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Subcompartmentalization by cross-membranes during early growth of *Streptomyces* hyphae

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Bacteria of the genus *Streptomyces* are a model system for bacterial multicellularity. Their mycelial life style involves the formation of long multinucleated hyphae during vegetative growth, with occasional cross-walls separating long compartments. Reproduction occurs by specialized aerial hyphae, which differentiate into chains of uninucleoid spores. While the tubulin-like FtsZ protein is required for the formation of all peptidoglycan-based septa in *Streptomyces*, canonical divisome-dependent cell division only occurs during sporulation. Here we report extensive subcompartmentalization in young vegetative hyphae of *Streptomyces coelicolor*, whereby 1 μm compartments are formed by nucleic acid stain-impermeable barriers. These barriers possess the permeability properties of membranes and at least some of them are cross-membranes without detectable peptidoglycan. Z-ladders form during the early growth, but cross-membrane formation does not depend on FtsZ. Thus, a new level of hyphal organization is presented involving unprecedented high-frequency compartmentalization, which changes the old dogma that *Streptomyces* vegetative hyphae have scarce compartmentalization.
Streptomyces are filamentous Gram-positive bacteria that are of great importance for biotechnology given their ability to produce a large array of natural products, including antibiotics, anticancer agents and immunosuppressants, as well as a plethora of industrial enzymes. 

The *Streptomyces* life cycle has largely been studied during the growth of surface-grown cultures along the transverse axis of the plate (Fig. 1). The life cycle starts with the germination of a spore, which expands out via tip growth and hyphal branching to form a vegetative mycelium consisting of multinucleate compartments. When dispersal is required, for example, after nutrient depletion, the vegetative mycelium eventually differentiates into a new so-called aerial mycelium, which grows into the air. The aerial hyphae are also initially multinucleated, but these eventually develop sporogenic structures that differentiate into chains of unigenomic spores. The lysis of the substrate mycelium and, later, the early aerial mycelium, exhibits the hallmarks of programmed cell death (PCD), with the involvement of specific lytic enzymes (nucleases, proteases and muramidases). New features affecting early development have been described over the last decade. An early compartmentalized mycelium (MI) undergoes an early PCD-like process affecting the substrate and aerial hyphae. Remarkably, live and dying cells are alternately observed in the MI hyphae. The lifespan of this young mycelium is very short under laboratory conditions, but it is likely the predominant mycelium in cultures grown under natural conditions, such as in non-amended soils. The substrate and aerial hyphae in the MI phase are physiologically different from those in the MI phase. MI corresponds to the vegetative mycelium, whereas the substrate and aerial mycelia are the reproductive stages driving towards sporulation. Secondary metabolism is typically restricted to the MI phase.

The study of cell division in *Streptomyces* has primarily focused on sporulation-specific cell division, in which ladders of Z-rings are formed, resulting in chains of spores. In contrast, during vegetative growth, the hyphae are compartmentalized by occasional cross-walls, which delimit adjacent elongated compartments containing multiple copies of the chromosome. Consequently, streptomyces are rare examples of multicellular bacteria. During both sporulation and vegetative growth, the GTPase FtsZ polymerizes to form a dynamic ring-like structure known as the Z-ring. Surprisingly, in the model organism *Streptomyces coelicolor* A3(2), ftsZ is required for cell division (and thus for sporulation) but not for growth, and the deletion of ftsZ results in hyphae that are devoid of septa. The process of vegetative cell division differs mechanistically from canonical division, as illustrated by the fact that many of the other canonical cell division genes (for example, ftsI, ftsL and ftsW) are required for sporation but not for cross-wall formation. Recently, a novel mechanism of cell division was established in vegetative hyphae of *Streptomyces*, based on membranous structures instead of peptidoglycan-based cross-walls. So far, the mechanisms of cell division and hyphal compartmentalization during early vegetative growth (MI stage) have been poorly characterized. Here we provide insights into the ultrastructure of MI hyphae, the regulation of MI compartmentalization, and the kinetics of membrane permeability alteration during PCD in *Streptomyces*. Our data also reveal a surprisingly high frequency of subcompartmentalization of the MI hyphae by cross-membranes.

**Results**

**MI hyphae exhibit differential membrane permeability.** PCD leads to changes in the membrane permeability of cells and to the alternation of propidium iodide (PI) membrane-permeable and non-permeable cellular segments in the same continuous hyphae (Fig. 2a) in the same continuous hyphae (compare fluorescence and phase-contrast images in Supplementary Fig. 1). The average size of MI segments demonstrating differences in permeability was 1.1 ± 0.38 and 0.9 ± 0.25 for PI- and SYTO9-stained segments, respectively, and compartmentalization affected 100% of the analysed hyphae (Fig. 2a; Supplementary Fig. 1). In addition to SYTO9 and PI, we used the non-permeable nucleic acid stain YOPRO-1, which selectively stains eukaryotic apoptotic cells. YOPRO-1 permits the visualization of cells with altered selective permeability that have not yet undergone complete lysis and are therefore not stained by PI. We applied this new technique here to determine whether partial lysis (as observed in eukaryotic apoptotic cells) also occurs in *Streptomyces*. Cells that were stained with PI were also stained with YOPRO-1 (Fig. 2b), whereas live cells were not stained (compare Fig. 2a with Fig. 2b). However, when the samples were processed for microscopy in a more rapid manner (within seconds instead of minutes), the dying cells in the MI hyphae were stained only with YOPRO-1 and not with PI (Supplementary Fig. 2), resembling eukaryotic apoptosis. The average size of the segments stained with SYTO9, PI, and YOPRO-1 at the MI stage was 1.1 ± 0.41 µm (Fig. 2a,b), similar to the spacing observed during sporulation-specific compartmentalization (0.99 ± 0.16 µm) (Fig. 2e). At later time points (transition MI-MII, 18 h), SYTO9-stained cells (live cells) grew as multinucleated compartments, and the average compartment size increased from 1.0 µm (Fig. 2a, b, curves in black) to 1.5 ± 0.55 µm (Fig. 2c, curves in red). Dying cells were no longer stained with either PI or YOPRO-1, most likely due to the complete DNA degradation, but compartmentalization was still observed, and the average length of these unstained segments (18 h; average size of 0.91 ± 0.17 µm) (Fig. 2c) was the same as that observed in early dying cells stained with PI (Fig. 2a,b). The regular pattern of cellular segments with differential, alternating permeabilities to PI and YOPRO-1 in the same hyphae indicates the existence of permeability barriers to these two vital stains separating cellular segments in the MI hyphae.
A regular pattern of permeable and non-permeable cells was not observed during the MI stage, once the dying MI cells disintegrated, while live cells grew out to form non-septated multinucleated hyphae (Fig. 2d). This indicates that the regular pattern of YOPRO-1/PI staining observed in the MI hyphae is attributable to the nature of this mycelium, which differs from that of the MI hyphae.

During the last developmental stages, the hyphae compartmentalized into spores with an average diameter of 0.99 ± 0.16 μm and a size distribution comparable to that of the MI segments (compare Fig. 2a–c with Fig. 2e). Sporulating septa consisted of very thick cell walls that were not stained by nucleic acid-binding stains and were observed as unstained regions (Fig. 2e; notice that the length of the unstained regions

Figure 2 | Confocal laser scanning fluorescence microscopy analysis of S. coelicolor growing on GYM agar. (a) SYTO9 (green) and PI (red) staining (MI, 10 h). (b) YOPRO-1 (green) and PI (red) staining (MI, 10 h). (c) SYTO9-PI staining (transition from MI to MII, 18 h). (d) SYTO9-PI staining and YOPRO-1-PI (MII, 48 h). YOPRO-1 and PI were used simultaneously; notice that not all of the hyphae stained with YOPRO-1 were stained with PI. (e) SYTO9-PI staining (spores, 72 h). The scale bars correspond to 8 μm. The arrows in a–c highlight dying cells in ‘MI’ and ‘transition MI-MII’ hyphae. Histograms of the stained and unstained segments are shown. Two distributions were observed: one from the MI stained and unstained segments in a and b and from the unstained segments in c (black lines), and the second from the living segments stained with SYTO9, which begin to enlarge as multinucleated hyphae in c (red lines).
corresponding to thick cell walls is much shorter than that of the unstained regions corresponding to MI cellular segments).

Visualization of compartmentalization by electron microscopy.

To obtain detailed insight into the discontinuities along the MI mycelium (12 h), the hyphae were analysed by performing cryo-correlative light/electron microscopy (cryo-CLEM; Fig. 3a–d) and high-pressure freezing and freeze substitution electron microscopy (Fig. 3e,f). Two types of cells/compartments were observed to alternate and contained either weakly FM5-95-stained and electron-dense cytoplasm, or strongly FM5-95-stained and electron-lucent cytoplasms (Fig. 3b–d). In the latter, vesicles and membrane invaginations were frequently observed (arrows in Fig. 3). Samples for cryo-CLEM were flash-frozen within milliseconds in liquid ethane, without chemical fixation, minimizing the possibility that the membranous structures observed may have been the result of chemical artifacts.

Two types of barriers delimiting MI cellular segments were detected: cross-membranes without detectable peptidoglycan under the tested conditions (Fig. 3e); and peptidoglycan-based cross-walls (Fig. 3f).

FtsZ expression and Z-ring formation in MI hyphae. MI is a transitory stage in laboratory cultures, and has been ignored in most studies examining *Streptomyces* development. However, previous transcriptome analysis of RNA isolated from young hyphae of *S. coelicolor* suggested that *ftsZ* is overexpressed at this stage of the life cycle. We performed quantitative reverse transcription–PCR (qRT–PCR) analysis of RNA isolated from solid-grown cultures on GYM agar plates to show that *ftsZ* transcript levels are higher after spore germination (MI, 15 h) and are even higher those observed during sporulation (that is, 63–70 h; solid line in Fig. 4a). The lowest *ftsZ* transcript levels were observed at ~39 h, corresponding to the formation of multinucleated substrate/pre-sporulating aerial hyphae. *ftsZ* gene expression correlates well with FtsZ protein abundance (dashed line Fig. 4a), as quantified by tandem mass tag (TMT) protein labelling and LC-MS/MS. FtsZ was more abundant at the MI stage than during sporulation.

There is a strong correlation between the frequency of septation—and thus compartment sizes—and the expression level of FtsZ: high levels of FtsZ are required to support sporulation-specific cell division. Therefore, we examined whether high *ftsZ* transcription and protein levels also correspond to the septation frequency during the earliest stages of growth after spore germination. The cellular localization of FtsZ-eGFP was analysed by performing confocal microscopy with *S. coelicolor* FM145, a derivative of the model strain M145 exhibiting low autofluorescence (Fig. 4b,c; Supplementary Fig. 1 and Supplementary Movie 1). Z-ring formation begins with the development of dynamic spiral-like structures of FtsZ, which are visualized as transitory spots that move rapidly inside the mycelium, and do not cross the entire diameter of the hypha (arrowheads in Fig. 4b, Supplementary Movie 1). These spots ultimately form Z-rings, which are more stable and cross the entire diameter of the hypha (arrows in Fig. 4b, Supplementary Movie 1). During the early MI stage, Z-rings formed asynchronously during MI growth (Supplementary Movie 1). Z-rings disappear once the septa are complete; consequently, they could not be observed at the same developmental time point in a single image (Supplementary Movie 1). However, the maximum projection of images acquired during an overnight time-lapse experiment (Fig. 4c and Supplementary Fig. 1) revealed that the Z-rings in the Z-ladders were spaced at an average of 1.1 ± 0.48 μm in all of the MI hyphae (Fig. 4d and Supplementary Fig. 1). This spacing and regularity are highly similar to that observed during sporulation-specific cell division.

To analyse the relationship between the Z-rings observed in *S. coelicolor* FM145 expressing FtsZ-eGFP as well as the differences in the PI permeability observed in the MI, both techniques were combined, staining the *S. coelicolor* FM145 strain expressing FtsZ-eGFP with PI. Z-rings are transitory, and PI permeability barriers can only be visualized when dying cells (stained with PI) alternate with living cells (not stained with PI). Consequently, it was difficult to detect Z-rings at a discrete time...
lipophilic membrane colourant FM4-64 was used to stain hyphae (used for normalization and consequently has a value of 1 and an s.d. of 0. All abundance values were significantly different with respect to the 15-h sample (dashed line). The average values of three biological replicates are presented (with SD). The MI sample (15 h in transcriptomics, 16 h in proteomics) was FtsZ at early developmental time points of *S. coelicolor*.

Membrane permeability of the *ftsZ* mutant. SYTO9/PI and YOPRO-1/PI staining were applied to the *ftsZ* null mutant HU133 (McCormick *et al.*). Surprisingly, the *ftsZ* mutant exhibited an alternating pattern of PI/YOPRO-1 permeable and impermeable segments comparable to that of the parental strain (Fig. 5). This pattern was observed at all time points in the mutant (the images shown in Fig. 5 correspond to a 48-h culture). The average size of the live segments, that is, those stained with SYTO9 but not with PI or YOPRO-1, was 0.85 μm ± 0.41 and 0.81 μm ± 0.36, respectively (red curves in Fig. 5), comparable to the sizes observed in the parental strain (see above and Fig. 2). As discussed below, the average length of dying cells, thta is, those stained with PI and YOPRO-1, was 1.83 μm ± 1.27 and 2.03 μm ± 1.3, respectively, with a maximum length of 6.12 μm (curves in black in Fig. 5), which was double the length observed in the parental strain. This pattern of PI/YOPRO1-permeable and -impermeable segments alternating in the same hypha was present in 100% of the mycelium (Supplementary Fig. 4).

Membrane and cell wall staining of *Streptomyces* hyphae. The lipophilic membrane colourant FM4-64 was used to stain hyphae of *S. coelicolor* M145 at the MI stage (Fig. 6a–d), and this staining was compared with HU133 (*ftsZ* mutant; Fig. 6e,f). As previously reported7, FM4-64 stained the *S. coelicolor* hyphae heterogeneously, and only a fraction of the hyphae were stained (compare the hyphae observed by phase-contrast with those stained with FM4-64 in Fig. 6a). Two types of internal membranes were detected: sharp cross-membranes continuous with the extracellular membrane and delimiting cellular segments (arrows in Fig. 6b) and large spots stained with FM4-64 (arrowhead in Fig. 6c). At the MI stage, some hyphae exhibited a regular pattern of cross-membranes, and/or FM4-64 stained spots (Fig. 6d), but this pattern was not observed in all hyphae. As discussed below, FM4-64 could not be used to quantify the proportion of cross-membranes in the MI hyphae, because it does not stain all membranes under the conditions employed in this work. FM4-64 also stained internal membranes in the *ftsZ* null mutant (Fig. 6e,f). The two types of internal membranes described above were observed, but with an obvious difference: the large spots stained with FM4-64 were much larger in the *ftsZ* null mutant than in the parental strain (compare Fig. 6c with Fig. 6e).

Cell wall stains such as fluo-wheat germ agglutinin (fluow-WGA) and boron-dipyromethene-vancomycin (BODIPY-vancomycin) stained 100% of the hyphae observed (Fig. 6g,j). Fluo-WGA stained the complete external hyphal walls and the cross-walls of the MI septa in the *S. coelicolor* parental strain (Fig. 6h). BODIPY-vancomycin stains nascent peptidoglycan25, and most of the cell walls were not visualized with this stain (Fig. 6k,l). D-amino acid pulse labelling of cell walls26 gave the
Figure 5 | Confocal laser scanning fluorescence microscopy analysis of the ftsZ mutant HU133. (a) SYTO9 (green) and PI (red) staining (48 h). (b) YOPRO-1 (green) and PI (red) staining (48 h). Histograms of the stained and unstained segments are shown. Two distributions were observed: one from viable segments stained with SYTO9 and not stained with YOPRO-1 or PI (red lines); the second from dying cells stained with YOPRO-1 and/or PI (black lines). The scale bars correspond to 8 μm.

Figure 6 | Membrane and cell wall staining of S. coelicolor and its ftsZ mutant HU133. (a-f) FM4-64 staining (membranes). (g-i) WGA staining (cell wall). (j-l) BODIPY-vancomycin staining (nascent peptidoglycan). Fluorescent images in a, g and j correspond to the maximum projection 10-μm series overlaid with their respective phase-contrast images, showing 100% of the stained hyphae. Arrows indicate cross-membranes and cross-cell walls. Arrowheads indicate membrane cellular segments filled with membrane vesicles. Scale bars, 4 μm.
same results as those observed for fluo-WGA staining (Supplementary Fig. 5). The frequency of cross-walls stained with all cell wall colourants was lower than the frequency of the PI and YOPRO-1 permeability barriers as previously described (Fig. 2). Specifically, most of the permeability barriers separating PI/YOPRO-1-permeable and -impermeable segments do not have sufficient cell wall to be observed by fluorescence microscopy. The use of fluorescent D-amino acids combined with PI in vivo showed that septa (membranes with thick cell walls) colocalize only with some of the PI permeability barriers (Supplementary Fig. 3). This provides further evidence that, at minimum, the PI permeability barriers colocalizing with cross walls in the MI correspond to cross-membranes. Fluo-WGA, fluorescent D-amino acids or BODIPY-vancomycin did not stain cross walls in the ftsZ null mutant (Fig. 6i,l), as expected for a mutant without cross walls.

S. coelicolor FM145 expressing FtsZ-eGFP was stained with FM4-64 (membrane stain) and HADA (cell wall stain), which indicated that at least a portion of the Z-rings colocalize with cross-walls and/or cross-membranes (Supplementary Fig. 3). As discussed below, Z-rings are transitory, and FM4-64 does not stain all membranes, but colocalization can be detected, providing further evidence that Z-rings may be involved in the formation of cross-membranes.

Compartmentalization correlates with protoplast formation. The ability to form protoplasts depends on the differentiation stage10, and this feature can be used to distinguish MI-compartmentalized hyphae from MII-multinucleated hyphae because MII hyphae do not form many protoplasts, likely due to the instability of the large protoplasts formed by multinucleated hyphae. We devised a method based on protoplast formation and flow cytometry measurements to quantify the number of protoplasts formed per unit of biomass. Protoplasts formed in high amounts during the MI stage (16 h), whereas their numbers progressively decreased during the MI and MII transition phase, and very few protoplasts were produced at the late MII stage (48 h) (Fig. 7a). The average protoplast size was 2.2 ± 1.13 μm, and there were no protoplasts with diameters larger than 5–6 μm (Fig. 7b; Supplementary Fig. 6). During the sporulation stages, unigenic spores were readily obtained, but no protoplasts were observed (data not shown) because Streptomyces spores are resistant to lysozyme.28 Protoplast formation correlated well with the compartmentalization observed in the MI but not MII hyphae (see above), thus providing a method to assess the degree of compartmentalization of the hyphae and to distinguish between the MI and MII phases.

The ftsZ null mutant (S. coelicolor HU133) formed protoplasts in numbers and with an average diameter (1.98 ± 0.8; Fig. 7c,d; Supplementary Fig. 6) comparable to those of the S. coelicolor parental strain at the MI stage. The S. coelicolor ftsZ null mutant grows very slowly, and its growth was therefore not comparable to the parental strain. S. coelicolor HU133 formed protoplasts at all time points (the protoplasts quantified in Fig. 7c,d were obtained from a 48-h culture).

Discussion

The alternation of PI/YOPRO1-permeable/impermeable segments in the MI hyphae demonstrates the existence of barriers impermeable to these viability stains, with the diffusion properties of membranes (outlined in Fig. 8). The frequency of these permeability barriers is much higher than the frequency of septa formed by cross walls. Here we applied cryo-CLEM and FM lipophilic styryl dyes (FM4-64/FM5-95) to reveal the existence of two types of internal membranes, which were not associated with detectable peptidoglycan: cross-membranes continuous with the extracellular membrane that delimit cellular segments and are difficult to be observed (Fig. 3 and Fig. 6b); and vesicles/membrane arrays, that are non-continuous with the extracellular membrane, and are easily visualized by CLEM (Fig. 3 and Fig. 6c). As discussed below, cross-membranes are also found in the ftsZ null mutant, which is not able to produce peptidoglycan-based septa.13 Importantly, recent FRAP and CLEM/cryo-electron tomography experiments performed on liquid-grown mycelia, revealed the existence of impermeable cross-membranes compartmentalizing vegetative hyphae of Streptomyces albus, and their existence was also corroborated in S. coelicolor.16 In the current work, experimentation was performed at the earliest stages of growth (MI stage) in solid-grown cultures of S. coelicolor, with the experiments aimed at quantifying the nature and the extent of hyphal compartmentalization. Our fluorescence and electron microscopy experiments failed to detect 1-μm spacing cross-membranes correlating with the 1-μm spacing permeability barriers observed with PI/YOPRO-1. This is most likely explained by the fact that fluorescence and electron microscopy do not permit the visualization of all Streptomyces membranes, as it happens in other microorganisms. For example, FM4-64 only stains vacuum membranes in yeast29, the inner membrane and membrane domains enriched in basic phospholipids in E. coli,30 the outer membrane in Agrobacterium31 and under the conditions employed in this work, only a fraction of S. coelicolor hyphae (Fig. 6a). Next-generation electron microscopy methodologies, such as CLEM and cryo-electron tomography, are enabling the detection of novel internal structures in bacteria, including membranes, but the existence of further undetected structures cannot be dismissed (reviewed in Jensen et al.32). Interestingly, the use of fluorescent D-amino acids combined with PI in vivo showed that the membranes associated with cross walls (septa), can be detected as...
PI permeability barriers colocalizing with cross walls (Supplementary Fig. 3). PI permeability barriers that are not associated with cross walls most likely also represent cross-membranes. The compartmentalization of MI hyphae correlates with the ability to form stable protoplasts, which again supports the existence of cross-membranes surrounding the compartments that are able to produce protoplasts (Fig. 7a,b). Further work employing new and improved microscopy techniques will be necessary to test whether all permeability barriers to PI and YOPRO-1 correspond to membranes.

In contrast to the cross-membranes delimiting cellular segments, internal membranous structures are easier to contrast against the cytoplasm of the MI hyphae, and were described long ago in S. coelicolor by Glauert and Hopwood33 and in dying cells of S. antibioticus by Miguelez et al.12. However, these authors applied chemical fixation, and thus they could not discard the possibility that these structures were chemical artifacts5; to this day, their discovery has remained unvalidated. Further work is necessary to characterize the biological function of vesicles/membrane invaginations in the MI hyphae, but their observation by cryo-electron microscopy supports the conclusion that they are not chemical artifacts. Celler et al.16 recently observed internal membranous structures in vegetative hyphae using cryo-electron tomography and CLEM. The authors showed that large membrane assemblies are formed creating DNA-free zones, often (but not always) associated with initiation of septum formation, suggesting a role of these structures in protecting the DNA during the onset of vegetative cell division.

Two different types of septa exist in Streptomyces, both of which consist of peptidoglycan and membranes: the cross walls in the substrate and early aerial mycelia and the sporulation septa in sporulating aerial hyphae. Although their formation depends on FtsZ, the localization of the Z-rings is regulated by entirely different mechanisms during these two growth phases14,15,34,35. The compartmentalization of MI hyphae is comparable to that of sporulation-specific cell division, with the formation of ladders of Z-rings, with an average spacing of 1 μm. A proportion of the Z-rings observed in the MI colocalize with cross-membranes, and some colocalize with PI permeability barriers (Supplementary Fig. 3). These results indicate that Z-rings might contribute to the formation of at least some of the permeability barriers separating the 1-μm cellular segments and again suggest that PI permeability barriers correspond to the cross-membranes observed by CLEM. One of the most intriguing peculiarities of Streptomyces cell division is that ftsZ null mutants are viable, resulting in long non-septated branched vegetative hyphae13. Counterintuitively, the hyphae of the ftsZ-null mutant can be fragmented without loss of viability, suggesting that the release of their contents is somehow prevented13. Here we have provided evidence for the existence of cross-membranes in the ftsZ-null mutant (Fig. 6e,f). The formation of membranous- rather than peptidoglycan-based ‘septa’ that are not dependent on FtsZ is so far unique in bacteria. Interestingly, a somewhat analogous case is known in archaea: most Crenarchaea lack ftsZ, but some such as Pyrobaculum islandicum, produce cross-walls that consist of an S-layer rather than peptidoglycan36. Further work should characterize the role of FtsZ (if any) in the formation of cross-membranes and identify any possible differences between the cross-membranes formed in the presence or absence of ftsZ.

In summary, this work provides evidence for the existence of an unprecedented high-frequency compartmentalization in MI hyphae based on cross-membranes. Cross-membranes may have developed to support the multicellular life style of streptomycetes, enabling subcompartmentalization to provide an additional level of organization in the long hyphae. It will be very interesting to determine whether similar membrane-based compartmentalization also exists in other (multicellular) bacteria.

**Methods**

**Strains and media.** Streptomyces coelicolor M145 (ref. 37) was obtained from the John Innes Centre strain collection and its ftsZ null mutant HU133 (ref. 13) was obtained from the Harvard University strain collection. GYM (glucose, yeast and malt)38 was used as the growth medium in both liquid and solid media. Agar plates were used with and without cellophane disks and were inoculated with 100 μl of an inoculum suspension (1 × 107 viable spores ml−1), followed by incubation at 30 °C. The reduced autofluorescence strain S. coelicolor FM145 harbouring a plasmid expressing FtsZ-eGFP was previously described22.

![Figure 8 | Model of compartment formation and PCD in vegetative hyphae of Streptomyces coelicolor.](image-url)
Real-time qRT–PCR. Total RNA was obtained by phenol extraction and using the RNeasy Midi Kit (Qiagen). RNA integrity was verified using a 2100 BioAnalyzer (Agilent). The RNA was resuspended in real-time RT–PCR buffer with the TURBO DNA-free kit (Ambion) to remove possible DNA contamination according to the manufacturer’s instructions. Briefly, 50 μl of 200 ng/ml RNA solution was treated with DNase I at 37 °C for 30 min. The samples were mixed with 0.2 volumes of inactivation reagent, incubated for 5 min at room temperature and recovered by centrifugation.

One microgram of RNA was used as the template for complementary DNA (cDNA) synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s specifications. The primers used for qRT–PCR were obtained from Integrated DNA Technologies. The reactions were performed in a 50-μl volume using 5 μl of cDNA dilution twofold, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM primers in a final volume of 20 μl. Three biological samples were analysed, and control reactions with RNA and water as templates were performed to verify the absence of DNA contamination and primer–dimer formation. The thermal profile was as follows: an initial stage at 50 °C for 2 min, a second stage at 95 °C for 10 min, a third stage of 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and a final dissociation profile of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 1 s. The abundance of transcripts was normalized to the expression of a reference gene. Relative quantification was performed using the ΔCt method39. SCO3878, which encodes the β-chain of DNA polymerase III, was used as an internal control to quantify the relative expression of the target gene employing experimental data. Thus the reported values for the change in FtsZ expression were obtained as described by Frias et al.26.

Membrane staining. The lipophilic styryl dye, N-(3-diethylamino phenylpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM4-64) (Molecular Probes, T-3166) was added directly to the culture medium at a final concentration of 1 mg ml−1 before the plates were poured. This concentration of FM4-64 does not affect growth. Samples were observed under a confocal laser scanning microscope at wavelengths of 550 nm for excitation and 700 nm for emission.

Cell wall staining. Cells were fixed for 15 min at room temperature using PBS (0.14 M NaCl, 2.6 mM KCl, 1.8 mM KH2PO4, and 10 mM Na2HPO4) containing 2.8% paraformaldehyde and 0.0045% glutaraldehyde. Texas Red WGA (Invitrogen W21405) was added at a concentration of 100 mg ml−1 in 2% BSA in PBS and the cells were incubated at room temperature for 3 h. BODIPY-vancomycin (Invitrogen V34850) was used at a concentration of 0.5 μg ml−1 in PBS for 15 min. The samples were washed with PBS and observed under a Leica TCS-SP8 confocal laser scanning microscope at excitation wavelengths of 595/505 and emission wavelengths of 615/513 for WGA and BODIPY-vancomycin, respectively. Fluorescent o-amine (HADA) was used as described by Kuru et al.28. Briefly, the HADA stock solution was prepared in dimethylsulphoxide at a concentration of 100 mM. In the case of the S. Cholerae paracrystals, confocal images were obtained as described by Frias et al.26. The samples were incubated at 30 °C and imaged with a Zeiss Observer confocal microscope. Images were acquired every 45 min for 15 h. Excitation was performed with a 488-nm laser, and detection was performed with a 505–530-nm bandpass filter. To minimize focal drift, the microscope stage and imaging chamber were allowed to equilibrate for 60 min before imaging. Time-lapse images were processed with ImageJ. Z-rings were detected using a Gaussian filter with a Sigma value of 2 followed by an Unsharp Mask filter with a radius of 3 and a mask weight of 0.6. Then, a Find Maxima process with a noise tolerance of 50 was used to obtain binary images of the Z-ring local maxima. Finally, the nearest distance between Z-rings was calculated using the Nearest Neighbour Distance plugin (Nnd; https://icme.hpc.msstate.edu/miawiki/index.php/Nearest_Neighbor_Distances_Calculation_with_ImageJ).

Cryo-correlative light and electron microscopy. For cryo-CLEM, an EM grid was positioned on a Streptomyces culture during growth and was vitrified directly by plunging it into liquid ethane using a Leica EM FE30 cryo light microscope stage (Linkam, Surrey, UK), in conjunction with a Zeiss Axiocam Mr2 Cryo-EM was performed on a Tecnai 20 FEG operated at 200 kV (FEI Company). Images were recorded on a 2k x 2k camera mounted behind a GIF energy filter (Gatan) operated at a slit width of 20 eV.

Viability staining. Culture samples were obtained and processed as previously reported45, for excised cellophane or agar pieces and were stained for a few minutes later. The LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, L-13152) was employed for staining. This kit uses SYTO9 and PI, two DNA-binding colourants. SYTO9 penetrates intact membranes and stains viable cells green, whereas PI only penetrates bacteria with damaged membranes. At the concentrations used in the kit, PI displaces SYTO9 in the cells of Streptomyces with an adjusted for multiple comparisons within the data set. The relative abundance package. Thus the reported values for the change in FtsZ expression were obtained as described by Frias et al.26.

High-pressure freezing and freeze substitution. To observe S. coelicolor cells by transmission electron microscopy (TEM), Epon-embedded thin sections were obtained as described by Fras et al.45. Briefly, bacterial cells were cryo-immobilized as quickly as possible using a Leica EMFac high-pressure freezer (Leica, Vienna, Austria). Frozen samples were freeze-substituted in a Leica EM automatic freeze substitution system (Leica, Vienna, Austria). The substitution was performed in pure acetone containing 2% (wt/vol) osmium tetroxide and 0.1% (wt/vol) uranyl acetate at 90°C for 72 h. The temperature was gradually decreased (5 °C h−1) to 4 °C, held constant for 2 h, and then finally increased to room temperature and recovered for 1 h. The samples were washed for 1 h in acetone at room temperature and infiltrated in a graded series of Epon-acetone mixtures: 1:3 for 2 h and 1:10 for 4 h, before being embedded in fresh Epon and polymerized at 60 °C for 48 h. Ultrathin sections were cut with a Leica UC7 ultramicrotome and mounted on Formvar carbon–nickel–palladium grids with a 200 mesh diameter. Sections were stained with 2% uranyl acetate and lead citrate and examined with a Tecnai Spirit electron microscopy (FEI Company, The Netherlands) at an acceleration voltage of 120 kV.

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Mycelium protoplasting. Protoplasts were obtained according to the method described by Okanishi et al.12,17 and Keser et al.22 with some modifications to ensure that the efficiency of protoplasting was close to 100% (as analysed by observing the disintegration of the hyphae by phase-contrast and confocal microscopy, Supplementary Fig. 6) and that there was no significant loss of protoplasts during manipulations, both of which are critical requirements for reproducible and significant flow cytometry measurements (see below).

Mycelia grown on collophane discs were scraped off, and 60 mg of mycelia (fresh weight) were resuspended in 1.12 ml of buffer P (0.6% TES buffer pH 7.2, 103% sucrose) in a 2-ml Eppendorf tube. Lysozyme was added from a freshly prepared stock at a final concentration of 2 mg ml⁻¹ and incubated for 30 min at 600 rpm and 37°C in an Eppendorf ThermoMixer. Protoplasts were drawn in and out twice in a 1-ml pipette, incubated for an additional 30 min, washed two times by sedimentation (1,000g) and resuspended in buffer P. After the final wash, the protoplasts were resuspended in 500 µl of buffer P.

The original buffer P described by Keser et al.12 included a trace element solution and other salts. These salts interfere with flow cytometry measurements, and consequently, it was necessary to use the modified buffer P described in this work, which only includes TES buffer and sucrose (K₂SO₄, MgCl₂ and the trace element solution) and other salts. These salts interfere with flow cytometry measurements, observing the disintegration of the hyphae by phase-contrast and confocal microscopy, Supplementary Fig. 6) and that there was no significant loss of protoplasts during manipulations, both of which are critical requirements for reproducible and significant flow cytometry measurements (see above).

Flow cytometry measurements were performed using BD Trucount Tubes (reference 340334), containing 500 FL, USA). In all cases, protoplasts from two biological replicates were quantified.

The number of protoplasts per the 5,000–10,000 range, which was close to the number of beads used as a standard.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding authors on request.

References
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Author contributions


Additional information

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