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Title: A multidimensional study of streptomyces morphogenesis and development

Issue Date: 2013-11-27

CHAPTER 4

Intracellular Membrane Structures Mediate Crosswall Formation and Nucleoid Occlusion in *Streptomyces*

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Manuscript in preparation.

ABSTRACT

During the bacterial cell cycle, chromosome replication and segregation is tightly regulated and coordinated with septum formation to guarantee the survival of progeny. The cytokinetic machinery is highly conserved, with subtle variations, and stabilizing and destabilizing factors cooperate to properly position the cell division apparatus at mid-cell. Yet the precise mechanisms that initiate divisome formation coordinated with nucleoid segregation are still largely unknown. Here we show that intracellular membrane structures play a major role in division-site selection and nucleoid occlusion in *Streptomyces* hyphae. Cross-membranes, consisting of a heterogeneous distribution of lipids, create a chromosome-free area to ensure that septum formation can occur without damaging DNA. Crosswalls are then seen to form in these cross-membrane structures, away from the nucleoid. The membranes fully colocalize with - and are dependent on - the division scaffold protein FtsZ, which highlights the subtle interplay between membrane and cell wall formation during the initiation of cell division.

INTRODUCTION

Within the bacterial cell, several control systems cooperate to ensure that chromosomes are faithfully separated and not damaged during the formation of a cell division septum. In rod-shaped bacteria, the combined action of the Min system and nucleoid (chromosome) occlusion ensures the precise positioning of the division machinery at mid-cell (Rothfield *et al.* 2005; Harry *et al.* 2006; Barak and Wilkinson 2007; Wu and Errington 2012). The Min system prevents FtsZ assembly at the cell poles, while nucleoid occlusion prevents the formation of the Z-ring over the nucleoids, with Z-ring formation therefore occurring at the mid-cell by default. Yet though division is much less efficient in cells lacking these systems, neither the Min system nor the Noc/SlmA nuclear occlusion proteins are essential in *E. coli* or *B. subtilis* (Wu and Errington 2004; Bernhardt and de Boer 2005). What exactly initiates the formation of the divisome at cell centre remains unclear, and several lines of evidence suggest that there are additional mechanisms at work.

A recently proposed model suggests that DNA replication at the chromosomal origin of replication (*oriC*) triggers the accumulation of an as-of-yet unidentified positive signal at mid-cell which activates Z-ring formation (Moriya *et al.* 2010). Complementary studies on the distribution of phospholipids in the bacterial membrane have shown that their heterogeneous distribution results in regions enriched in particular lipids at cell poles - as well as at the cell centre. In fact, lipid heterogeneity is critical for the spatial and temporal organization of cells (Fishov and Woldringh 1999; Nishibori *et al.* 2005; Barak *et al.* 2008; Fishov and Norris 2012). Lipid domains play a crucial role in the specific localization of proteins and protein complexes and are important for cellular function, influencing signal transduction, secretion, as well as cell division and development (Mileykovskaya and Dowhan 2005; Matsumoto *et al.* 2006; Barak and Muchová 2013). Phospholipid composition at a particular location affects the chemical and physical properties of a membrane, as well as its curvature (Lenarcic *et al.* 2009; Ramamurthi *et al.* 2009) and electric potential (Strahl and Hamoen 2010), effectively determining when and where proteins and protein complexes specifically localize. In fact, lipid domains may bind amphitropic proteins responsible for selection and recognition of the division site (Mileykovskaya and Dowhan 2005; Barak *et al.* 2008). Evidence exists that the phospholipid composition at mid-cell is optimal for initiation of DNA replication and Z-ring positioning, suggesting that a dynamic protein-lipid interaction may act as the positive trigger for Z-ring formation (Norris *et al.* 2004; Mileykovskaya and Dowhan 2005; Saxena *et al.* 2013).

In the long hyphae of streptomycetes there is no mid-cell. Positioning of the divisome therefore poses a difficult problem: where should a septum be formed in a branching, multinucleoid and filamentous cell? These multicellular soil-dwelling bacteria grow as a syncytial vegetative mycelium separated by crosswalls (Wildermuth and Hopwood 1970). During the reproductive phase, they produce chains of spores in aerial hyphae, following a complex cell division event whereby long ladders of Z-rings are produced in a short time span (Flårdh and Buttner 2009). Interestingly, while the *Streptomyces* cell division machinery resembles that of unicellular bacteria, the canonical control systems like Min, Noc and Sula are missing, perhaps redundant and therefore lost in hyphae because of the absence of a mid-cell reference point (McCormick 2009; Jakimowicz and van Wezel 2012). This implies that an entirely different mechanism of controlling septum-site localization and nucleoid occlusion is present in these bacteria. We recently showed that septum-site localization during sporulation-specific cell division in aerial hyphae is positively controlled, with FtsZ recruited by the SsgB protein (Willemse *et al.* 2011), which in turn depends on its paralogue SsgA. SsgA is a cell division activator protein, and the number of septa formed in the hyphae directly correlates to the expression level of SsgA (van Wezel *et al.* 2000a; van Wezel *et al.* 2006). In aerial hyphae, SsgA plays an important role in the correct localization of the FtsZ-recruiting SsgB in time and space (Willemse *et al.* 2011). SsgA and SsgB belong to the family of the SsgA-like proteins or SALPs, which occur exclusively in morphologically complex actinomycetes (Noens *et al.* 2005; Traag and van Wezel 2008). The way these proteins find the future sites of septation is as of yet unknown.

In vegetative hyphae, cross-walls form at irregular intervals to delimit multigenomic compartments. These compartments usually have an irregular number of chromosomes, and contain replication machinery in different stages of assembly and disassembly, sometimes within the same compartment (Ruban-Ośmiałowska *et al.* 2006). Unlike aerial septa, crosswalls do not constrict the cell, and do not result in cell fission, effectively constituting a completely different form of cell division in this microorganism. Notably, many canonical divisome components, such as FtsI and FtsW, which are required for septal peptidoglycan synthesis in *E. coli* (Wang *et al.* 1998), are not required for crosswall formation. This suggests an entirely different mechanism, where another pair of proteins consisting of an SEDS (shape, elongation, division, and sporulation) protein and a cognate class B penicillin-binding protein (PBP) may carry out septum synthesis (Bennett *et al.* 2009; McCormick 2009). Here we show that crosswall formation is preceded by the formation of large membrane structures delimiting hyphae. These membrane structures form during development, localizing either in small sites along the cell wall, at tips, or within hyphae,

compartmentalizing the vegetative mycelium. The large delimiting membranes, which we have dubbed cross-membranes, appear to be essential for FtsZ recruitment prior to the formation of a crosswall. The membranes play a dominant role in nucleoid occlusion, creating a chromosome-free area prior to septum synthesis and ensuring that cell division can occur without damaging the chromosomes.

RESULTS

Live imaging reveals dynamic membrane localization in Streptomyces vegetative hyphae

As the Min and Noc/SlmA control systems are missing in streptomycetes, and the SsgA-like proteins SsgA and SsgB play only a minor role in the control of crosswall formation, we set out to discover what governs vegetative division and how DNA damage is prevented. Lipid domains enriched in cardiolipin, an anionic negative-curvature phospholipid, have recently been implicated in cell division processes, hypothesized to be responsible for selection and recognition of the division site (Mileykovskaya and Dowhan 2005; Barak and Wilkinson 2007). We therefore performed live imaging experiments on *Streptomyces* hyphae grown in a medium containing FM 5-95 and 10-N-nonyl acridine orange (NAO) to investigate the dynamics of membrane and cardiolipin localization during crosswall formation. FM5-95 stains anionic phospholipids, including phosphatidylglycerol (PGL), while NAO allows for visualization of cardiolipin.

Imaging demonstrated that membrane localization is highly dynamic and complex. Membranes could be seen associating and dissociating along the hyphae within an hour, at times forming large cross-membrane structures, localizing to the tip, or forming small foci at the cell wall. Simultaneously, punctate localization of cardiolipin assemblies occurred throughout vegetative hyphae. Both membrane and cardiolipin phospholipids localized highly dynamically (Figure 1 and Supplemental Movie S1). Interestingly, typically after roughly 12 hours of growth, a flash of cardiolipin was seen propagating through the hyphae. Within 20 minutes this distributed localization faded, to be replaced by discrete membrane localization. Large membrane agglomerates (ranging from one to several μm in length, red arrow in Figure 1) grew and subsequently shrunk in the regions of localization; within the span of an hour, the membranes faded again, and were accompanied by forming cardiolipin assemblies.

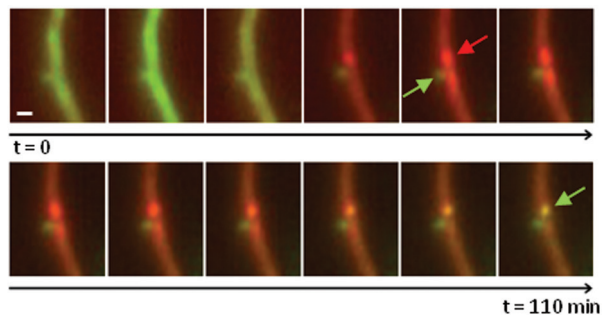


Figure 1. Membrane and cardiolipin localization within hyphae is highly dynamic (stills taken from Supplemental Video S1). Rapid translocation of cardiolipin (green) is seen within *Streptomyces coelicolor* hyphae, followed by diffused localization of this phospholipid. Membranes (red) next appear within the hyphae and are recruited to the future site of a crosswall (red arrow), while cardiolipin can be seen localizing steadily at the tip of a newly forming branch (green arrow). Membranes expand and then coalesce and disappear. By the end of the time lapse, membrane localization is replaced by cardiolipin. *S. coelicolor* was grown in a medium containing FM5-95 (staining negatively charged phospholipids in the plasma membrane) and NAO (preferentially staining cardiolipin) and live imaging was started after 12 hours of growth, with a time lapse of 10 minutes between images. Scale bar, 0.5 μm .

Cross-membranes localize to the sites where crosswalls are formed

Intrigued by the membrane localization dynamics, we set out to investigate the underlying mechanism and structure of the observed membrane patches in more detail and at higher resolution using cryo-electron microscopy (cryo-EM). Cryo-EM on vitrified *Streptomyces* liquid culture samples of early mycelia revealed the presence of a vast and intricate intracellular membrane system within hyphae. As in live imaging, membranes were seen either at small regions along the cell wall, at tips, or as large structures completely delimiting hyphae (the cross-membranes) (Figure 2). Often the observed membranes consisted of bundles of tube-like structures, extending from the cytoplasmic membrane. Searching for crosswalls, we discovered that in samples grown for 14 hours or more, crosswalls had completely formed (Figure 2I), whereas at earlier time points, membrane material was more plentiful, and crosswalls were scarce (Figure 2A-G). Interestingly, in these younger samples, the start of cell wall invagination could sometimes be seen within cross-membrane structures (Figure 2H), suggesting that these are the sites of crosswall formation. Examining samples at different time points suggested that crosswalls form rapidly, in line with earlier live imaging experiments (Jyothikumar *et al.* 2008). While in 10 hr samples hardly any crosswalls or cross-membranes were present, in 12 hr samples such membranes were abundant, sometimes associated with septum synthesis. After 14 hrs, many crosswalls had

been completed. In these and older (20-24 hr) samples, membranes could sometimes still be found, though they were not as abundant. This strongly suggests that once started, cell-wall formation is completed rapidly. Since we have never seen incomplete crosswalls in the absence of cross membranes, it is likely that the crosswalls are associated with - and likely formed within - membrane assemblies.

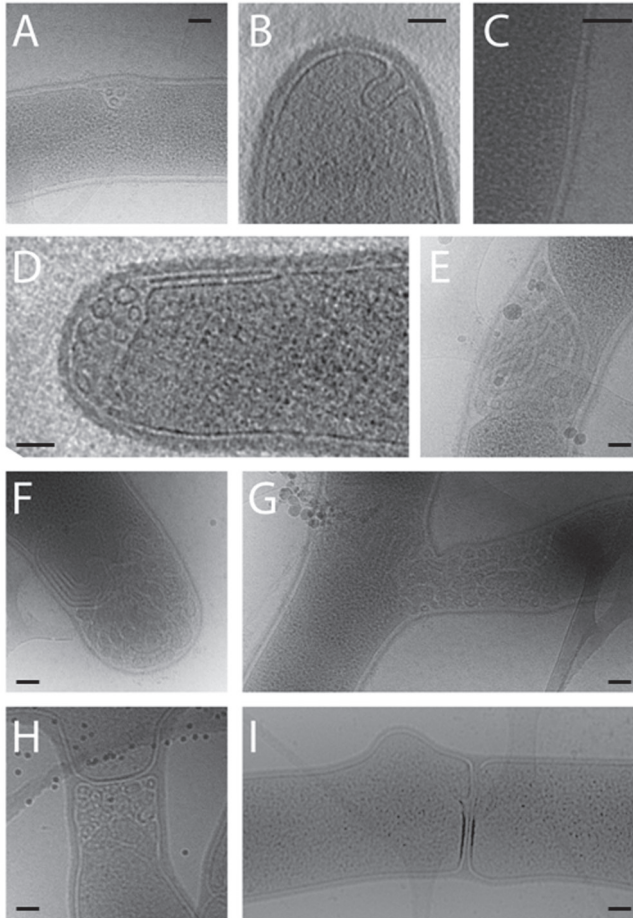


Figure 2. Diverse intracellular membrane structures are found in *Streptomyces* sp. Membranes localize at the sides of hyphae in small or large blurbs (A,C,E) or to tips (B,D,F). Cell delimiting cross-membranes can also be found, with membrane tubes creating large intracellular structures (E,G). In some instances, cell wall can be found within these membrane structures (H), suggesting that crosswalls are initiated in membranes. In later stages of growth, complete crosswalls are seen (I). Scale bars, 100 nm.

Membranes create a chromosome-free area, mediating nucleoid occlusion

Intrigued by the cell wall invaginations within cross-membrane structures, we sought to obtain a more detailed and three-dimensional view of the onset of crosswall formation. Since membrane patches, and specifically the CL rafts, localized transiently in time and space, depending on growth stage, and because the membrane and CL patches localized closely together, we developed correlative light and electron microscopy (CLEM) for use in streptomycetes. Cryo-CLEM correlates the images acquired by cryo-fluorescence light microscopy and cryo-electron microscopy, including tomography, enabling selective labeling and identification of membrane components, as well as DNA, and direct mapping of these lower resolution data onto high resolution tomograms.

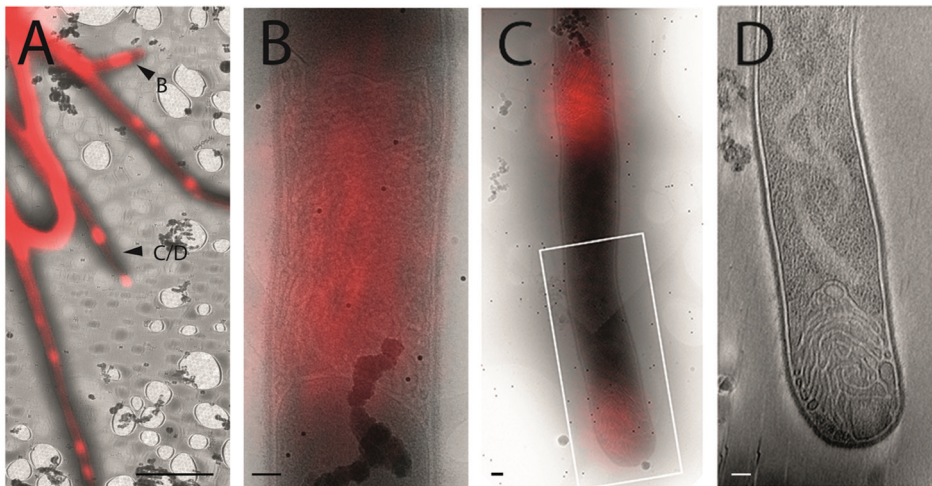


Figure 3. Cryo-CLEM on *Streptomyces* stained for membranes with FM5-95. An overlaid fLM (red) and cryo-EM overview image (A) shows FM5-95 staining patches along the hyphae. Cryo-EM of the locations identified by boxes in (A) indicates that at the positions of fluorescent staining, large internal membranes can be found (B,C). A tomographic slice through the membrane-filled tip shown in (C) reveals the partly tubular shape of the membranes (D). Chromosomal DNA can be seen forming a curve following the tip. Scale bars, 5 μm (A), 100 nm (B-D).

We stained for membranes using fluorescent dye FM5-95, and found that the large membrane formations observed in tomograms correlate directly to areas stained by FM5-95 in the light microscopy images, and are therefore likely to be enriched in PGL. Staining allowed us to identify cross-membranes as well as membrane-filled apical sites (Figure 3). Chromosomal DNA, in tomograms evidenced by a finely textured region devoid of ribosomes and other macromolecular complexes, could be seen between small membrane blebs, or

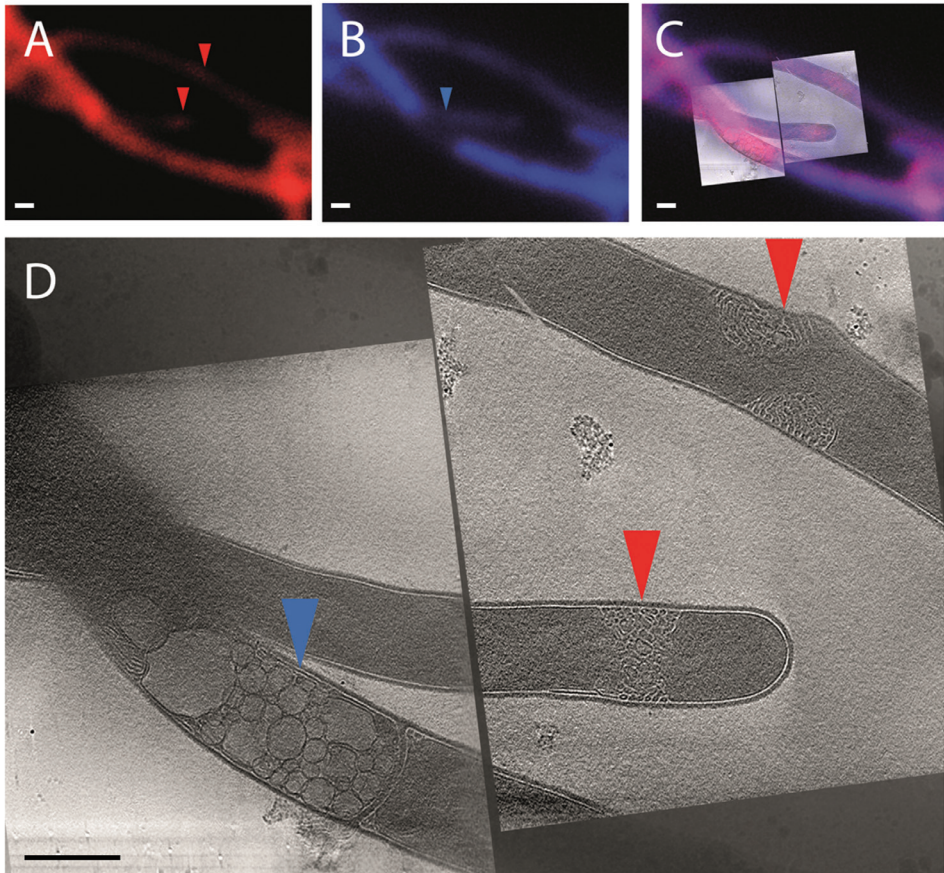


Figure 4. Cryo-CLEM on *Streptomyces* stained for membranes (using stain FM5-95) and DNA (using stain DAPI) demonstrates that patches of membrane stain coincide with tubular membrane structures in hyphae (red arrows in A and D). Large gaps in the DNA staining coincide with vesicles near a crosswall (blue arrows in B and D). An overlay of the fLM and cryo-EM images is given in (C). Tomographic slices through two adjoining tomograms show the membranes localizing at the sides of a hyphae, near a forming branch (upper right), or near a tip (lower right) (D). At the new branch point, new cell wall invagination can be seen within the membranes. Scale bars, 0.5 μm .

behind a membrane-filled tip (Figure 3D and Supplemental Video S2). This provided the first evidence that DNA and membranes structures may be mutually exclusive.

To analyze this further, membrane staining was combined with 4',6-diamidino-2-phenylindole (DAPI) to visualize chromosomes. As in the case of the membrane-filled apical sites, all areas where large cross-membrane or membrane-enriched vesicle structures localized were devoid of DNA, evidenced by a distinct gap in DAPI fluorescence (Figure 4).

Where membranes localized to the hyphal wall, but did not delimit a hyphae, DNA could still be seen passing through the channel between membranes. Together with direct evidence from the native tomograms (Supplemental Video S2), this suggests that the cross-membranes create DNA-free zones, occluding the chromosomal DNA from sites where active cell-wall restructuring takes place in *Streptomyces* vegetative hyphae.

We also stained for cardiolipin with NAO and discovered many cardiolipin-rich zones in vegetative hyphae, which invariably correlated to membrane vesicles observed in the tomograms (Figure 5). These multilayered vesicles appeared in many different shapes and forms and were clearly distinct from the tubular and unilamellar vesicles in cross-

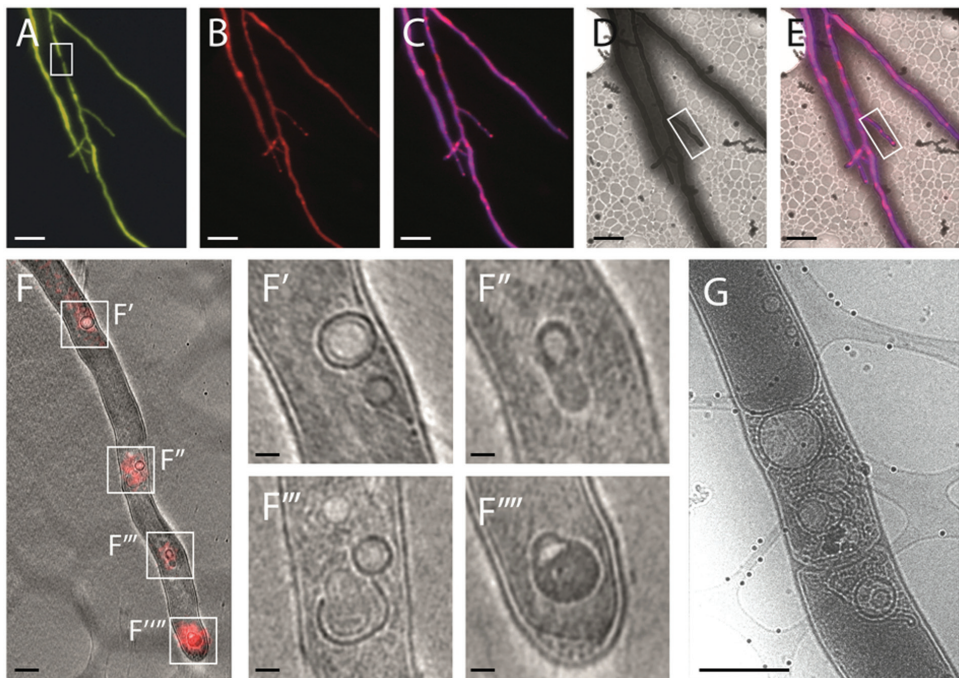


Figure 5. Cryo-CLEM on *Streptomyces* stained for cardiolipin (using stain NAO) and DNA (using stain DAPI), demonstrates that cardiolipin forms vesicle-like structures within the hyphae. fLM images of DAPI staining, shown in yellow, for clarity (A) and cardiolipin staining (B) have been overlaid in (C). The EM picture is shown in (D) and an overlay of fLM and EM is provided in (E). At higher magnification, direct correlation can be seen between the staining and intracellular structures rich in cardiolipin (F) - Hyphae shown in (F) is the one denoted by a square in (D,E). A gap in the DNA (denoted by a square in (A)) is filled with membranes and cardiolipin, forming tubes and vesicle-like structures (G). A crosswall can be seen at this location. Scale bars, 5 μm (A-E), 0.5 μm (F,G), 100 nm (F'-F''').

membranes. Co-staining with DAPI revealed that cardiolipin vesicles obstruct DNA localization. Cardiolipin localized either diffusely, or in the vesicle-like structures, directly corresponding to focal points seen in the hyphae (Figure 5B,F). Such structures were also routinely seen in unstained samples; intracellular formations are therefore not an artifact of staining, which was performed immediately prior to vitrification.

Vesicularization occurs when crosswalls are completed

In addition to membranes, we observed large vesicles adjacent to a newly formed layer of peptidoglycan (PG) in cryo-electron tomograms (Figures 4D, 5G). PG deposition occurred preferentially at one side of the membrane structures. These vesicles were low in electron density as compared to the cytosol, and hence of different composition. In all cases where PG had formed, vesicles were highly disorganized, forming large and small bubble-like structures, seemingly lodged between a membrane and PG layer exerting pressure on one side, and cytosol on the other. Apparently, once PG is deposited, vesicularization of the lipid tubes occurs as excess membrane is gathered, with vesicles coalescing to one another and into the cytoplasmic membrane. The appearance of cardiolipin at the final stage of crosswall formation (as evidenced by our live imaging experiments) likely coincides with vesicularization, with non-bilayer forming cardiolipin acting to help remodel the membrane. This likely marks the final stage in crosswall formation.

FtsZ and cross-membranes colocalize

If membrane structures indeed mark the sites of crosswall formation, that would predict that they may also mark the sites Z-ring formation is initiated. In a previous study, we demonstrated that during sporulation-specific cell division in *Streptomyces*, FtsZ is actively recruited by the membrane-associated divisome component SsgB (Willemse *et al.* 2011). Interestingly, though the *S. coelicolor* *ssgB* mutant does not form sporulation septa, cross-membranes do still form in vegetative hyphae (Figure 6A), similarly to the wild-type (Figure 6B) (see also (Willemse *et al.* 2011)). However, in the *ftsZ* mutant, cross-membranes are missing or scarce (Figure 6C), suggesting that membrane localization is largely dependent on FtsZ, and occurs at a greatly decreased rate in the absence of FtsZ. Immunofluorescence microscopy allowed visualization of membranes in a strain harboring FtsZ-eGFP, revealing complete colocalization of FtsZ and phospholipids and further demonstrating their interdependence (Figure 6D). Attempts to covisualize FtsZ-eGFP and cross-membranes in CLEM experiments were as yet unsuccessful, as visualizing eGFP in the cryo-fluorescent stage is not yet possible, and hampered by the green autofluorescence of *Streptomyces* hyphae.

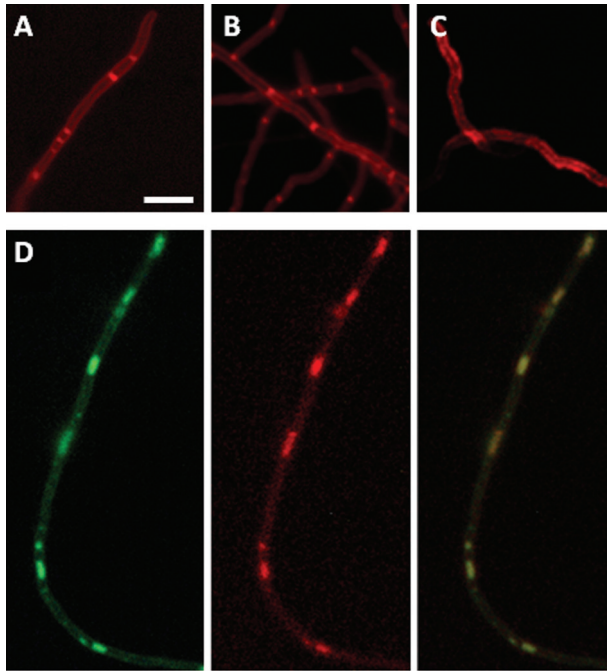


Figure 6. Membrane localization depends on FtsZ.

In an *ssgB* deletion strain of M145, which does not form sporulation septa in aerial hyphae, cross-membranes still occur (A) in the vegetative mycelium, as evidenced by staining with FM5-95. These membranes are similar to those that form in wild type hyphae (B), though *SsgB* is not present to bind FtsZ at the sites of septation. In the absence of FtsZ, however, cross-membranes no longer form (C). Colocalization of FtsZ-eGFP (green), with FM5-95 (red) indicates interaction between FtsZ and membranes (D). An overlay of both channels is also shown. Scale bar, 5 μm .

DISCUSSION

The accurate localization of the cell division machinery in time and space is a tightly controlled process that is critical for growth and survival. The canonical view of bacterial cell division is binary fission, which involves finding the precise mid-cell position and coordinating septum synthesis with chromosome replication and segregation to avoid damage to the DNA (Harry *et al.* 2006). The bulk of the experimental information available to us today comes from genetic and biochemical experiments, combined with fluorescence microscopy-based cell biology. Using these techniques, the Min, SulA and NOC negative control systems were discovered, as well as a large number of septum localizing and stabilizing proteins, including FtsA, ZipA, ZapA and SepF (Romberg and Levin 2003; Goehring and Beckwith 2005; Harry *et al.* 2006). All of these proteins are absent in *Streptomyces*, which is readily explained by the absence of a mid-cell reference point in the long multinucleoid hyphae. While sporulation-specific cell division requires a divisome that is highly similar to that in other bacteria, crosswalls require FtsZ and to a lesser extent FtsQ, but their formation is not hampered by the absence of FtsI or FtsW, peptidoglycan-synthesizing proteins in *E. coli*, suggesting that an entirely different mechanism is in place. Using cryo-electron tomography, which allows the visualization of intracellular structures in three dimensions, we discovered an important if not essential role for intracellular membranes in division-site selection and nucleoid occlusion in *Streptomyces* vegetative hyphae. These cross-membranes create a DNA-free zone during the initiation of division, allowing crosswall formation inside these membrane-protected areas. Thus our work for the first time provides insight into the mechanism of nucleoid occlusion in streptomycetes. Furthermore, fluorescence and time-lapsed microscopy demonstrated that the membranes form dynamically and play a major role in the localization of FtsZ. We previously showed that the SsgB protein recruits FtsZ during sporulation-specific cell division in aerial hyphae (Willemse *et al.* 2011), but like for many cell division genes, null mutants of *ssgB* were apparently not affected in crosswall formation. Indeed, during vegetative cell division lipids and FtsZ fully colocalize, suggesting that this is how FtsZ is recruited to septum sites in vegetative hyphae.

Live imaging of young vegetative hyphae grown in a medium containing FM5-95 and NAO demonstrated that localization of P_{GL} and CL-enriched lipids is remarkably rapid and dynamic. After roughly 12 hours of growth, cardiolipin was seen propagating through the vegetative hyphae and localizing diffusely prior to P_{GL} localization, suggesting that cardiolipin is required to recruit membrane synthases to areas where membrane deposition should occur. Some of the larger membranes then created cross-hyphal structures, expanded,

and then began to dissolve, accompanied by renewed CL localization. Examining these formations using CLEM revealed large tubular membrane structures forming in hyphae, completely obstructing DNA localization, and vesicular-like CL deposits associated with the membranes during late stages of division. Thus, by staining for specific membrane components, we may for the first time have identified the structures previously seen in early transmission electron micrographs of *Streptomyces* hyphae and dubbed ‘mesosomes’ (Glauert and Hopwood 1959; Stuart 1959; Glauert and Hopwood 1960), and which have also been seen in other bacteria (Reusch and Burger 1973). Researchers hypothesized that proliferation of membranes plays a role in enhancing enzymatic activity and is involved in crosswall formation, but the so-called mesosomes were later taken to be an artifact of fixation (Nanninga 1971; Higgins *et al.* 1976; Silva *et al.* 1976). We show that intracellular membranes in fact exist and are prevalent in early vegetative hyphae, playing an important role in cell division.

Although large cross-membranes have a clear role in vegetative cell division, the abundance of lipid structures localized in small blebs adjacent to the cell wall or at apical sites - in addition to those delimiting the hyphae. This implies that lipid structures could also be involved in other cellular functions. Tip-localizing membranes likely tether DivIVA and the tip growth complex, analogous to the fungal Spitzenkörper (Steinberg 2007), and membranes at the sides of hyphae may be actively involved in localizing the secretome (involving the Tat or Sec secretion machineries).

Based on these results, we propose a model of membrane formation in which small membrane tubes found at the hyphal wall either grow in size to enable crosswall formation, or resorb back and disappear (Figure 7). Those that grow in size at sites of crosswall formation coalesce in an asymmetric fashion, fusing from one side of a hyphal wall across to the other. This change of conformation decreases the channel connecting neighboring hyphal compartments, until complete convergence of membrane is seen. Coalescing membranes not only localize a large amount of FtsZ, but also form a barrier to prevent damage to chromosomal DNA as evidenced by the complete gap in DAPI fluorescence whenever cross-membranes are seen. The membranes seem therefore to mediate nucleoid occlusion during crosswall formation. DNA can still be seen passing through this channel at late stages of constriction, until at some point it no longer can pass, and a membrane-filled, chromosome-free area is formed. Our high resolution three-dimensional tomograms support the notion that the structure of the chromosomes controls division site selection (Mannik *et al.* 2012).

Coupled transcription-translation-insertion (transertion) of proteins from segregating chromosomes creates heterogeneous phospholipid domains at the cell centre which allow

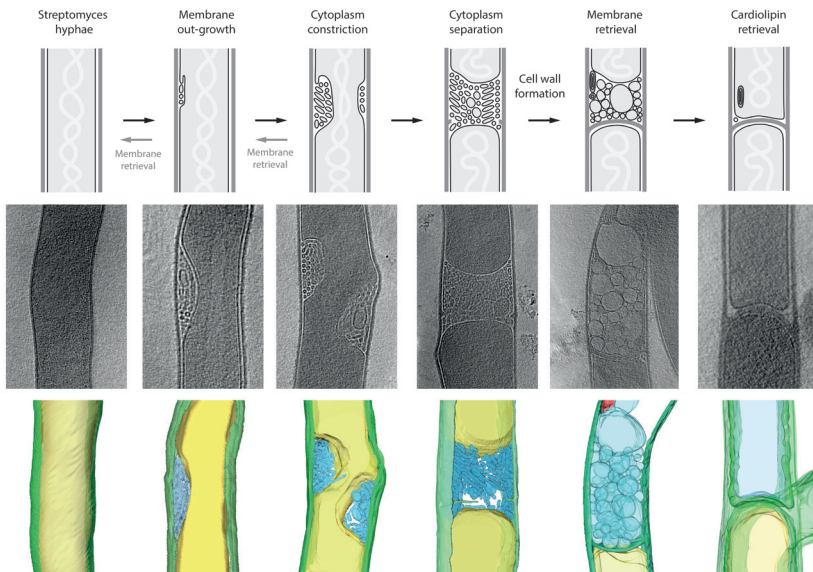


Figure 7. Model to explain crosswall formation in *Streptomyces* vegetative hyphae.

Initially, during early growth, no intracellular membranes can be seen. Chromosomal DNA can be found uncondensed within hyphae (gray helix). Small membrane blebs at the sides of hyphae appear and disappear continuously (gray arrow). Those that grow, constrict the hyphae, including the DNA, from one side. When membranes have completely converged, the DNA is obstructed. At this point, cell wall deposition can occur. A crosswall forms, and membrane tubes disintegrate into small vesicles that coalesce into larger vesicles. These vesicles eventually coalesce with the cytoplasmic membrane, while simultaneously cardioliolip rich membrane vesicles appear. A fully formed crosswall remains.

membranes to extend into the cytoplasm (Norris *et al.* 2004). The rapid changes observed in membrane formation are likely necessary to create an optimal phospholipid composition - with suitable membrane potential and curvature - for protein recruitment (Matsumoto *et al.* 2006). We show that these membrane tubes are substrate for the dynamic assembly and disassembly of FtsZ. The colocalization of FtsZ and membranes in vegetative hyphae supports a two-way relationship, whereby membranes create an optimal environment for FtsZ localization, while at the same time FtsZ is required for cross-membrane formation. This correlates to observations in *B. subtilis*, where localization of phospholipid synthases to cardioliolip and phosphatidylethanolamine-rich septa also depends on FtsZ (Nishibori *et al.* 2005). Other membrane-associated proteins and phospholipids show the same synergy: overproduction of the proteins results in a compensatory overproduction of membrane,

enriched with the phospholipid composition preferred by the overexpressed protein (Arechaga *et al.* 2000; van den Brink-van der Laan *et al.* 2003; Eriksson *et al.* 2009). In these cells, similar membrane structures have also been observed at high resolution. It is unclear how exactly in *B. subtilis* FtsZ interacts with membranes to induce tube formation, as all proteins which interact with a membrane bilayer do so via an amphipathic α -helix which is missing in FtsZ (Norris *et al.* 2004). It has been hypothesized that C-terminus residues can make intrasubunit contacts in an FtsZ protofilament, or that these residues may extend to make a contact to the next adjacent subunit in a protofilament fostering intersubunit interaction (McCormick 2009). Nevertheless, *in vitro* studies have clearly demonstrated FtsZ interaction with phospholipid structures: FtsZ polymerization is facilitated by cationic lipids (Erickson *et al.* 1996; Lu *et al.* 2001) and in a phosphatidylethanolamine monolayer system, FtsZ can create large rings of up to 500 nm in diameter (the upper limit of a *Streptomyces* vegetative hypha) as well as linear polymers of several microns (Alexandre *et al.* 2002; Lafontaine *et al.* 2007). These observations directly connect to the colocalization of (and likely interaction between) FtsZ and the membrane assemblies we observe in vegetative hyphae.

Sporulation-specific cell division in *Streptomyces* is positively controlled, mediated by SsgA and SsgB, whereby SsgB directly recruits FtsZ to division sites and stimulates its polymerization (Willemse *et al.* 2011). Overproduction of the cell division activator protein SsgA enforces a physical change of vegetative hyphae to structures much more resembling sporogenic aerial hyphae (van Wezel *et al.* 2000a; van Wezel *et al.* 2006). Sporulation-specific cell division is mechanistically similar to binary fission in e.g. *Bacillus*, except that the control of septum-site localization requires different proteins (Jakimowicz and van Wezel 2012). Conversely, vegetative crosswalls do not result in cell fission, and these structures do not depend on *ssgA* or *ssgB*. Remarkably, the same is true for mutants lacking the canonical divisome proteins *ftsI* or *ftsW*, required for septal PG synthesis in most bacteria. Perhaps because crosswalls consist of only a single layer of PG, and not the thicker double-layer which is present in sporulation septa, crosswall formation is an entirely different process as compared to sporulation-specific cell division in aerial hyphae. As discussed above, FtsZ and membranes have a synergistic relationship, and only very few cross-membranes occur in *ftsZ* null mutants of *S. coelicolor*. This apparently correlates to the fact that in rare occasions crosswalls are formed in the same *ftsZ* null mutants (McCormick *et al.* 1994). It is likely that in these cases, crosswalls are the result of fortuitous conditions in which membranes converge unaided and septum synthesis results if the required SEDS and PBP proteins are present.

An important question that needs to be addressed in the future is what controls the spectacular intracellular membranes in *Streptomyces*. Candidate proteins are dynamins and flotillins. Dynamins are GTPases which mechanochemically interact with lipids to assist in membrane remodeling, and two dynamin homologues exist in *Streptomyces* (SCO2684 and SCO2685 in *S. coelicolor*). Flotillins are associated with lipid rafts in eukaryotes and characterized by the SPFH (Stomatin, Prohibitin, Flotillin and HflK/C) domain of unknown function and extended heptad repeat regions (Hinderhofer *et al.* 2009). *S. coelicolor* encodes for at least six flotillin-domain proteins. In *B. subtilis*, the dynamin ortholog DynA plays a role in cell division, colocalizing with FtsZ and affecting Z-ring formation, while flotillin generates a local environment favoring membrane curvature, perhaps assisting in the recruitment of cell division proteins to the division site (Dempwolff *et al.* 2012). It would be interesting to see what role dynamin and flotillin play in the formation of crosswalls, and these proteins are good candidates for generating the membrane tubes that are favored by FtsZ.

Taken together, our work provides evidence that intracellular membranes play a critical role during *Streptomyces* vegetative growth, assisting in positioning of the division machinery in the branching, filamentous hyphae, in the absence of canonical control systems such as Min and Noc/SlmA. We believe that membrane-mediated division is not unique to *Streptomyces*. In fact, though membrane structures are not always evident in high resolution images of *E. coli* and *B. subtilis*, studies show they also play a critical role in the cell division of these organisms. *E. coli* and *B. subtilis* cells compromised in lipid production produce long filamentous cells, indicative of defective cell division (Mileykovskaya *et al.* 1998; Salzberg and Helmann 2008). In *E. coli* cells that are unable to produce phosphatidylethanolamines, FtsZ spirals and ladders are formed not dissimilar to those seen in *Streptomyces* hyphae, but cell constriction does not take place. This aberrant non-fission type of cell division is reminiscent of crosswall formation. It is likely that variations in lipid composition correlate to the different lifestyles of microorganisms and the approaches they have evolved to ensure the correct timing and localization of cell division. It will be interesting to see whether the intracellular membranes implicated in *E. coli* and *B. subtilis* division function in a similar manner as the cross-membranes in *Streptomyces*, and perhaps also play a role in triggering divisome formation, e.g. by activating Z-ring formation. Regardless of how widespread cross-membranes are, the next step will be to discover how their formation is initiated and what dictates their function and dynamics.

EXPERIMENTAL PROCEDURES

Bacterial strains and constructs

E. coli strains were routinely grown in Luria-Bertani medium. *S. coelicolor* and *S. albus* strains were grown on R2YE agar or soya flour mannitol (SFM) agar. Liquid cultures were grown in a 50:50 mixture of tryptone soya broth (TSB; Oxoid) and yeast extract-malt extract (YEME), or 2YT medium supplemented with 10% sucrose. All of the growth medium recipes used are those of (Kieser *et al.* 2000). For fluorescence microscopy, samples from liquid cultures were spotted onto a glass microscope slide before microscopy analysis. Images of vegetative hyphae from solid growth samples were collected from samples that had been inoculated at the acute-angle junction of coverslips inserted at a 45° angle in SFM agar plates.

Strains used in this study include *S. coelicolor* M145 (Kieser *et al.* 2000), obtained from the John Innes Centre strain collection, the *S. albus* subspecies *albus* G. ATCC 25426, as well as strains GSB1 (M145 Δ *ssgB* (:::aacC4)) (Keijser *et al.* 2003) and K202 (M145 + KF41) (Grantcharova *et al.* 2005).

Microscopy

Fluorescence microscopy

Fluorescence and corresponding light micrographs were obtained with a Zeiss Axioscope A1 upright fluorescence microscope (with an AxioCam Mrc5 camera at a resolution of 37.5 nm/pixel), with, for the green channel, 470- to 490-nm excitation and 515 long-pass detection; and for the red channel, 530- to 550-nm excitation and 590 long-pass detection. The green fluorescent images were created using 470/40-nm bandpass excitation and 525/50 bandpass detection; for the red channel, 550/25-nm bandpass excitation and 605/70 bandpass detection were used. For staining of the cell wall (peptidoglycan), we used TRITC-WGA; for membrane staining, we used FM5-95 (both obtained from Molecular Probes). All images were background-corrected, setting the signal outside the hyphae to 0 to obtain a sufficiently dark background. These corrections were made using Adobe Photoshop CS5.

Time-lapsed (live) imaging

Uncoated μ -Slide 2 \times 9 dishes (Ibidi GmbH) were placed at an angle and the central wells semi-filled with SFM medium containing spore solution as well as FM5-95 and NAO stains. The remaining wells were filled with water to maintain humidity in the slide chamber, and the lid was left partially open, allowing for gas exchange, sealed off by two layers of Parafilm to prevent drying of the medium. Samples were incubated at 30 °C in the microscope stage

overnight and imaged with a Zeiss Observer A1 with a Hamamatsu EM-CCD C9100-02 camera. Images were taken with 10 min intervals for 60 min.

Sample preparation for electron microscopy

A small drop (3 μ L) of *Streptomyces* liquid culture was applied to EM grids and vitrified using a vitrobot Mark IV (FEI Company, USA) operated at 22 °C and 100% humidity using 1-2 seconds blotting. Subsequently, the cells were cryo-fixed by plunging into a liquid ethane/propane mixture. Plunge-frozen grids were stored in liquid nitrogen until further use. For cryo-electron tomography, 15 nm colloidal gold particles coupled to protein A (CMRC, Utrecht, The Netherlands) were added to samples as fiducial markers. For correlative light and microscopy work, finder grids were used and samples were stained with FM5-95, NAO and/or DAPI directly before sample application to the grid and subsequent plunge freezing.

Electron microscopy

Cryo electron tomography was performed on a Tecnai 20 FEG operated at 200 kV and a Titan Krios operated at 300 keV (FEI Company). Images were recorded using Explore 3D software on a 2k x 2k camera mounted behind a GIF energy filter (Gatan) operated at a slit width of 20 eV. Cryo electron tomograms of membrane vesicles were recorded with 2° tilt steps between -60° to +60° at a defocus of -5 μ m, at magnifications between 1850x (6.6 nm pixels) and 8000x (1.64 nm pixel size).

Cryo-correlative light and electron microscopy

Plunge-frozen grids containing fluorescently labeled *Streptomyces* were imaged using a fluorescence microscope equipped with a THMS600 or CMS196 cryo light microscope stage (Linkam, Surrey, UK), in conjunction with a Leitz DMRB (Leica, Wetzlar, Germany), with a 100x dry objective with a working distance of 4.7mm and a numerical aperture (NA) of 0.75. Digital images were recorded with a Leica DFC350FX CCD camera. Following cryo-FM imaging, sample grids were stored in liquid nitrogen until they were used for cryo-EM.

Image analysis and visualization

Tomographic tilt series were processed using IMOD version 4.5 (Kremer *et al.* 1996). Projection images were preprocessed by hot pixel removal and rough alignment by cross-correlation. Final alignment was done using fiducial gold markers. The tomograms were obtained using a weighted back-projection or a simultaneous iterative reconstruction technique. Cryo-electron tomograms were Fourier filtered and denoised with a non-linear

anisotropic diffusion (Frangakis and Hegerl 2001) to enhance the visibility of structures. For 3D surface rendering, the tomographic volumes were imported into AMIRA (FEI) for further processing and representation.