM4-64 does not cross membranes (Sharp and Pogliano, 1999), the forespore is no longer accessible to the dye. Thus, an engulfed forespore had a CFP signal but no FM4-64 staining (Fig. 4A, panel 'T5', yellow arrow), whereas a forespore that has not completed engulfment and remains attached to the mother cell was stained with FM4-64 (Fig. 4A, panel 'T4.5', green arrow). This assay therefore distinguishes cells at the pre-separation step and cells where the membranes had completed fission and separated.

Inhibition of muropeptide synthesis blocks engulfment membrane fission

This fusion assay was used to determine the effect of inhibiting peptidoglycan synthesis on completion of engulfment. Addition of fosfomycin (5 mM) after asymmetric septation at T2 of sporulation allowed membrane migration but blocked membrane fission (i.e. the detachment of the forespore from the mother cell; Fig. 4B, bottom images). When fosfomycin was added at different times after the start of sporulation, fosfomycin blocked engulfment membrane fission only if it was added before T3, but had no effect when added at T3 (Fig. 4C). A decreasing fraction of cells containing a forespore that

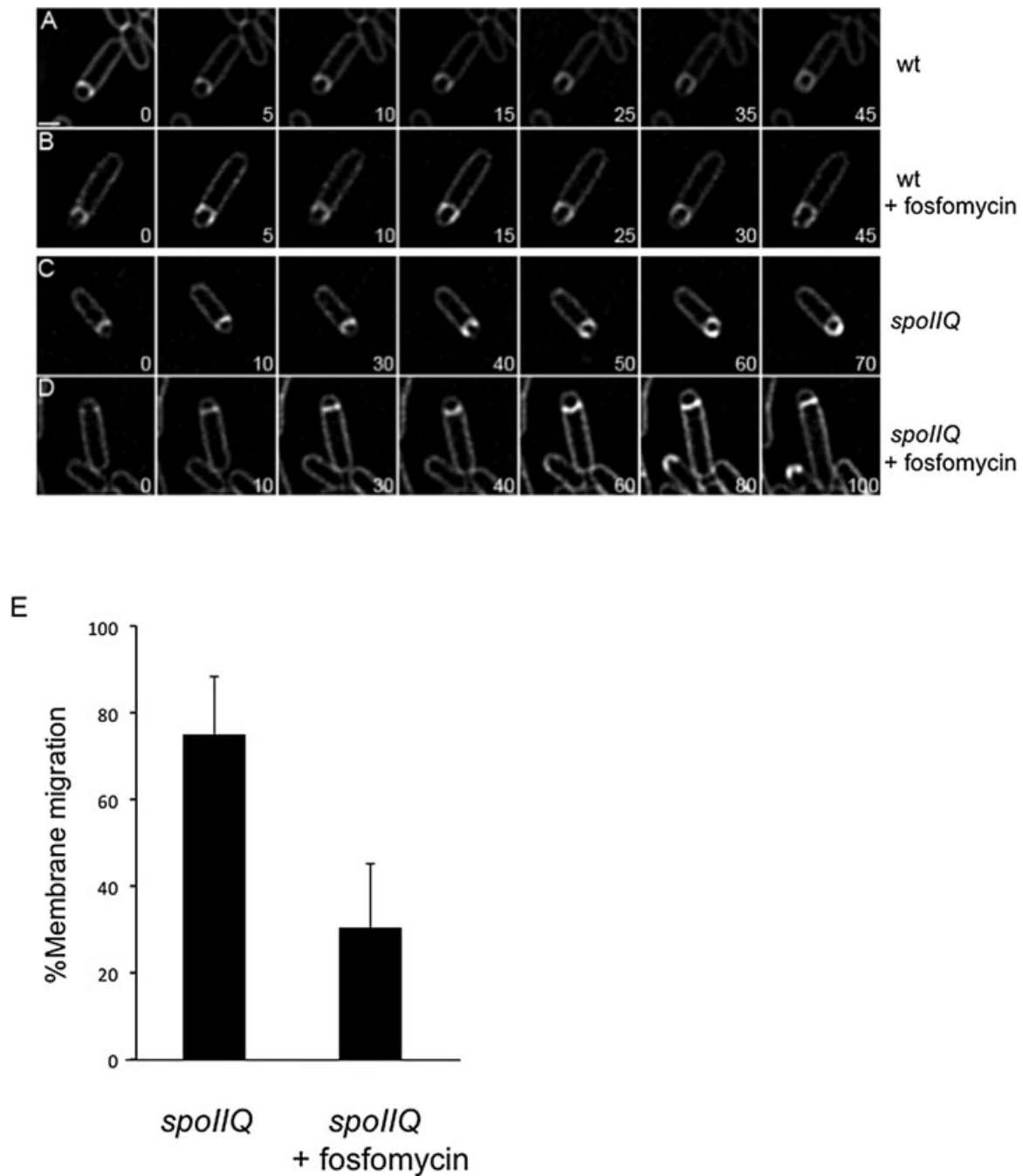


Fig. 3. Inhibition of muropeptide synthesis blocks membrane migration in a *spoIIQ* mutant. All cultures were grown on agarose pads composed of A+B medium at 30°C and stained with FM4-64. The initial image in each sequence was taken at approximately T2 after sporulation initiation and arbitrarily set to t = 0 min. The time of subsequent images of the membrane stain is indicated in minutes in the lower right corner.

A. Wild type (PY79).

B. Wild type + 5 mM fosfomycin added at T1.5 after initiation of sporulation.

C. *spoIIQ* (KP575).

D. *spoIIQ* + 5 mM fosfomycin added at T1.5.

E. Histogram showing the percentage of cells where membrane migrated in $\Delta spoIIQ$ cells with (17 out of 55 cells or 75 ± 5%) or without (54 out of 72 cells or 30 ± 6%) 5 mM fosfomycin added at T1.5 after initiation of sporulation. Means are significantly different by *t*-test ($P < 10^{-5}$). For complete movies, see Fig. S3. Scale bar = 1 μ m.

had detached from the mother cell was observed at fosfomycin concentrations ranging from 1 mM to 10 mM (Fig. S4).

When 5 mM fosfomycin was added at T2 to a strain that expressed an inducible fosfomycin-resistant allele of MurAA (C117D), the fraction of sporulating cells that completed engulfment was significantly higher than a culture where this allele was not expressed (Fig. 4D, Fig. S5). Since the presence of this MurAA mutant allele by itself did not effect engulfment (Fig. S6), fosfomycin must be blocking engulfment membrane fission by directly inhibiting muropeptide synthesis.

Inhibition of peptidoglycan synthesis blocks completion of engulfment

The requirement of muropeptide synthesis to complete engulfment suggests that polymerization of these monomers during formation of mature peptidoglycan was also necessary for engulfment. Vancomycin inhibits transpeptidation and thereby blocks the transglycosylation step necessary for polymerization. Addition of vancomycin at T2 after resuspension blocked engulfment membrane fission (Fig. 5A). Importantly, this was not an effect of blocking septal synthesis since the assay uses a fluorescent reporter whose activity is dependent on the completion of asymmetric septation. This inhibition did depend on when vancomycin was added in sporulation although it still blocked completion of engulfment in 50% of cells when it was added as late as T3.5 (Fig. 5B). Thus, muropeptide polymerization is necessary for completion of engulfment defined as the release of the forespore from the mother cell.

Topology of membrane bulges

Sporulating cells lacking either the SpoIIIP or the SpoIID autolysins fail to initiate engulfment and instead produce septal membrane bulges (Fig. 1A, bottom, Fig. S7; Lopez-Diaz *et al.*, 1986; Illing and Errington, 1991; Smith and Youngman, 1993; Bylund *et al.*, 1994; Frandsen and Stragier, 1995). Time-lapse microscopy of single cells revealed that bulge formation was continuous and occurred over a period of ~30 min (Fig. 6A, Fig. S7). To characterize the topology and origin of these bulges in living cells, we expressed CFP under control of a forespore specific promoter (P_{spoIIQ}). We observed a CFP signal in membrane bulges of a *spoIID* null mutant indicating that they had been, at least transiently, contiguous with the forespore (Fig. 1B, 'bulge'). In some cells, the CFP signal in the forespore and the bulge was not continuous, and the presence of an FM4-64 signal surrounding these discontinuous bulges suggests that the bulge

was transformed into a physically distinct membrane-bounded vesicle (Fig. 1B, 'vesicle'; Fig. S8).

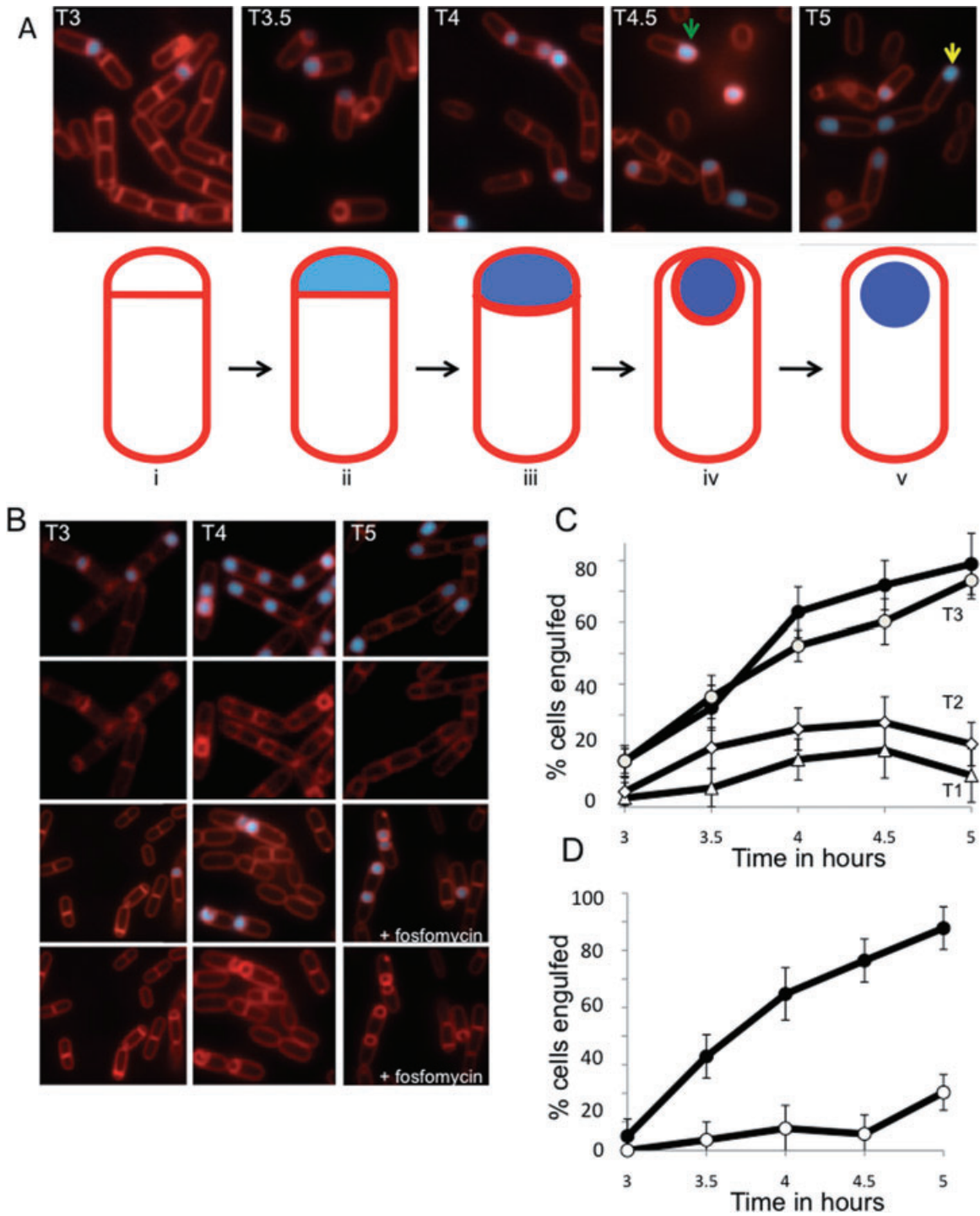
Peptidoglycan synthesis in membrane bulges

What is the driving force underlying the formation of these bulges and vesicles? They originated from the septum, a site of prior peptidoglycan synthesis. Bulge-forming *spoIID* cells stained with ramoplanin-FL at T4 of sporulation had a fluorescent signal associated with the septum, indicating the presence of active peptidoglycan synthesis (Fig. 6B). We examined whether peptidoglycan synthesis was necessary for bulge formation by blocking it using fosfomycin. When 5 mM fosfomycin was added at T2 of sporulation to either *spoIID* (Fig. 6C, red) or *spoIIP* (Fig. S9) mutants, many fewer bulges were observed as compared with untreated cells (Fig. 6C, Fig. S9, black). Furthermore, addition of fosfomycin at T2 also blocked formation of vesicles that presumably originated as bulges (Fig. 6D). This effect of fosfomycin was due to a direct inhibition of peptidoglycan synthesis because a *spoIID* strain carrying the MurAA(C117D) allele exhibited bulge formation during sporulation even in the presence of 5 mM fosfomycin (Fig. S10).

While the inhibition of muropeptide synthesis results indirectly in decreased levels of mature, polymerized peptidoglycan, the experiments using fosfomycin do not address the possibility that direct inhibition of synthesis of peptidoglycan polymers from muropeptide monomers would similarly block formation of bulges and vesicles. We used vancomycin to answer this question, and observed that addition of vancomycin ($0.5 \mu\text{g ml}^{-1}$) at T2 of sporulation blocked bulge (Fig. 6E) and vesicle formation (Fig. S11) in a strain lacking *spoIID*, indicating that synthesis of mature peptidoglycan is necessary for bulge formation. This inhibition by vancomycin could be through an indirect effect on lipid synthesis if reducing lipid synthesis blocked bulge formation. We addressed this possibility by using cerulenin, an inhibitor of an essential enzyme (FadD) in the fatty acid biosynthetic pathway. However, addition of cerulenin had no effect on bulge formation when it was added to *spoIID* cells at the same time in sporulation (T3.5) where the inhibitory effect of vancomycin was observed (Fig. S12). While addition of cerulenin at earlier time points (T2; data not shown) prevented bulge formation in *spoIID* cells, this may be due to the membrane growth that is evident in time-lapse movies of bulge formation (Fig. S7).

A peptidoglycan transpeptidase is necessary for bulge formation

The muropeptide monomer is incorporated into mature peptidoglycan by enzymes mediating transpeptidation



and transglycosylation reactions. During sporulation, the transpeptidase SpoVD is necessary for the production of mature peptidoglycan (Vasudevan *et al.*, 2007). A complementing GFP–SpoVD fusion localizes to septal membrane bulges and vesicles (Fig. 7A), similar to the pattern observed with ramoplanin-FL (Fig. 6B). This similarity suggested that SpoVD is involved, and perhaps neces-

sary, for the peptidoglycan synthesis occurring in the bulges. In fact, bulge formation is reduced in a *spoIIIP* strain lacking SpoVD (Fig. 7B, red) as compared with the parent *spoIIIP* strain (black). The result is consistent with the inhibition of bulge formation by the transpeptidase inhibitor vancomycin in *spoIID* (Fig. 6E) and *spoIIIP* cells (data not shown) and demonstrates that a protein neces-

Fig. 4. Inhibition of muropeptide synthesis blocks membrane fission.

A. Assay of membrane fission. Top, sporulating wild-type cells (AES574) express CFP (blue) under control of a forespore specific promoter (P_{spoIIQ}). Membranes of cells at intermediate stages of engulfment (T3–T4.5, green arrow) are also stained by a membrane impermeant dye (FM4-64, red) just before imaging; upon completion of membrane fission, cells are no longer stained by this dye (yellow arrow). Time after resuspension is indicated. Bottom, cells at different stages of sporulation. Prior to the completion of asymmetric septation (i), no CFP (blue) signal is observed. Following completion of asymmetric septation, a faint CFP signal is observed in the forespore (ii). As the cells proceed through engulfment (iii), the CFP signal increases. When membrane movement is complete (iv), the forespore remains attached to the mother cell and stained with FM4-64 (red). When the forespore is released from the mother cell (v), the CFP signal is no longer surrounded by an FM4-64 signal.

B. Fosfomycin blocks engulfment before membrane separation. Sporulating wild-type cells were either treated with fosfomycin (5 mM) at T2 (bottom panels) or not treated (top panels). Time after resuspension is indicated.

C. Inhibition of membrane fission by fosfomycin is dependent on time of addition. Wild-type cells (AES574) were untreated (filled circles) or fosfomycin (5 mM) was added at T1 (triangles), T2 (diamonds) or T3 (open circles) and the fraction of cells with a CFP signal not surrounded by an FM4-64 signal was determined.

D. Fosfomycin-resistant *murAA* mutant engulfs normally in the presence of fosfomycin. The effect of fosfomycin on engulfment of a merodiploid strain (JDB2426) expressing an IPTG-inducible *C117D murAA* allele and YFP under a forespore-specific promoter (P_{spoIIQ} -*yfp*) was determined. Filled circles, both 1 mM IPTG was added at T0 and 5 mM fosfomycin was added at T2; open circles, only 5 mM fosfomycin at T2.

sary for the synthesis of mature peptidoglycan is also necessary for bulge formation.

Discussion

Muropeptide polymerization during engulfment

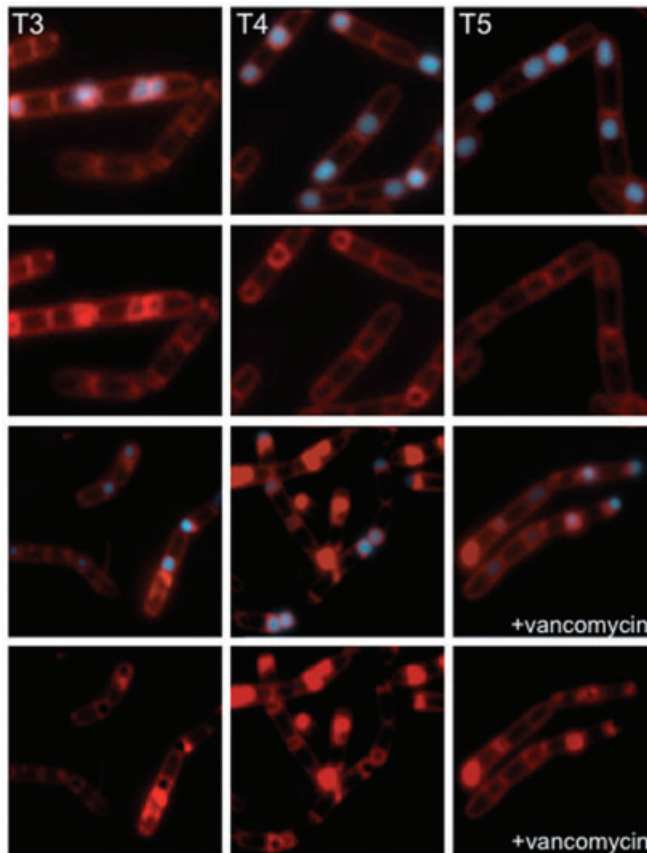
Our results allow historical experiments concerning the role of peptidoglycan biosynthesis in engulfment to be put into a mechanistic context. Among the earliest hints of such a role was the observation that removal of the cell wall by lysozyme treatment leads to a block in sporulation, but only when this treatment occurs before engulfment (Fitz-James, 1964). Consistent with this observation, *B. subtilis* exposed to vancomycin during engulfment do not reach a phase bright state, suggesting that peptidoglycan synthesis is required towards the end of engulfment (Dancer, 1979). During engulfment, a thin layer of peptidoglycan known as the germ cell wall is generated adjacent to the inner forespore membrane (Tipper and Linnett, 1976; Meador-Parton and Popham, 2000). Thus, the inhibitory effect of vancomycin on engulfment (Fig. 5) could be due to an inhibition of germ cell wall synthesis.

Consistent with this possibility, our observation that sites of active peptidoglycan synthesis are distributed around the engulfing forespore (Figs 2A and 8A, top) suggests that muropeptide polymerization could be mediating earlier stages of engulfment. Protoplasts of *B. subtilis* that lack peptidoglycan are still able to undergo this membrane movement in a mechanism that depends on the SpoIIQ–SpoIIIAH zipper, which is dispensable for engulfment in intact cells, but which is independent of the SpoIID–SpoIIP peptidoglycan hydrolase proteins that are essential in intact cells (Broder and Pogliano, 2006). Our results demonstrate that the inhibition of muropeptide synthesis blocks membrane migration in intact cells lacking the SpoIIQ–SpoIIIAH zipper (Fig. 3), suggesting that peptidoglycan polymerization comprises a mecha-

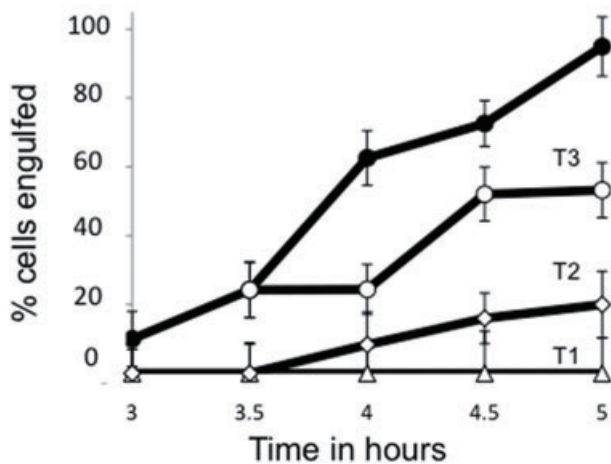
nism for membrane migration during engulfment that is essential in the absence of the Q–AH zipper. We therefore propose that peptidoglycan polymerization and the SpoIIQ–SpoIIIAH zipper comprise two redundant mechanisms for force generation (Fig. 8B). Peptidoglycan hydrolysis is required early in engulfment in order to release the asymmetric septum from the cellular cross-wall, and proteins that mediate this event are therefore required for the initiation of engulfment (Abanes-De Mello *et al.*, 2002; Morlot *et al.*, 2010). Peptidoglycan hydrolysis is required throughout membrane migration (Abanes-De Mello *et al.*, 2002; Gutierrez *et al.*, 2010), but it remains unclear if it provides force for membrane migration or plays some other role, such as removing steric barriers to the advancing membranes.

During the last step of engulfment, membrane fission, peptidoglycan synthesis was restricted to the zone of contact between the advancing membranes (Fig. 2, panel iii) and inhibition of muropeptide synthesis (Fig. 4) or polymerization (Fig. 5) prevented detachment of the forespore from the mother cell (engulfment membrane fission). The production of a rigid peptidoglycan polymer in this zone of contact could generate a mechanical force that drives elongation of the bud neck, distorting it into a tube, that eventually breaks, leading to cell separation. Then, localized peptidoglycan hydrolysis would allow the membranes to meet and rearrange (Fig. 8C). A membrane protein, SpoIIIE, is necessary for mediating this fission event during engulfment (Sharp and Pogliano, 2003) and our results suggest that SpoIIIE could interact with membrane proteins involved in peptidoglycan synthesis and organize their spatial distribution at the point of membrane fission. Consistent with this possibility, the membrane domain of SpoIIIE is necessary for its localization to the septum and its function in membrane fission (Sharp and Pogliano, 2003) and for the final steps of cytokinesis at the asymmetric septum (Liu *et al.*, 2006).

A



B



Muropeptide polymerization during bulge and vesicle formation

Membrane bulges in a *spoIID* or *spoIIP* strain form initially as a deformation of the membrane and then develop into

a clear invagination that, at least in some cases, becomes a membrane-bounded vesicle whose cytoplasmic contents are visibly separated from that of the forespore (Fig. 1A, bottom; Fig. 1B). These bulges are sites of active peptidoglycan formation (Fig. 6B) and inhibiting synthesis

Fig. 5. Inhibition of muropeptide transpeptidation blocks completion of engulfment.

A. Vancomycin blocks engulfment before membrane fission. At T2 of sporulation, cells (AES574) expressing CFP (blue) under control of a forespore-specific promoter (P_{spoIIA}) were either treated with vancomycin ($0.5 \mu\text{g ml}^{-1}$; bottom rows) or not treated (top rows). Membranes were visualized with FM4-64 (red). Time after resuspension is indicated.

B. Inhibition of membrane fission depends on the time of addition. The percentage of sporulating cells completing membrane fission was determined for untreated cells (filled circle) or cells incubated with vancomycin ($0.5 \mu\text{g ml}^{-1}$) at T1 (triangles), T2 (diamonds) or T3.5 (open circles) of sporulation by resuspension.

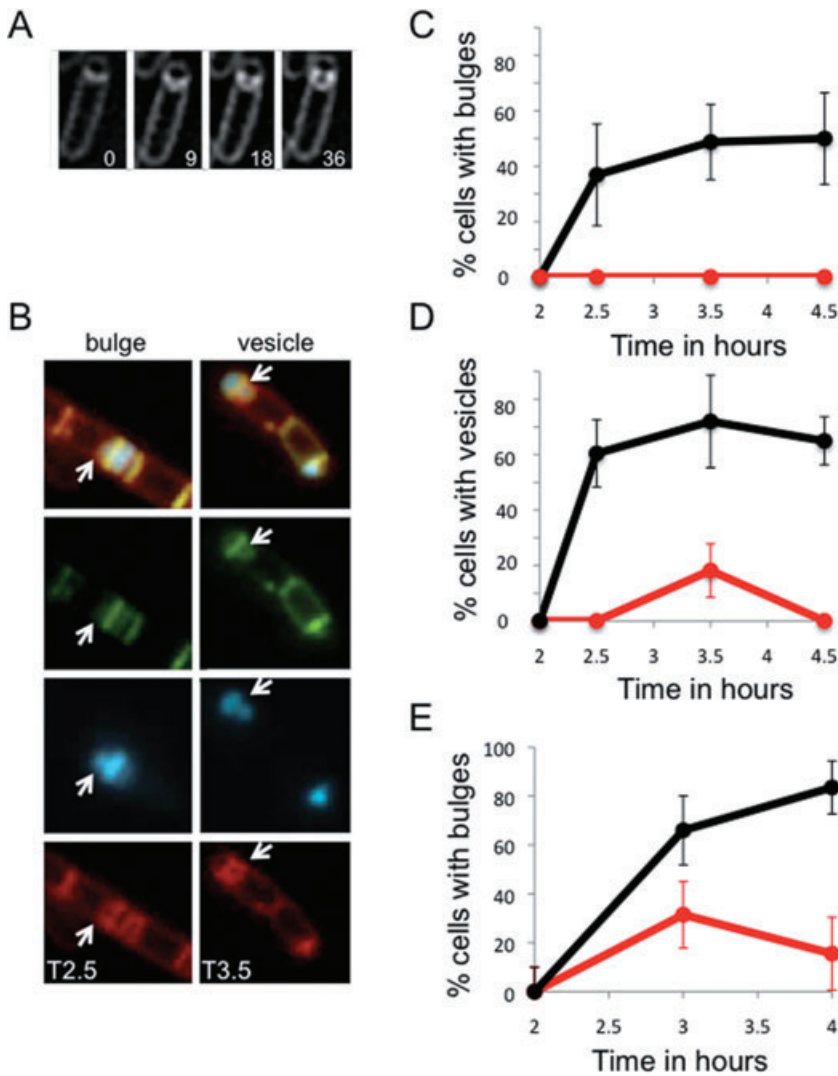


Fig. 6. Muropeptide synthesis and polymerization are required for bulge and vesicle formation.

A. Time-lapse images of a bulge progressively forming in a *spoIID* mutant strain (JDB2395) sporulating in a pad at 30°C and stained with FM4-64. Initial image was taken at approximately T2 after sporulation initiation and arbitrarily set to $t = 0$ min. The time of subsequent images is indicated in minutes in the lower right corner. See movie Fig. S7.

B. In a *spoIID* mutant strain (JDB2494) expressing CFP (blue) in the forespore, bulge and vesicles (white arrows) are sites of peptidoglycan synthesis. Membranes were visualized with FM4-64 (red) and newly synthesized peptidoglycan identified by ramoplanin-FL, a fluorescent ramoplanin derivative (green). Time after start of sporulation is indicated.

C. Fosfomyicin blocks bulge formation. Bulges do not form in a strain (JDB2494) lacking *spoIID* when 5 mM fosfomyicin was added at T2 of sporulation (red line) compared with cells where no fosfomyicin was added (black line).

D. Fosfomyicin blocks vesicle formation. Percentage of cells with vesicles in strains carrying mutations in *spoIID* and expressing CFP at the forespore (JDB2494) in the absence (black) or in the presence of 5 mM fosfomyicin added at T2 of sporulation (red).

E. Vancomycin blocks bulge formation. Percentage of *spoIID* cells (JDB2494) with bulges in the absence (black) or in the presence of 0.5 μg ml⁻¹ vancomycin (red) added at T2 of sporulation. Bulges and vesicles were identified as defined in Fig. 1B.

of the muropeptide precursor using fosfomyicin or of mature peptidoglycan using vancomycin blocks formation of the bulges (Fig. 6C and E). The effect of fosfomyicin was on MurAA since a merodiploid strain expressing a resistant mutant protein (MurAAC117D) generated vesicles even in the presence of fosfomyicin (Fig. S10). The effect of vancomycin was likely on an inhibition of polymerization directly and not on an inhibition of peptidoglycan hydrolases since inhibition of polymerization in *E. coli* leads to increased muramidase activity (Kohlrausch and Holtje, 1991). Thus, septal peptidoglycan polymerization could exert a mechanical force on the septal membrane that, by analogy to actin polymerization during lamellipodial motility (Mogilner and Oster, 1996), would drive membrane deformations (Fig. 8A, bottom).

The requirement of the SpoVD transpeptidase (Fig. 7A and B) is consistent with the observed inhibition of bulge formation by vancomycin (Fig. 6E) and demonstrates that a protein necessary for the synthesis of mature pepti-

doglycan is also necessary for bulge formation. In addition, the presence of bulges only on the mother cell side can be explained by this result as SpoVD is under control of σ^E , which is only active in the mother cell. If the forespore had a higher osmotic pressure than the mother cell because of the presence of equally sized chromosomes in the very differently sized compartments, this pressure could push the bulges from the middle of the septum (Perez *et al.*, 2000). However, we did not observe a significant difference in forespore size between the *spoIIP* and the *spoIIP spoVD* strains despite their observed differences in bulge formation (Fig. S13).

Rod-shaped *E. coli* depleted for proteins necessary for cell shape transform into spherical cells that contain large intracellular, membrane-bounded vesicles (Bendezu and de Boer, 2008). The inability of these mutant cells to couple the rate of phospholipid synthesis to changes in shape suggests that vesicle production is caused by overproduction of phospholipids. In *B. subtilis*, a similar coupling is not

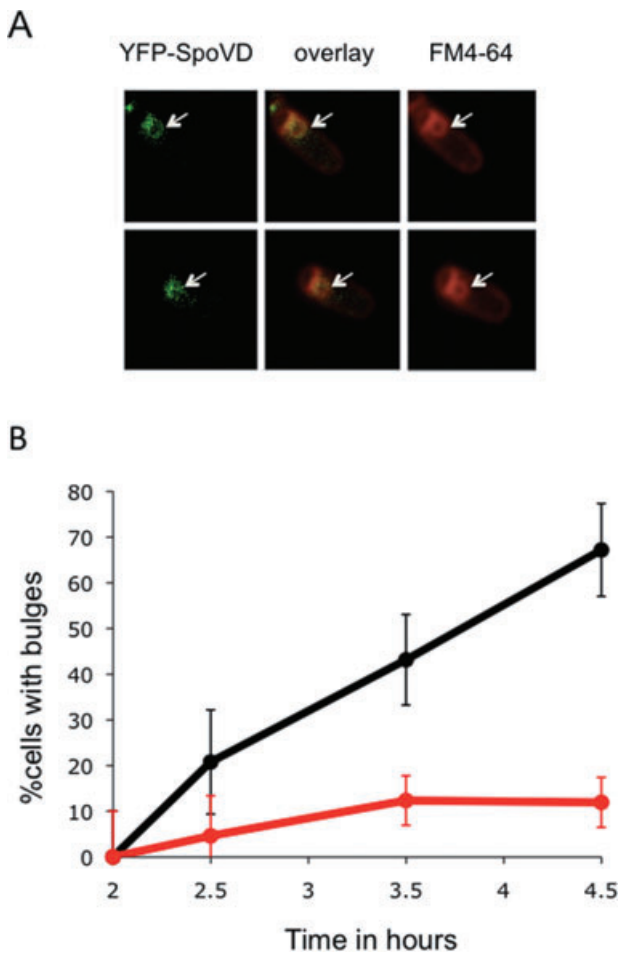


Fig. 7. Muropeptide-polymerizing enzyme is required for bulge formation.

A. The transpeptidase SpoVD localizes to the bulges. A strain lacking *spoIIP* and expressing a YFP–SpoVD fusion (JDB2553) was sporulated by resuspension and images acquired at T4. Two cells are shown. Left, YFP–SpoVD signal; middle is overlay of the YFP–SpoVD and FM4-64 signals; right, FM4-64. White arrows show the bulge.

B. Bulge formation is suppressed by a *spoVD* mutation. Percentage of *spoIIP* (JDB2396; black) and *spoIIP spoVD* (JDB2537; red) cells with bulges was determined. Bulge formation was assessed as defined in Fig. 1B.

known to exist (Paoletti *et al.*, 2007), consistent with our observation that fatty acid synthesis was not required at later time points where peptidoglycan synthesis was required for bulge and vesicle formation (Fig. S12) even though it was necessary for initial bulge formation.

Peptidoglycan polymerization as a force-generating mechanism during cytokinesis

A basic question in microbiology is the origin of the force underlying cell division. Although there are well-conserved proteins known to be involved, including the tubulin homologue FtsZ that forms constricting polymeric

rings at mid-cell, the source of the force generating these constrictions remains unknown (Weiss, 2004; Margolin, 2005). It has been suggested on theoretical grounds (Lan *et al.*, 2007; 2009; Ghosh and Sain, 2008; Allard and Cytrynbaum, 2009) as well as from electron microscopic observations of FtsZ polymer structure *in vivo* (Li *et al.*, 2007) that the energetics of FtsZ ring depolymerization could provide such a force. In tubular vesicles, FtsZ forms rings that produce visible constrictions (Osawa *et al.*, 2008) consistent with the ability of the constricting Z ring to generate a force. The absence of complete septal closure despite formation of physically distinct compartments seen in some *E. coli* amidase mutants suggests that, at least in these cells, membrane invagination could be mediated solely by constriction of the divisome (Priyadarshini *et al.*, 2007).

Gradients of peptidoglycan synthesis generate cell curvature in the bacterium *Caulobacter crescentus* (Cabeen *et al.*, 2009), supporting the notion that spatially differentiated peptidoglycan synthesis produces physical force sufficient to generate changes in cell shape that occur during cytokinesis (Huang *et al.*, 2008). High-magnification images of dividing *S. aureus* demonstrated the presence of peptidoglycan lying in tight apposition to the membrane. In fact, careful measurement of the diameter of the very tip of the in-growing septum indicated that this site was the locus of peptidoglycan synthesis (Matias and Beveridge, 2007). Thus, this synthesis could provide a driving force for the ‘iris-like’ movement of the septum from the initial stages of membrane invagination to the completion of septation.

Cell wall polymerization could be used in other systems to generate active and locally controlled forces. Although peptidoglycan is only found in bacteria [and perhaps in the chloroplasts of some plant cells (Machida *et al.*, 2006)], yeast and other fungi have rigid cell walls composed of long polysaccharides (Cabib *et al.*, 2001) that, similar to peptidoglycan, are polymerized by transglycosylases (Cabib *et al.*, 2008). In fact, growing *S. pombe* exerts a large mechanical force at its cell tips that is independent of actin cables and sufficient to play a role in piercing membranes during host invasion (Minc *et al.*, 2009). Finally, a variety of cell types such as budding yeasts can undergo cytokinesis in the absence of an actin–myosin contractile ring (Bi *et al.*, 1998). Thus, polymerization of fungal polysaccharides could serve a similar function as muropeptide polymerization in providing a force-generating mechanism for cytokinesis.

Experimental procedures

Microbiological methods

Bacillus subtilis strains are derivatives of PY79 (Table S1) and details of their construction are described in *Supporting infor-*

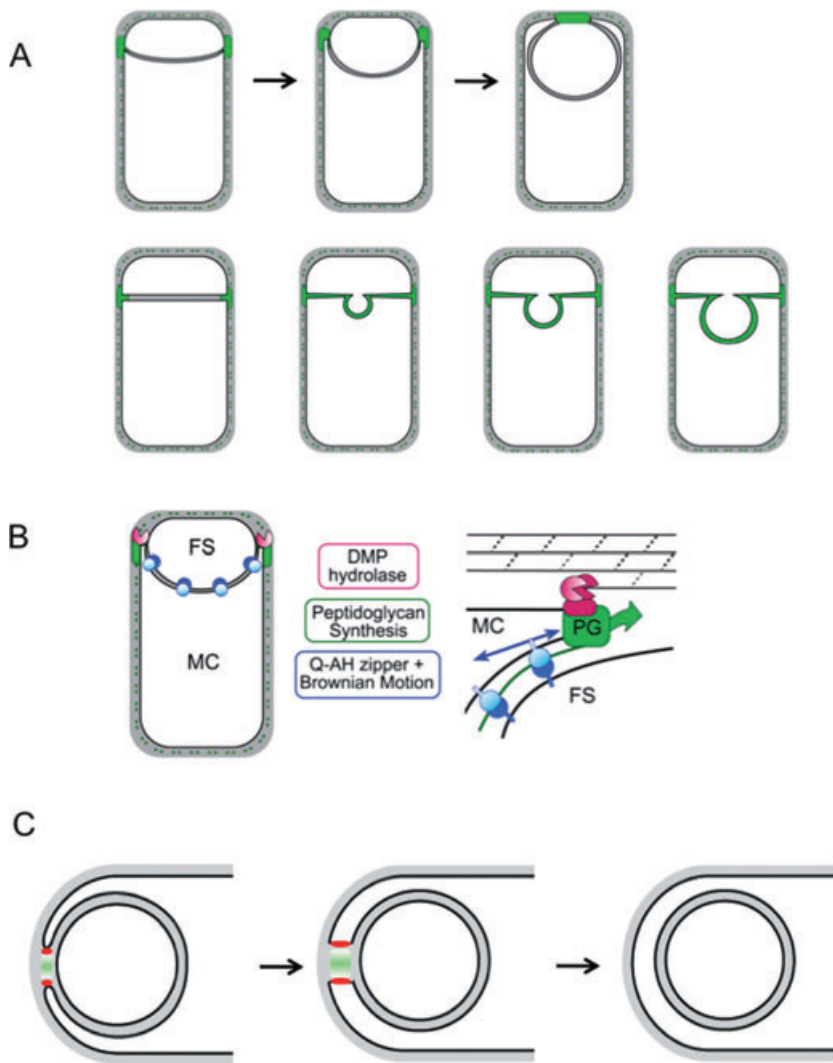


Fig. 8. Role of peptidoglycan polymerization in engulfment.

A. Diagram shows distribution of active peptidoglycan synthesis (green) during membrane migration and before detachment of the forespore from the mother cell (top) as well as during bulge formation (bottom).

B. The polymerization of these newly synthesized muropeptides partially drives membrane movement and results in the separation of the two cells. Our data suggest that membrane movement can be mediated either by peptidoglycan synthesis (PG; green) or by the SpoIIQ–SpoIIIAH proteins (blue) and that peptidoglycan hydrolysis mediated by the DMP proteins (red) plays a necessary role in detaching the septal peptidoglycan from the transverse peptidoglycan. FS, forespore; MC, mother cell.

C. Polymerization of newly synthesized muropeptides into peptidoglycan (green) at the point of contact between the migrating arms of the membranes pushes the septal membranes apart. Following detachment, the forespore becomes an independent membrane-bounded compartment in the cytoplasm. This polymerization is mediated by an unidentified transpeptidase-transglycosylase (red).

ation. Standard procedures were used to prepare and handle recombinant DNA and to transform *E. coli*. *B. subtilis* was transformed using competent cells made by the two-step method (Cutting and Vander Horn, 1990). Sporulation for microscopy was conducted at 30°C and used CH medium or 25% LB for growth and A+B medium for resuspension (Sterlini and Mandelstam, 1969). To measure completion of engulfment, membranes from sporulating cells expressing CFP under control of a forespore-specific promoter (P_{spoIIA}) were stained with FM4-64 (1 $\mu\text{g ml}^{-1}$). Every half hour starting at T3 a sample of cells was observed under the microscope. Cells expressing CFP were counted and the fraction of those not exhibiting FM4-64 staining of the forespore was determined.

Reagents

Fosfomycin and vancomycin were obtained from Sigma, cerulenin was obtained from Cayman Chemical, and FM4-64 was from Invitrogen. Ramoplanin-FL and ramoplanin-2c were gifts of Dr Suzanne Walker (Harvard Medical School).

Fluorescence microscopy

One microlitre of FM4-64 (Molecular Probes; 100 $\mu\text{g ml}^{-1}$) was added to each sample of 100 μl of sporulating cells that were taken at designated times after resuspension, immediately prior to collection by centrifugation. The pellet was resuspended in 10 μl of PBS, and added to a poly-L-lysine pre-treated coverslip. All microscopy (except Fig. 2 and Fig. S2) was performed on a Nikon Eclipse 90i with a 100 \times objective using phase contrast and captured by a Hamamatsu Orca-ER camera using Nikon Elements BR software. CFP, YFP, FITC (Ramoplanin-FL) and TRITC (FM4-64) exposures were 400 ms. Other microscopy was performed using an Applied Precision Spectris microscope.

Dynamics of engulfing forespores

Time-lapse microscopy was performed as described (Becker and Pogliano, 2007). Briefly, FM4-64 was added to a final concentration of 0.5 $\mu\text{g ml}^{-1}$ in a 1.2% solution of molten

agar/media (A+B) and added to the well of a culture slide and covered with a glass slide. After cooling, the slide was removed and two air pockets were cut out of the agar leaving a 3–5 mm agar bridge in the centre of the well. Sporulating cells suspended in A+B with FM4-64 ($0.5 \mu\text{g ml}^{-1}$) were added at T2 to the agar bridge and covered by a glass coverslip. To prevent drying during the experiment, 50% glycerol was applied to the region of contact between the slide and the coverslip. The slide equilibrated in an environmentally controlled chamber at 30°C (Precision Control Weather Station) for at least 10 min prior to visualization. Images were acquired using an Applied Precision Spectris microscope.

Finally, the following metric was used for defining membrane migration: 'No migration' is defined as: 40 min of observation with no advancement of the membranes (curvature is acceptable as long as the distance between septum edge and cell pole does not decrease). 'Migration' is defined as: advancement that is maintained for at least 20 min of observation. In addition, cells must be observed for 40 min without going off the screen or lysing.

Acknowledgements

We thank Suzanne Walker (Harvard Medical) for generously supplying fluorescent derivatives of ramoplanin, Avigdor Eldar and Michael Elowitz (Caltech) for strains and Allison Fay for constructing pAF54. This work was supported by NIH R01GM83468 (J.D.) and R01 R01GM57045 (K.P.). P.M. is a Helen Hay Whitney Fellow and J.D. is an Irma T. Hirsch Scholar.

References

- Abanes-De Mello, A., Sun, Y.L., Aung, S., and Pogliano, K. (2002) A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore. *Genes Dev* **16**: 3253–3264.
- Allard, J.F., and Cytrynbaum, E.N. (2009) Force generation by a dynamic Z-ring in *Escherichia coli* cell division. *Proc Natl Acad Sci USA* **106**: 145–150.
- Becker, E.C., and Pogliano, K. (2007) Cell-specific SpoIIIE assembly and DNA translocation polarity are dictated by chromosome orientation. *Mol Microbiol* **66**: 1066–1079.
- Bendezu, F.O., and de Boer, P.A. (2008) Conditional lethality, division defects, membrane involution, and endocytosis in *mre* and *mrd* shape mutants of *Escherichia coli*. *J Bacteriol* **190**: 1792–1811.
- Bi, E., Maddox, P., Lew, D.J., Salmon, E.D., McMillan, J.N., Yeh, E., and Pringle, J.R. (1998) Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *J Cell Biol* **142**: 1301–1312.
- Broder, D.H., and Pogliano, K. (2006) Forespore engulfment mediated by a ratchet-like mechanism. *Cell* **126**: 917–928.
- Bylund, J.E., Zhang, L., Haines, M.A., Higgins, M.L., and Piggot, P.J. (1994) Analysis by fluorescence microscopy of the development of compartment-specific gene expression during sporulation of *Bacillus subtilis*. *J Bacteriol* **176**: 2898–2905.
- Cabeen, M.T., Charbon, G., Vollmer, W., Born, P., Ausmees, N., Weibel, D.B., and Jacobs-Wagner, C. (2009) Bacterial cell curvature through mechanical control of cell growth. *EMBO J* **28**: 1208–1219.
- Cabib, E., Roh, D.H., Schmidt, M., Crotti, L.B., and Varma, A. (2001) The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J Biol Chem* **276**: 19679–19682.
- Cabib, E., Farkas, V., Kosik, O., Blanco, N., Arroyo, J., and McPhie, P. (2008) Assembly of the yeast cell wall. Crh1p and Crh2p act as transglycosylases *in vivo* and *in vitro*. *J Biol Chem* **283**: 29859–29872.
- Cutting, S.M., and Vander Horn, P.B. (1990) Genetic analysis. In *Molecular Biological Methods for Bacillus*. Harwood, C.R. and Cutting, S.M. (eds). New York, NY: John Wiley & Sons, pp. 24–74.
- Dancer, B.N. (1979) Requirement for peptidoglycan synthesis during sporulation of *Bacillus subtilis*. *J Bacteriol* **140**: 786–797.
- Elbaum, M., Kuchnir Fygenon, D., and Libchaber, A. (1996) Buckling microtubules in vesicles. *Phys Rev Lett* **76**: 4078–4081.
- Fitz-James, P.C. (1964) Sporulation in protoplasts and its dependence on prior forespore development. *J Bacteriol* **87**: 667–675.
- Francius, G., Domenech, O., Mingeot-Leclercq, M.P., and Dufrene, Y.F. (2008) Direct observation of *Staphylococcus aureus* cell wall digestion by lysostaphin. *J Bacteriol* **190**: 7904–7909.
- Frandsen, N., and Stragier, P. (1995) Identification and characterization of the *Bacillus subtilis* *spoIIIP* locus. *J Bacteriol* **177**: 716–722.
- Ghosh, B., and Sain, A. (2008) Origin of contractile force during cell division of bacteria. *Phys Rev Lett* **101**: 178101.
- Gutierrez, J., Smith, R., and Pogliano, K. (2010) SpoIID peptidoglycan hydrolase activity is required throughout engulfment during *Bacillus subtilis* sporulation. *J Bacteriol* (in press).
- Hamburger, J.B., Hoertz, A.J., Lee, A., Senturia, R.J., McCafferty, D.G., and Loll, P.J. (2009) A crystal structure of a dimer of the antibiotic ramoplanin illustrates membrane positioning and a potential Lipid II docking interface. *Proc Natl Acad Sci USA* **106**: 13759–13764.
- Hayhurst, E.J., Kailas, L., Hobbs, J.K., and Foster, S.J. (2008) Cell wall peptidoglycan architecture in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **105**: 14603–14608.
- van Heijenoort, J. (2001) Recent advances in the formation of the bacterial peptidoglycan monomer unit. *Nat Prod Rep* **18**: 503–519.
- Huang, K.C., Mukhopadhyay, R., Wen, B., Gitai, Z., and Wingreen, N.S. (2008) Cell shape and cell-wall organization in Gram-negative bacteria. *Proc Natl Acad Sci USA* **105**: 19282–19287.
- Illing, N., and Errington, J. (1991) Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of sigma E and sigma F in prespore engulfment. *J Bacteriol* **173**: 3159–3169.
- Inoue, S., and Salmon, E.D. (1995) Force generation by microtubule assembly/disassembly in mitosis and related movements. *Mol Biol Cell* **6**: 1619–1640.
- Kim, S.J., Cegelski, L., Stueber, D., Singh, M., Dietrich, E., Tanaka, K.S., *et al.* (2008) Oritavancin exhibits dual mode

- of action to inhibit cell-wall biosynthesis in *Staphylococcus aureus*. *J Mol Biol* **377**: 281–293.
- Kohlrausch, U., and Holtje, J.V. (1991) Analysis of murein and murein precursors during antibiotic-induced lysis of *Escherichia coli*. *J Bacteriol* **173**: 3425–3431.
- Komeili, A., Li, Z., Newman, D.K., and Jensen, G.J. (2006) Magnetosomes are cell membrane invaginations organized by the actin-like protein MamK. *Science* **311**: 242–245.
- Lan, G., Wolgemuth, C.W., and Sun, S.X. (2007) Z-ring force and cell shape during division in rod-like bacteria. *Proc Natl Acad Sci USA* **104**: 16110–16115.
- Lan, G., Daniels, B.R., Dobrowsky, T.M., Wirtz, D., and Sun, S.X. (2009) Condensation of FtsZ filaments can drive bacterial cell division. *Proc Natl Acad Sci USA* **106**: 121–126.
- Li, Z., Trimble, M.J., Brun, Y.V., and Jensen, G.J. (2007) The structure of FtsZ filaments *in vivo* suggests a force-generating role in cell division. *EMBO J* **26**: 4694–4708.
- Liu, N.J., Dutton, R.J., and Pogliano, K. (2006) Evidence that the SpoIIIE DNA translocase participates in membrane fusion during cytokinesis and engulfment. *Mol Microbiol* **59**: 1097–1113.
- Lopez-Diaz, I., Clarke, S., and Mandelstam, J. (1986) *spoIID* operon of *Bacillus subtilis*: cloning and sequence. *J Gen Microbiol* **132**: 341–354.
- Machida, M., Takechi, K., Sato, H., Chung, S.J., Kuroiwa, H., Takio, S., *et al.* (2006) Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss. *Proc Natl Acad Sci USA* **103**: 6753–6758.
- Margolin, W. (2005) FtsZ and the division of prokaryotic cells and organelles. *Nat Rev Mol Cell Biol* **6**: 862–871.
- Matias, V.R., and Beveridge, T.J. (2007) Cryo-electron microscopy of cell division in *Staphylococcus aureus* reveals a mid-zone between nascent cross walls. *Mol Microbiol* **64**: 195–206.
- Meador-Parton, J., and Popham, D.L. (2000) Structural analysis of *Bacillus subtilis* spore peptidoglycan during sporulation. *J Bacteriol* **182**: 4491–4499.
- Minc, N., Boudaoud, A., and Chang, F. (2009) Mechanical forces of fission yeast growth. *Curr Biol* **19**: 1096–1101.
- Mitchison, T.J., and Cramer, L.P. (1996) Actin-based cell motility and cell locomotion. *Cell* **84**: 371–379.
- Miyata, H., Nishiyama, S., Akashi, K., and Kinosita, K., Jr (1999) Protrusive growth from giant liposomes driven by actin polymerization. *Proc Natl Acad Sci USA* **96**: 2048–2053.
- Mogilner, A., and Oster, G. (1996) Cell motility driven by actin polymerization. *Biophys J* **71**: 3030–3045.
- Morlot, C., Uehara, T., Marquis, K.A., Bernhardt, T.G., and Rudner, D.Z. (2010) A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis*. *Genes Dev* **24**: 411–422.
- Osawa, M., Anderson, D.E., and Erickson, H.P. (2008) Reconstitution of contractile FtsZ rings in liposomes. *Science* **320**: 792–794.
- Paoletti, L., Lu, Y.J., Schujman, G.E., de Mendoza, D., and Rock, C.O. (2007) Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*. *J Bacteriol* **189**: 5816–5824.
- Pelham, R.J., and Chang, F. (2002) Actin dynamics in the contractile ring during cytokinesis in fission yeast. *Nature* **419**: 82–86.
- Perez, A.R., Abanes-De Mello, A., and Pogliano, K. (2000) SpoIIIB localizes to active sites of septal biogenesis and spatially regulates septal thinning during engulfment in *Bacillus subtilis*. *J Bacteriol* **182**: 1096–1108.
- Phillips, R., Kondev, J., and Theriot, J. (2009) *Physical Biology of the Cell*. New York: Garland Science.
- Priyadarshini, R., de Pedro, M.A., and Young, K.D. (2007) Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. *J Bacteriol* **189**: 5334–5347.
- Qualmann, B., Kessels, M.M., and Kelly, R.B. (2000) Molecular links between endocytosis and the actin cytoskeleton. *J Cell Biol* **150**: F111–F116.
- Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev* **32**: 234–258.
- Scheffel, A., Gruska, M., Faivre, D., Linaroudis, A., Plitzko, J.M., and Schuler, D. (2006) An acidic protein aligns magnetosomes along a filamentous structure in magnetotactic bacteria. *Nature* **440**: 110–114.
- Sharp, M.D., and Pogliano, K. (1999) An *in vivo* membrane fusion assay implicates SpoIIIE in the final stages of engulfment during *Bacillus subtilis* sporulation. *Proc Natl Acad Sci USA* **96**: 14553–14558.
- Sharp, M.D., and Pogliano, K. (2003) The membrane domain of SpoIIIE is required for membrane fusion during *Bacillus subtilis* sporulation. *J Bacteriol* **185**: 2005–2008.
- Smith, K., and Youngman, P. (1993) Evidence that the *spoIIIM* gene of *Bacillus subtilis* is transcribed by RNA polymerase associated with sigma E. *J Bacteriol* **175**: 3618–3627.
- Sterlini, J.M., and Mandelstam, J. (1969) Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochem J* **113**: 29–37.
- Theriot, J.A. (2000) The polymerization motor. *Traffic* **1**: 19–28.
- Tipper, D.J., and Linnett, P.E. (1976) Distribution of peptidoglycan synthetase activities between sporangia and forespores in sporulating cells of *Bacillus sphaericus*. *J Bacteriol* **126**: 213–221.
- Tiyanont, K., Doan, T., Lazarus, M.B., Fang, X., Rudner, D.Z., and Walker, S. (2006) Imaging peptidoglycan biosynthesis in *Bacillus subtilis* with fluorescent antibiotics. *Proc Natl Acad Sci USA* **103**: 11033–11038.
- Vasudevan, P., Weaver, A., Reichert, E.D., Linnstaedt, S.D., and Popham, D.L. (2007) Spore cortex formation in *Bacillus subtilis* is regulated by accumulation of peptidoglycan precursors under the control of sigma K. *Mol Microbiol* **65**: 1582–1594.
- Walsh, C.T. (2003) *Antibiotics*. Washington, DC: ASM.
- Weiss, D.S. (2004) Bacterial cell division and the septal ring. *Mol Microbiol* **54**: 588–597.
- Yao, X., Jericho, M., Pink, D., and Beveridge, T. (1999) Thickness and elasticity of gram-negative murein sacculi measured by atomic force microscopy. *J Bacteriol* **181**: 6865–6875.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied

by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.