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How sisters grow apart: mycobacterial growth and division

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Abstract

Mycobacterium tuberculosis, which is the aetiological agent of tuberculosis, owes much of its success as a pathogen to its unique cell wall and unusual mechanism of growth, which facilitate its adaptation to the human host and could have a role in clinical latency. Asymmetric growth and division increase population heterogeneity, which may promote antibiotic tolerance and the fitness of single cells. In this Review, we describe the unusual mechanisms of mycobacterial growth, cell wall biogenesis and division, and discuss how these processes might affect the survival of *M. tuberculosis in vivo* and contribute to the persistence of infection.

Mycobacterium tuberculosis is an extraordinarily successful human pathogen that infects up to one-third of the global population[1] and causes one of the most recal-citrant bacterial infections of the modern era. Infection begins with the inhalation of aerosols that contain *M. tuberculosis*, which are primarily taken up by alveolar macrophages, although other host cells can phagocytose the bacterium, including dendritic cells, monocytes and neutrophils[2,3]. A local pro-inflammatory response occurs in the lungs, which results in the recruitment of additional monocytes and lymphocytes[3,4]. The assembly of these cells around infected macrophages generates a cellular mass, known as a granuloma, which is a pathological hallmark of tuberculosis (TB).

Granuloma histology is heterogeneous, even within an individual, and ranges from cellular to caseous to fibrotic lesions[5]. Granulomas are traditionally considered to be an attempt by the host to control the infection, but the ability to restrict mycobacterial proliferation varies[6,7]; for example, caseous granulomas, which contain hypoxic centres[8], often rupture and release bacteria, which are then free to invade the surrounding host cells[4]. Furthermore, *M. tuberculosis* localizes to distinct regions in the granuloma[5], which also contain different subsets of immune cells. Thus, depending on the bacterial location and the immune cells that are present, the inflammatory response is likely to differ between distinct sites of the granuloma[9], which probably affects clearance of the infection. Inside macrophages, *M. tuberculosis* is usually found within phagosomes of variable maturation states[3] and encounters a range of host defences, including phagosome acidification, antimicrobial

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peptides and reactive oxygen species[4]. This range of distinct microenvironments exert discrete selective pressures on individual bacterial cells, which suggests that mycobacteria need to adapt to ensure survival. How is such adaptation achieved? Recent work has shown that mycobacterial cell growth and division produces heterogeneous daughter cells[10–13], which also undergo substantial cell wall remodelling during infection[14–17], possibly enabling the bacterium to withstand the stresses that are encountered during infection.

The proliferation of bacterial cells can be divided into two stages: elongation of the mother cell and division of the elongated mother cell into two daughter cells. In many bacteria, cell division is a symmetric process and produces daughter cells of the same size[18]. However, mycobacteria do not adhere to the ‘one size fits all’ rule and grow and divide in an asymmetric manner, which produces daughter cells of unequal sizes[10–13]. This trait might have been selected for, as cells of different sizes might have distinct survival advantages in the highly variable host environment.

As discussed below, the unique mode of mycobacterial elongation and division produces a population of daughter cells that vary in size, growth rate and cell wall composition[10–13], which functionally diversifies the population. This phenotypic heterogeneity is further increased by cell wall remodelling processes that occur within the host[14–17]. Mycobacteria have an elaborate cell envelope that is comprised of several layers (BOX 1). Each of these layers has different chemical modifications, and the architecture of the cell wall is also moulded by complex regulation. In the host, further remodelling occurs[14–17], which generates a population of cells that differ not only in size and growth properties but also in the composition of their cell walls. This diversity is predicted to increase survival *in vivo* and has the potential to influence disease progression and clinical latency.

In this Review, we discuss the mechanisms and regulation of mycobacterial cell wall biogenesis, cell elongation and cell division. We also describe how these processes produce heterogeneity at the population level and consider the implications of single cell diversity for survival in the host.

Peptidoglycan synthesis and polar growth

Traditional model organisms, such as *Escherichia coli* and *Bacillus subtilis*, are rod-shaped bacilli in which the elongation of a mother cell occurs along the cell body, followed by division at mid-cell to generate two equally sized daughter cells. By contrast, elongation occurs at the cell poles in mycobacteria and results in the production of unequally sized daughter cells (FIG. 1).

Is polar growth symmetric or asymmetric?

All rodshaped bacteria contain two poles: an old pole, which is inherited from the mother cell; and a new pole, which is synthesized during division. In mycobacteria, polar growth results in daughter cells of different sizes, and two hypotheses were recently proposed to explain how this occurs[10,13] (FIG. 1). One model reported that growth is unipolar, owing to the extremely slow growth of the new pole relative to the old pole[10], and it is now recognized that both poles elongate, but at different rates[10,13], thus giving rise to daughter

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cells of different sizes. The second model suggests that the growth rate of the old and new poles is symmetric for most of the cell cycle but that growth predominantly occurs at the old pole after cytokinesis[13], which results in unequally sized daughter cells. The use of different experimental conditions and methods to determine the timing of cell division could account for the deviating observations.

The evidence for the symmetric model was derived from the study of *Mycobacterium smegmatis*, in which polar elongation was measured between successive cytokinesis events[13] (FIG. 1a). As cytokinesis partitions the mother cell by separating the cytosolic contents, the authors reason that it is during this process that the two daughter cells are generated. To visualize cell growth, cells were pulse labelled with a fluorescent amine-reactive dye that stains the cell surface. The dye does not diffuse during cell growth and enables the visualization of newly incorporated, unlabelled cell wall material over time[10,13]. Using this cell surface dye and a fluorescently tagged Wag31 (which is a protein that localizes to the division septum), the authors assessed polar growth in single cells over time, using a microfluidics approach[13]. After cytokinesis (which is detected by the localization of Wag31 to the septum) but before the physical separation ofthe cells, growth occurs predominantly at the old pole. After the physical separation of the cells, both the new and old poles of the daughter cells elongate at the same rate until the following cytokinesis event[13] (FIG. 1a).

In the second model, polar elongation of *M. smegmatis* was measured between successive cell separation events (FIG. 1b). Similarly to the previous study, a microfluidics device was used to monitor single cells that were stained with the fluorescent amine-reactive dye[10]. The marker does not obscure the initiation of cell constriction[10], which indicates the beginning of physical cell separation. Using physical separation as the readout for cell division, the authors found that cells elongate preferentially at the old pole throughout the entire cell cycle (which the authors term unipolar growth), and this was also observed in *M. tuberculosis*[10]. Subsequent studies that have also used the physical separation of daughter cells to define cell division have also shown that polar growth occurs predominantly at the old pole throughout the cell cycle[11,12]. Whether polar elongation is symmetric or asymmetric depends, in part, on whether cell division is defined as cytokinesis[13] or as the physical separation of cells[10]. The use of a fluorescently labelled allele of Wag31 may also mask wild-type elongation, as has recently been reported[19].

However, irrespective of the methods used, both models are in agreement that the cell cycle produces daughter cells of different sizes, which elongate at different rates. Daughter cells that inherit the old pole are larger than their siblings that inherit the new pole, and they also elongate faster after the cells separate[10,13]. Hence, cell size, cell age and the nature of the cell poles (old versus new, which correlates with cell size) determine the elongation rate (which is referred to as velocity in REF 13) of newly divided cells. Thus, although daughter cells are genetically identical, they are phenotypically different. This programme of cell growth is in marked contrast to *E. coli*[10,12], and even to *Corynebacterium glutamicum*, which undergoes polar growth but produces daughter cells of a similar size and with similar elongation rates[11]. Asymmetric growth leads to intrinsic heterogeneity in the mycobacterial population, which can have important consequences; for example, large and small daughter cells within microcolonies have been reported to differ in their susceptibility to antibiotics,

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such that large cells (that have inherited the old pole) are more sensitive to cell wall synthesis inhibitors than their smaller sibling cells[10]. However, this has been challenged by studies in which antibiotic sensitivity was tested across populations instead of within microcolonies[13,20]. Furthermore, asymmetric growth affects the distribution of cell wall components, as one of the daughter cells inherits mostly newly synthesized cell wall (FIG. 1). The production of phenotypically distinct daughter cells may confer an advantage when the population is challenged with the variable environments that are encountered in the host, and it could also contribute to the heterogeneous bacterial population that is observed in patients with TB, including cells that are recalcitrant to antibiotics.

Peptidoglycan synthesis.

Polar growth requires the synthesis of new cell wall material, which is particularly complicated for mycobacteria as they must simultaneously synthesize peptidoglycan, arabinogalactan and mycolic acids (BOX 1). Although the arabinogalactan and mycolic acid biosynthetic machineries have been characterized, we focus on peptidoglycan synthesis in this Review, as more is known about how peptidoglycan synthesis and remodelling contribute to cell proliferation. For both elongation and division, peptidoglycan precursor subunits (which are known as lipid II)[18] are shuttled from the cytosol to the periplasm, probably by MviN, which is a transmembrane protein that links precursor synthesis to cell wall incorporation. MviN activity is promoted by FhaA, which contains a forkhead-associated (FHA) domain that recognizes phosphorylated threonines and binds to a phosphorylated domain of MviN[21]. FhaA localizes to the poles and septa[21], where active cell wall synthesis occurs, so it may be involved in localizing MviN. After transport to the cell exterior, lipid II is covalently inserted into the expanding cell wall (BOX 1).

The elongation complex.

Penicillin-binding proteins (PBPs) coordinate peptidoglycan synthesis, and pepti-doglycan hydrolases coordinate peptidoglycan hydrolysis; they are therefore key players in peptidoglycan remodelling and require stringent regulation. A macromolecular machine known as the elongation complex regulates peptidoglycan remodelling during cell elongation and comprises proteins that have scaffolding, hydrolytic and synthetic roles[18] (FIG. 2a). To ensure that elongation is directed to the cell poles, cytoplasmic proteins anchor the elongation complex at these sites. In Actinobacteria, the coiled-coil protein DivIVA is used as an anchor[22,23], rather than cytoskeletal-like proteins, which are used to guide cell wall synthesis in other bacteria[18] (BOX 2). In *B. subtilis*, DivIVA recognizes membrane curvature[22], which ensures its localization to the poles. Recent data indicate that the recognition of membrane curvature also targets Wag31, which is the mycobacterial DivIVA homologue, to the poles[19].

It is likely that Wag31 anchors the nascent elongation complex at the pole and functions as a base for the recruitment of the remaining components of the complex[19]. As optimal Wag31 localization at the pole[24,25] requires interaction with cell wall synthesis protein A (CwsA)[26], CwsA may recruit or stabilize Wag31 at the pole (FIG. 2a). Indeed, overexpression of CwsA increases the polar localization of Wag31 in *M. smegmatis*[27], whereas the deletion of cwsA decreases Wag31 polar localization[26]. Expression of a phosphomimetic version of Wag31

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stimulates the enzymatic activity of MurG and MraY[25] (which are involved in lipid II synthesis), which suggests that Wag31 may interact with lipid II synthases and link lipid II biosynthesis to cell wall incorporation. This hypothesis is supported by the close proximity of *wag31* to the lipid II biosynthetic cluster (they are separated by a distance of 6 kb) in the mycobacterial genome. After the establishment of the Wag31 anchor, it is likely that peptidoglycan remodelling enzymes are then recruited. Wag31 might interact directly with transmembrane peptidoglycan synthases, such as PonAl, which would ensure that cell wall synthesis is connected to cytoplasmic cues. Genetic evidence indicates that such interactions exist; for example, Wag31 is required for shape maintenance and peptidoglycan incorporation at the pole[19,24,25,28], its depletion produces round cells that have growth defects[24] and its overexpression produces branching[28]. These phenotypes are consistent with PonA1 depletion[29] and overexpression, respectively (K.J.K. and E.J.R., unpublished observations).

M. tuberculosis PonA1 and PonA2 are predicted to be essential for growth during infection[30]. PonA1 localizes to the poles and division septum in *M. smegmatis*[29], which suggests that it may synchronize the elongation and division complexes. Indeed, PonA1 interacts with the hydrolase RipA, which localizes to the septum and is required for cell separation[29]. Mycobacterial elongation does not seem to require monofunctional PBPA[31] — the putative homologue of PBP2 — which is required for cell elongation in *E. coli*[18]. Deletion of pbpA in *M. smegmatis* produces cells that are viable but that form filaments[31], which suggests that PBPA has a role in division instead of elongation in mycobacteria. As mycobacterial peptidoglycan is heavily 3–3 crosslinked (BOX 1), non-canonical transpeptidases (such as LdtA and/or LdtB (also known as LdtMt2)) might be recruited to the elongation complex to substitute for PBPA. Recent evidence has shown that the absence of LdtA and LdtB leads to the production of bulging, short cells, which suggests that they have a defect in cell elongation[32]. The coordination of 4–3 and 3–3 crosslinking during elongation may be facilitated by cofactors that modulate the activity of peptidoglycan synthases[18] (FIG. 2a).

Mycobacterial cell division

Following elongation, mycobacteria divide at approximately mid-cell to form two daughter cells, and a large multiprotein complex that is known as the divisome coordinates this step (FIG. 2b). Crosstalk between components of the elongation complex and the divisome probably triggers peptidoglycan synthesis to move from the poles to mid-cell[33]. The divisome contains cytoskeletal-like proteins, structural factors, and peptidoglycan synthases and hydrolases[18,23], which coordinate the synthesis, and then the splitting, of the septum during cell division.

Asymmetric septation.

Septal site selection contributes to the inherent phenotypic heterogeneity of mycobacterial cells. Unlike other species, such as *E. coli* and *C. glutamicum*, several studies have shown that septa are placed over a surprisingly wide zone within the cell body of *Mycobacterium bovis* Bacille Calmette–Guerin (BCG), *Mycobacterium marinum* and *M. smegmatis*[11,12].

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What are the functional consequences of promiscuous septal placement? Irregular septation increases the size difference between daughter cells and results in the differential distribution of proteins and small molecules to daughter cells, which further increases population heterogeneity[34].

Formation of the Z ring.

In almost all bacteria, septation is initiated by polymerization of the self-activating GTPase FtsZ into a ring-like structure that is known as the Z ring[35] (FIG. 2b). The Z ring determines the site of septation and provides the energy for membrane constriction[36] in addition to functioning as the scaffold for downstream peptidoglycan remodelling enzymes[18]. As the Z ring determines the site of eventual cell division, its proper placement is crucial. Mycobacteria regulate Z-ring formation at multiple levels by modulating FtsZ polymerization and guiding Z-ring localization. The MinD homologue Rv3660c is a putative regulator of Z-ring formation, and its overexpression reduces *ftsZ* expression and inhibits septum formation, which results in highly elongated cells[37]. Unlike other bacteria, mycobacterial FtsZ is phosphorylated by the action of the serine/threonine protein kinase (STPK) PknA, which reduces GTP hydrolysis by FtsZ, and hence, inhibits FtsZ polymerization *in vitro*[38]; both activities are required for the constriction of the Z ring to promote division[36]. The expression of mycobacterial PknA in *E. coli* results in the phosphorylation of *E. coli* FtsZ and the production of elongated cells[38], which suggests that FtsZ phosphorylation perturbs polymerization and dysregulates septum formation in the cell. ClpX, which is an integral member of the ClpXP protease, regulates FtsZ polymerization via a direct interaction with FtsZ, and overexpression of ClpX decreases the production of Z rings, which results in elongated cells[39]. A similar phenotype has been observed following the overexpression of ChiZ, which is a lysin motif (LysM) domain-containing protein[40]. As overexpression of any of these factors individually does not abolish Z-ring formation, this suggests that these systems function in parallel to coordinate Z-ring formation.

The divisome.

After the Z ring forms at approximately mid-cell, other proteins assemble to form the divisome. In addition to FtsZ, the structural elements of the divisome are FhaB (also known as FipA), FtsW, FtsQ, CrgA and CwsA (FIG. 2b). The small FtsZ-interacting protein FhaB forms a bridge between FtsZ and FtsQ, and its deletion increases cell length[41], which suggests that FhaB is crucial for mediating interactions between FtsZ and FtsQ to ensure proper Z-ring formation. The FtsZ-FhaB-FtsQ ternary complex has been shown to be required for Z-ring formation under oxidative stress, and consequently, loss of FhaB inhibits bacterial replication in macrophages[41]. FtsZ also interacts with the cytoplasmic tail of FtsW, which is unique to mycobacteria[42]. PBPB (which is a homologue of *E. coli* PBP3) joins the complex by interacting with the extracytoplasmic loops of FtsW[42], and the FtsZ-FtsW-PBPB complex links cytoplasmic structural cues (such as polymerization of the Z ring) to septum synthesis[42]. FtsQ associates with the actinomycete-specific cell division protein CrgA[43], which, in turn, interacts with FtsZ, PBPA and PBPB and thereby stabilizes the whole complex. CrgA localizes to the divisome after FtsZ and has a role in recruiting PBPB. Depletion of CrgA results in elongated cells, which is probably due to the inability to recruit PBPB to the divisome[43]. Furthermore, CrgA interacts with the elongation complex protein

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CwsA[26], and this interaction might coordinate elongation and division during the cell cycle to ensure that septum formation occurs after elongation.

These structural components stabilize the divi-some and promote the synthesis of a septum. Three peptidoglycan synthases — PBPA, PBPB and PonAl —synthesize the septal peptidoglycan. PonA1 is the sole bifunctional peptidoglycan synthase that is known to localize to the septum[29]. As described above, PBPA functions predominantly in septation[31], and the other mono-functional transpeptidase, PBPB, is required for septal synthesis and growth *in vitro*[44]. The chemical inhibition of PBPB produces branched cells[45]. Continued cell elongation can occur even with abnormal septation, which suggests that elongation and division are discrete, but interconnected, processes. PBPB also interacts with the polar anchor protein Wag31 (REF 46), probably when the division septum is transitioning to the pole. This physical crosstalk with the elongation complex may enable elongation to occur even when the synthesis of septal peptidoglycan by PBPB is chemically deregulated. The Wag31-PBPB interaction is particularly important for survival during oxidative stress[46] and, similarly to the CrgA-CwsA interaction, might have a role in relaying information between the elongation and division machineries. Recent evidence suggests that Wag31 localizes to the division site after the formation of the septum, and its concentration increases as division commences[13]. This would enable Wag31 to form an anchor at the nascent cell pole immediately after cell division to facilitate the recruitment of the elongation complex for the next division cycle.

Cell division.

After the completion of septum synthesis, the septum is cleaved to physically separate daughter cells. Peptidoglycan contains various chemical bonds that require cleavage by distinct enzymes[18]. In other bacterial systems, multiple peptidoglycan hydrolases, which show functional redundancy, participate in cell division[18]. By contrast, mycobacteria have at least one essential peptidoglycan hydrolase, RipA[47], and its depletion results in the generation of cell chains that fail to grow[47]. RipA interacts with another peptidoglycan hydrolase, known as resuscitation promoting factor B (RpfB)[48], and *in vitro* analysis has shown that these two peptidoglycan hydrolases synergistically cleave peptidoglycan[47]. This synergy is counteracted by the peptidoglycan synthase PonA1, probably owing to competition with RpfB for binding to RipA[29]. The balance between PonA1-RipA and RipA-RpfB interactions may be used as a mechanism to ensure that the synthesis of the septum precedes its hydrolysis. Septal hydrolysis in other species, such as *E. coli*, requires amidases and FtsE and FtsX[18], but the activity of mycobacterial amidases and other hydrolases in septation is poorly understood[49,50]. Recent evidence suggests that FtsX regulates the hydrolytic activity of RipC[51], although the role of RipC in mycobacterial cell division remains unclear. At the completion of cytokinesis, mycobacteria exhibit a ‘v-snapping’ phenotype[23] (FIG. 1). This may result from asymmetric splitting of the septum or from uneven shearing of the arabinogalactan and mycolic acid layers on the cell surface[23].

Does dissolution of the septum affect cell shape? Although cell length is heterogeneous in mycobacteria, cell width seems to be uniform. This may be due to the retention of unsalvageable peptidoglycan in the polar cap. RipA, homologues of which are not essential

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in other bacterial species, generates peptide fragments[47] that cannot be used in future crosslinking reactions, which may inhibit the incorporation of new peptidoglycan monomers. Thus, the static polar cap might restrict the insertion of nascent material to the subpolar region[19], which results in cells of uniform width.

Regulation of growth and division

Mycobacteria encode many regulatory pathways to control cell wall synthesis, cell growth and division, and the enzymes and processes that are involved are another source of heterogeneity[52].

Transcriptional regulation.

Cell wall biosynthesis can be modulated at the level of gene expression. The three transcription factors EmbR, MabR and FasR regulate the expression of the cell wall synthases EmbA, EmbB, EmbC and the FASI and FASII complexes (fatty acid synthase I and II complexes)[53–55]. The transcriptional activator EmbR binds to the promoter of the *embCAB* operon, which encodes arabinosyltransferases in *M. tuberculosis*, to increase the synthesis of arabinoga-lactan[53] (FIG. 3a). The FHA domain of EmbR is phosphorylated by the serine/threonine kinase PknH, which promotes the binding of EmbR to the *embCAB* promoter, and this is further promoted in macrophages, as PknH expression is known to increase[53]. This increase in arabinogalactan synthesis may facilitate adaptation to the stresses that are encountered inside macrophages.

Large and complex lipids, such as the immunomodulatory mycolic acids, are energetically expensive to produce, and the cell has evolved to tightly regulate their synthesis. The transcription factors MabR and FasR, both of which are required for cell proliferation, regulate mycolic acid synthesis[54,55]. MabR binds to the promoter of the FASII locus and negatively regulates the expression of *fabD*, *acpM*, *kasA* and *kasB*, thereby decreasing the production of mycolic acids[54]. Overexpression of MabR also decreases *fas* expression, which suggests that feedback between the FASI and FASII complexes (BOX 1) regulates transcript levels[54]. Mycolic acid production is also positively regulated by the activity of FasR, which binds to conserved sites in the *fas* promoter[55]. Depletion of FasR results in decreased expression of fas and a reduction in mycolic acid synthesis in *M. smegmatis*[55]. The opposing regulation that is carried out by MabR and FasR enables the fine-tuning of mycolic acid production.

Two component systems (TCSs) are also involved in regulating gene expression. Mycobacteria encode 12 TCSs that function analogously to the STPK system. The TCS DNA-binding response regulator MtrA is required for growth of *M. tuberculosis*[56], and deletion of its cognate sensor kinase, MtrB, in *M. smegmatis* causes cells to swell and form chains[57], which indicates that this TCS is essential for the maintenance of cell shape and division. MtrA binds to the promoters of several cell division factors, including those that drive the expression of the mycolic acid transferases FbpA, FbpB and FbpC[56] and the divisome-specific peptidoglycan hydrolase RipA[57]. Phosphorylation of MtrA negatively regulates *fbpB* expression[56]. By contrast, MtrA positively regulates *fbpA* expression, irrespective of its phosphorylation status[56]. The *mtrA* gene is upregulated by low pH[58],

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which suggests that MtrA can regulate cell cycle progression and the production of mycolic acids in response to the pH changes that occur inside macrophages.

Post-translational modifications.

Mycobacteria have a surprisingly large complement of eukaryotic-like STPKs that regulate important steps in cell morphogenesis[59,60]. As mycobacteria lack canonical regulatory systems for the coordination of cell division (BOX 2), the large complement of STPKs and TCSs may substitute for such systems. Phosphorylation modulates growth by two main mechanisms: by directly controlling protein activity; and by stimulating and balancing protein-protein interactions between cell envelope biogenesis[61–63], elongation[21] and divisome proteins[41,60]. The two kinases PknA and PknB are essential for growth *in vitro*[59]; overexpression of either PknA or PknB produces bulging cells that have reduced growth rates, whereas depletion of either kinase generates elongated, narrow cells[59].

Assembly and disassembly of the divisome is regulated, in part, by phosphorylation. Phosphorylation of FtsZ by PknA reduces polymerization of FtsZ *in vitro* and probably promotes its interaction with FhaB, which contains an FHA domain that is required for interaction with FtsZ[41]. As FhaB interacts with other divisome components, its interaction with FtsZ might stabilize the interactions between the Z ring and the rest of the divisome complex. FhaB is also phosphorylated by PknA, which is necessary for the formation of the FtsZ-FhaB-FtsQ ternary complex[41]. In addition, FtsQ is phosphorylated[60], but whether this is required for interactions with FhaB or CrgA remains unexplored. FtsZ, FhaB and FtsQ are key structural components of the divisome, and the high level of phosphorylation may regulate the formation of the divisome in response to environmental cues.

Synthesis of all components of the cell wall is affected by phosphorylation. The transmembrane protein MviN, which probably shuttles lipid II precursors across the periplasm, is phosphorylated by PknB, and this recruits its essential interacting partner, FhaA[21]. Phosphorylation of Wag31 by PknA is required for correct polar localization and peptidoglycan biosynthesis[25]. Hyper-phosphorylation of Wag31 can stimulate lipid II synthesis[25] and probably peptidoglycan incorporation. The peptidoglycan synthase PonA1 is also phosphorylated[60]. As PonA1 functions as part of the elongation complex and as part of the divisome[29], its phosphorylation status may direct PonA1 to the distinct complexes.

Arabinogalactan and mycolic acid synthesis are also subject to phosphorylative control. As described above, EmbR is phosphorylated by PknH[53], which results in the stimulation of arabinogalactan synthesis. Several factors that are involved in mycolic acid precursor synthesis are phosphorylated, including FabD, FabH, MabA, KasAB, InhA, Pks13 and PcaA[61,63,64]. Phosphorylation of KasB[64] and MabA[62] leads to an increase in their activity and increased production of mycolic acids. Phosphorylation of FabH by PknF, which shuttles mycolic acid precursors from FASI to FASII, decreases its catalytic activity[64], which probably decreases the production of mycolic acids (FIG. 3b).

Protein processing and degradation.

Several studies have shown that the proteolytic processing of cell division proteins is required for normal mycobacterial growth[46,65–67]. The essential hydrolase RipA requires

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cleavage for full catalytic activity to separate daughter cells[65,66]. The essential septal synthase PBPB also undergoes cleavage by the metalloprotease Rv2869 during oxidative stress[46], which may decrease cell division during unfavorable conditions.

In addition to processing, cell division proteins are subject to degradative control. Mycobacteria encode a system that is analogous to eukaryotic ubiquitylation, which is known as pupylation[67,68]. Conjugation of the prokaryotic ubiquitin-like protein (Pup) to target proteins by proteasome accessory factor A (PafA) results in their proteasomal degradation. In addition, Pup can also be removed by the depupylase Dop (which is functionally analogous to de-ubiquitylases)[69], which provides another layer of regulation. Several factors that are involved in cell wall assembly are pupylation targets, including MurA, KasB and MtrA[68]. MtrA is targeted for proteasomal degradation[68], and its directed turnover may regulate cell wall assembly and cell division; for example, degradation of MtrA might relieve repression of *fbpB*—a member of the mycolic acid transferase complex —which would promote cell wall incorporation of mycolic acids (FIG. 3c). However, not all pupylated proteins are targeted to the proteasome[68], and it remains to be determined what effect, if any, this modification has—for example—on the function of MurA and KasB and the processes that they control.

Cell wall remodelling and asymmetry

Cell wall plasticity facilitates bacterial survival.

Cell envelope biogenesis and cell division are tightly regulated, and the many levels of regulation provide ample opportunity for stochastic phenotypic differences to arise between individual cells. Such differences are likely to amplify the heterogeneity that is produced by asymmetric cell elongation and division. Moreover, restructuring of the cell wall is a key mechanism that *M. tuberculosis* uses to adapt to the changing host environment[14–17] and subvert host defences during infection[70]. In addition, the cell wall is an important target for antibiotics, and mutations in components of the cell wall synthesis machinery lead to clinical drug resistance[71], which highlights the importance of this structure as an interface with the host and as a clinical drug target.

The three major cell wall components form irregular structures. Peptidoglycan and arabinogalactan are extensively modified with glycolyl, amide, succinyl and galactosamine residues[72–74], and peptidoglycan contains unusual linkages between monomers[72,75] (BOX 1). The three forms of mycolic acids (that is, α-meroacids, methoxy-meroacids and keto-meroacids) are synthesized with different acyl chain lengths and varying levels of saturation and are also modified by cyclo-propanation, all of which vary between molecules[76,77] (BOX 1). The combination of these alterations induces physicochemical changes in the cell wall, which have been found in bacteria that are grown *in vivo*, suggesting that they are part of an adaptive response for the generation of a heterogeneous bacterial population. A potential consequence of this population diversity is that subpopulations of bacteria are equipped to survive the dynamic stresses that are encountered *in vivo*, such as the host immune response and antibiotics.

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Several studies have shown that the composition of the cell wall changes during infection (FIG. 4). Bacilli that have been isolated from chronically infected patients are no longer acid-fast, which suggests that gross structural and/or compositional changes occur during extended periods of *in vivo* growth[14]. Cells that are isolated from infected guinea pigs show differences in mycolic acid composition compared with cells that are grown *in vitro*[15]. Transcriptional analysis of bacteria that have been isolated from diverse regions of the lungs has revealed substantial changes in the expression of cell wall biosynthetic genes and membrane proteins compared with *in vitro* cultures[16], including the upregulation of lipid synthesis genes. For example, *pcaA*, *desA1* and *desA3*, which are involved in mycolic acid processing, are upregulated and are required for virulence *in vivo*[78]. Furthermore, the length and abundance of the cell wall lipids phthiocerol dimycocerosate (PDIM) and sulpholipid 1 (SL1) increase during the growth of *M. tuberculosis in vivo*[17]. Upregulation of lipid biosynthesis could confer multiple benefits to the bacterium: a thicker cell wall restricts the transit of toxic molecules (such as antibiotics) across the cell membrane; mycolic acids can manipulate the immune system[79] and absorb oxidative radicals[80]; and lipids function as a sink for toxic by-products from cholesterol catabolism during growth in macrophages[81,82], which is required for bacterial survival in a mouse model of TB[83].

Mycobacterial peptidoglycan is also extensively modified to interact with, and adapt to, host conditions (FIG. 4). The non-canonical transpeptidases LdtA and LdtB catalyse the predominant 3–3 peptide crosslink in peptidoglycan. LdtB expression peaks during late log phase and late stationary phase, whereas LdtA expression increases only during late stationary phase[84]. LdtB mutants are unable to persist, and they decrease in number during the course of infection[84]. These data indicate that heavily 3–3-crosslinked peptidoglycan is necessary for maintenance of the infection and resistance to immune clearance. LdtB mutants are also more susceptible to amoxicillin[84], which suggests that these unique crosslinks may have a clinically relevant role in resisting antibiotic stress.

In addition to unconventional crosslinks, transcriptomic analysis of *M. tuberculosis* isolated from granulomas has shown that several steps of peptidoglycan biosynthesis are upregulated *in vivo*, including the genes that are responsible for precursor production (such as *murE* and *ddl*) and remodelling (such as *Rv1730c* and *rpfA*)[16]. In addition, PonA1 is required for *M. tuberculosis* survival during hypoxia[85], and PonA2 is required during infection[86] and to support growth of *M. smegmatis* in conditions of reduced replication[87,88]. Furthermore, peptidoglycan remodelling by low molecular weight PBPs, such as DacBl and DacB2, which hydrolyse the terminal D-alanine residues from the peptidyl *N*-acetyl muramic acid (NAM) subunits, is required for survival in the host, as DacBl is predicted to be essential for the infection of non-human primates[89]. DacBl remodelling of pepti-doglycan may maintain the 3–3 crosslinks that are crucial for persistence *in vivo*, as removal of the terminal D-alanines inhibits 4–3 crosslinking. These data correlate with genetic evidence that shows that additional cell wall assembly enzymes become essential under host-like conditions[30,44,90–92]. Taken together, these data suggest that cells must undergo peptidoglycan remodelling to adapt to various stresses, many of which reflect conditions that are thought to be encountered in the host during latent or active infection.

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The wide range of microenvironments that are imposed by the host induce various metabolic changes[81,82,93] that, combined with stochastic variation in cell wall composition, such as variable peptidoglycan modifications and asymmetric cell division, could result in a substantially heterogeneous bacterial population. Changes in metabolism and growth rate[93–96] have consequences for the cell wall; for example, the mutation of cell wall genes generates low-fitness mutants under slow growth rates in a carbon-limited chemostat, which suggests that peptidoglycan synthesis, mycolic acid synthesis and the synthesis of the cell surface lipid PDIM[97] are all required under these conditions. These requirements probably result in gross changes in the cell wall, such as the thickening that is observed by electron microscopy in low oxygen environments[98]. Many of these changes might be closely linked to overall lipid metabolism, as the lipid-rich cell wall obtains its precursors from normal metabolism[17,81,82]. If lipid production decreases, which occurs during hypoxia[99], the lipid composition of the cell wall changes (FIG. 4). As many of these lipids are pro-inflammatory molecules[78,79], changes in their abundance will also affect interactions between the pathogen and the host. Indeed, deletion of *kasB*, which is a component of the mycolic acid synthase complex FASII, attenuates virulence in a clinical strain of *M. tuberculosis*[100]. Immunocompetent mice that are infected with a *kasB* mutant seem to remain healthy for up to 600 days post-infection, whereas mice that are infected with the wild type or a *kasB*-complemented strain succumb to infection within 356 days. The persistence of *kasB* bacteria is probably due to their shortened and altered mycolic acid chains[100], which are predicted to reduce immunogenicity.

Asymmetric growth may increase population heterogeneity.

Asymmetric growth and the resulting heterogeneity in cell size may have a role in the life cycle of *M. tuberculosis* in the human host, including its ability to actively grow in the range of host microenvironments, as well as its ability to persist in a quiescent state[5].

Several studies have modelled how asymmetric growth and division influence cell fate during antibiotic therapy *in vitro*[10,20]; for example, as mentioned above, analysis of microcolonies suggests that cells that have inherited the old pole are more recalcitrant to cell wall synthesis inhibitors than sibling cells that have inherited the new pole[10]. In addition to the heterogeneity that accompanies cell growth and division, recent work suggests that stochastic changes in the expression of *katG*, which encodes the catalase-peroxidase enzyme KatG that is required for the activation of isoniazid, are responsible for mycobacterial survival during isoniazid treatment[20]. It has been shown that individual *M. smegmatis* cells vary in their susceptibility to isoniazid, and this is independent of the genetic background and cell age[20]. Unlike *E. coli*, these stochastic processes are also independent of growth rate and cell size[101]. Variation in gene expression is also likely to influence cellular responses other than antibiotic resistance; this would also contribute to the ability to adapt to different conditions, which is an essential requirement for persisting in hypoxic conditions *in vivo*[93] as well as for antibiotic tolerance[102–104]. Deterministic and stochastic means of tolerance to antibiotics, combined with diversity in cell size and cell wall composition, could contribute to the notoriously long course of antibiotic therapy that is required to treat *M. tuberculosis* infections.

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Outlook

The mycobacterial cell wall is a complex structure, and the mechanisms that are required for its biogenesis, including regulation, reflect this complexity. The regulation of cell wall synthesis occurs at the level of transcription and post-translational mechanisms, including proteolytic processing, pupylation and widespread phos-phorylation. This enables mycobacteria to manipulate the composition of the cell wall in response to changing environmental conditions in the host. Thus, it seems reasonable to propose that dynamic remodelling of the cell wall[14–17] and the unusual mechanism of asymmetric elongation and division[10–13] are important contributors to the ability of the bacterium to evade immunity and persist in the host.

The findings that are presented in this Review offer insights into how cell–cell heterogeneity can arise in the population. The mechanisms that control asymmetric growth and its regulation remain to be elucidated, but it is likely that interplay between the elongation and division machineries is important. Finally, population diversity is further increased by stochastic changes in gene expression, which lead to variation in growth, particularly following exposure to antibiotics[20].

Biochemical and genetic techniques have made substantial progress in elucidating the details of cell wall synthesis during growth *in vitro*; however, several obstacles remain. Studying the mechanistic basis and the importance of heterogeneity in individual bacteria will require more advanced single-cell techniques, such as microfluidics, which is an area that is just emerging. Similarly, progress in our understanding of bacterial adaptation during infection is required; although *in vitro* models and animal models can replicate some aspects of infection, their correlation with infection of the human host remains mostly unknown. Thus, we still require technical breakthroughs to gain a more complete picture of the mycobacterial cell wall, as well as of cell growth and division in the most important environment —us.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Granuloma

An organized cellular structure that forms owing to the immune response to an invading pathogen. An array of immune and fibrotic cells ‘wall-off’ the invading pathogen in an attempt to limit its spread within the host

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Latency

An asymptomatic disease state in which the bacterial burden is low or undetectable

Non-traditional 3–3 peptide crosslinks

Peptide bonds between the third residues (*meso*-diamino-pimelic acid (mDAP) in mycobacteria) of two peptide tails in peptidoglycan; a traditional crosslink is a peptide bond between the third and fourth residues (between DAP and D-Ala in mycobacteria) of two peptide tails

Cytokinesis

The process in which a plasma membrane forms in the mid-cell region of a mother cell during cell division

Septum

A layer of peptidoglycan that is formed at approximately mid-cell (in mycobacteria) that generates a physical barrier between nascent daughter cells

Microfluidics

A technology in which small fabricated chambers are used to isolate and image single cells

Microcolonies

Groups of progeny cells that are generated from the growth and division of single cells

Periplasm

The extracellular space between the plasma membrane and the outer membrane of Gram-negative bacteria. The mycolic acid layer of mycobacteria functions analogously to an outer membrane, and thus, the space between the plasma membrane and the mycolic acid layer is known as the periplasm in mycobacteria

Actinobacteria

A phylum of bacteria to which mycobacteria belong. This phylum also includes species such as *Streptomyces coelicolor* and *Corynebacterium glutamicum*

Phosphomimetic

An amino acid substitution, typically by an aspartic acid or glutamic acid, at the site of a phosphorylated residue in a protein, which mimics constitutive phosphorylation

MinCDE system

A protein system in *Escherichia coli* that regulates placement of the division septum in the cell. MinCDE oscillate from pole-to-pole and establish a concentration gradient to prevent Z-ring formation at the cell poles

SOS response

A response to DNA damage in which bacteria halt cell cycle progression and induce DNA repair mechanisms in an effort to repair the chromosome before continuing division

FASI and FASII complexes

(Fatty acid synthase I and II complexes). Complexes that are involved in mycolic acid synthesis. FASI, which is composed of the protein Fas, synthesizes the initial C_{20}-C_{26} a-branch of mycolic acids. This branch is joined to a C_{60}-C_{90} meromycolate branch that is synthesized by the FASII complex, which is composed of MabA, HadABC, InhA, KasAB and an unidentified isomerase

Pupylation A

system that typically targets proteins for proteasomal degradation by the addition of a small prokaryotic ubiquitin-like protein (Pup). Pupylation directs some, but not all, of its targets to the proteasome, which suggests that pupylation may have additional roles in regulating protein activity

Chemostat

A closed system for bacterial growth that is used to control the bacterial replication rate and/or metabolism by the use of chemically defined media

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The cell envelope of mycobacteria

The mycobacterial cell wall is a complex structure that is required for cell growth, resistance to antibiotics and virulence[76,105,106]. It is composed of three distinct macromolecules — peptidoglycan, arabinogalactan and mycolic acids—which are surrounded by a non-covalently linked outer capsule of proteins and polysaccharides[23,76,105,107] (see the figure). The high density of lipids in the cell wall prevents accurate Gram staining, and mycobacteria are known as acid-fast, as they can be stained by acid-fast dyes, such as Ziehl-Neelsen stain[23]. The cell wall is the most common target of antituberculosis drugs, and many compounds that are in clinical use or under development target enzymes that synthesize distinct layers of the cell wall[108].

The peptidoglycan layer surrounds the plasma membrane and comprises long polymers of the repeating disaccharide N-acetyl glucosamine-N-acetyl muramic acid (NAG-NAM) that are linked via peptide bridges. The peptidoglycan precursor lipid II is generated in the cytoplasm[18,23] and is probably transported across the periplasm by the transmembrane protein MviN[21]. Unidentified hydrolases are required to open the peptidoglycan mesh for the insertion of new precursors[18], which are added in an 'inside to outside' manner[109]. The penicillin-binding proteins (PBPs) PonA1 and PonA2 incorporate new subunits into the existing structure. Transpeptidases, such as PBPA, PBPB, LdtA and LdtB, crosslink the newly inserted material[23]. Compared with other model bacteria, such as *Escherichia coli* and *Bacillus subtilis*, mycobacterial peptidoglycan is heavily crosslinked. Up to 80% of the peptidoglycan contains non-traditional 3–3 peptide crosslinks instead of traditional 4–3 crosslinks[72,75]. The peptidoglycan polymer also has modifications, such as glycolylation of NAM residues[73,110] and amidation of the D-Glu and meso-diaminopimelic acid (mDAP) residues of the peptide side chain[72,111] (see the figure). Amidation may mask recognition by the innate immune receptor nucleotide-binding oligomerization domain-containing 1 (NOD1), which has been observed in B. *subtilis*[112]. However, glycolylated NAM is efficiently recognized by NOD2 and induces the production of inflammatory cytokines in *Mycobacterium tubercuiosis*-infected macrophages[113,114].

A layer of arabinogalactan surrounds the peptidoglycan layer (see the figure). Galactan comprises a repeating disaccharide unit of 6-D-Gal*f*β–5-D-Gal*f*β and is synthesized by the galactofuranosyl transferases Glf, GlfT1 and GlfT2. Galactan is modified with long arabinan polymers that are synthesized by the successive actions of DprE1 and DprE2[115] and the arabinofuranosyltransferases AftA, EmbA, EmbB and AftB[105]. There is variability in arabinosylation, and some galactan chains remain free of arabinan[116]. Arabinan chain termini are branched, and this motif is generated by the actions of AftC[105], AftD[117] and Rv1459 (REF. 105). Arabinan can also be further modified by the addition of succinyl or unusual non-*N*-acetylated galactosamine (GalN) moieties[105]. The GalN modifications are mostly present in pathogenic mycobacteria and may promote efficient infection, as has been observed in *Francisella tularensis*[74].

Most arabinan is ligated with long-carbon-chain mycolic acids[76,77,105], which form the characteristic thick waxy lipid coat of mycobacteria[23,118] and are major contributors to

the impermeability of the cell wall[23,76,77] and to virulence[76,100]. Mycolic acids are formed from two fatty acids, a saturated shorter C_{20}-C_{26} α-branch that is connected to a C_{60}–C_{90} meromycolate branch. These branches are generated by the FASI and FASII complexes[76,77,119]. FASI products are transferred to FASII for extension by the combined action of 3-oxoacyl acyl carrier protein synthase 3 (FabH), acyl carrier protein AcpM and malonyl CoA-acyl carrier protein transacylase FabD[76,77]. Mycolic acids are then processed and matured by a cascade of enzymes[76,77,120,121], which results in three distinct meromycolate variants: α-meroacids, methoxy-meroacids and keto-meroacids[76,77]. All three variants are required for full virulence during infection and have varying levels of saturation, cyclopropanation and oxygenation[76,77]. The inner membrane transporter MmpL shuttles mycolic acids to the cell surface[122–124], where the FbpABCD (also known as antigen 85) complex ligates the mycolic acid monomers to arabinogalactan[76,77,105]. Although the coordination of mycolic acid and arabinogalactan synthesis and insertion in the cell wall remains unknown, recent data suggest that these macromolecules are produced and exported at the cell pole[19,125], where new cell growth occurs in mycobacteria.

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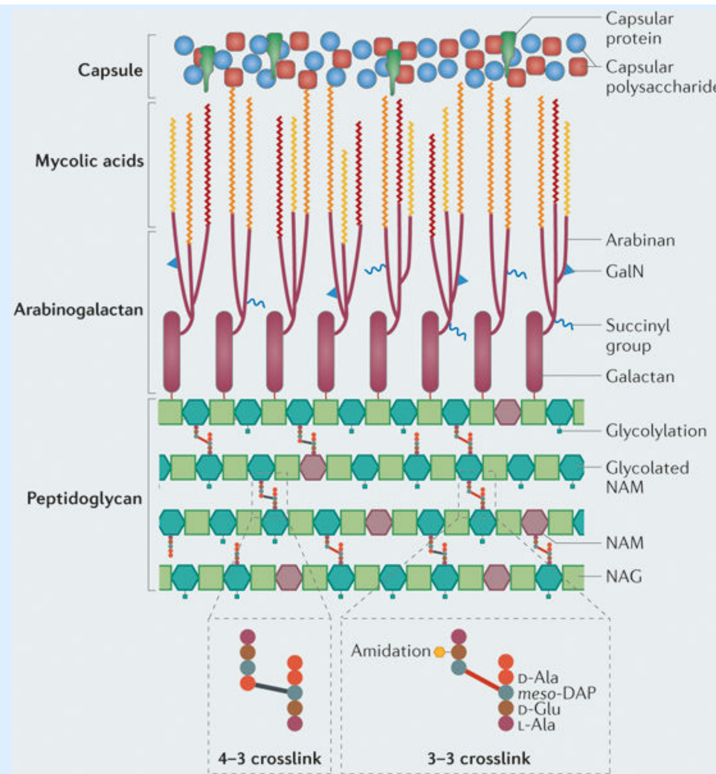

Canonical cell growth in model organisms

The traditional model bacteria *Escherichia coli* and *Bacillus subtilis* have vastly different cell wall architectures compared with mycobacteria, and as such, cell wall synthesis and cell division rely on a different set of proteins (Supplementary information S1 (table)). In *E. coli* and *B. subtilis*, elongation occurs along the lateral cell wall and the elongation complex is comprised of peptidoglycan synthases, lipoprotein modulators of penicillin-binding protein (PBP) activity, cytoskeletal-like proteins and peptidoglycan hydrolases[18,126–128]. Peptidoglycan synthesis is coordinated by bifunctional PBPs (such as PBPla and perhaps PBP1b[18] in *E. coli* and PBP1 in *B. subtilis*[129]) and monofunctional PBPs (such as PBP2 in *E. coli*[18] and PBP2a and PbpH in *B. subtilis*[126]). The actin homologue MreB forms filaments that underlie the elongation complex and facilitate elongation in *E. coli* and *B. subtilis* (which has two additional MreB homologues, MreBH and Mbl[130]) by guiding elongation complexes along the lateral wall[18,130]. It has also been reported that interactions between FtsZ and MreB are necessary for appropriate cell division in *E. coli*[131], which suggests a potential mechanism for the coordination of elongation and division.

Similarly to mycobacteria, polymerization of FtsZ initiates cell division in *E. coli* and *B. subtilis*. The divisome is mainly composed of peptidoglycan synthases that construct the septum (such as PBP1b and PBP3 (and possibly PBP1a) in *E. coli*[18] and PBP1 and PBP2b in *B. subtilis*[35]), structural proteins (such as FtsW, FtsN and FtsQLB[18] in *E. coli* and FtsW and DivIBC in *B. subtilis*[132]) and hydrolases that physically separate daughter cells (such as AmiABC in *E. coli*[18] and LytCDF in *B. subtilis*[133]). Other factors that regulate the formation of the divisome are also involved[18,35,134]. The placement of the Z

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ring is carefully controlled, and in *E. coli*, this is regulated by the MinCDE system during normal growth and by the cell division inhibitor SulA when the SOS response is induced[134]. In *B. subtilis*, the functional homologues of MinCDE (that is, MinCD and DivIVA), together with septation-ring formation regulator EzrA, are responsible for determining the site of Z-ring formation[35]. EzrA interacts with FtsZ and PBP1, which suggests that it may synchronize elongation and cell division in *B. subtilis*. The *B. subtilis* ClpXP protease also regulates Z-ring formation by inhibiting FtsZ polymerization[35]. The UDP-glucose transporter UgtP inhibits FtsZ polymerization in nutrient-limiting conditions and thereby couples growth rate to cell division in *B. subtilis*[35]. The DNA-binding nucleoid occlusion factors (such as SlmA in *E. coli*[134] and Noc in *B. subtilis*[35]) also function in FtsZ placement and regulate Z-ring formation via direct interactions with FtsZ and coordination with the replication machinery, respectively[134]. For a complete list of the main proteins involved in cell elongation and division in mycobacteria, *E. coli* and *B. subtilis*, see Supplementary information S1 (table).

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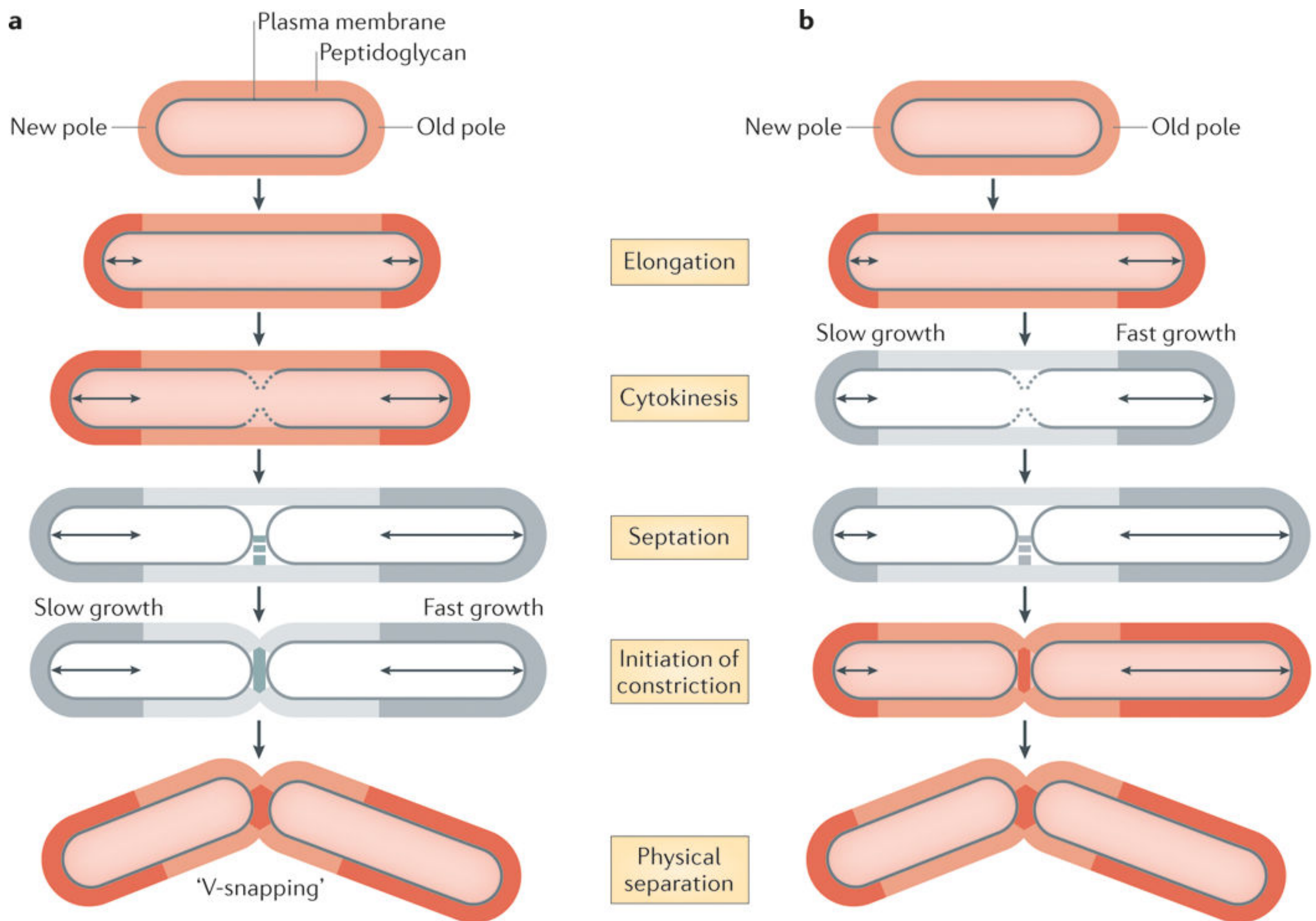

Figure 1 |. Elongation and division of mycobacteria generates asymmetric daughter cells.

Mycobacterial cells elongate by incorporating new cell wall material (dark orange and dark grey) at their cell poles, and all mother cells contain an old pole (which was inherited from the previous cell division) and a new pole (which was synthesized during the previous cell division). The process of cell division can be subdivided into several stages: synthesis of the plasma membrane roughly at mid-cell during cytokinesis; synthesis of a peptidoglycan septum at mid-cell (which is known as septation); constriction of the cell wall; and hydrolysis of the septum as the two cells physically separate. Cell separation in mycobacteria is characterized by a 'v-snapping' phenotype. Two models have been proposed to explain how daughter cells of different sizes are generated, and the models differ in terms of their definitions of cell division. **a** | In the first model[13], in which cytokinesis is used to define cell division, growth at both cell poles is symmetric until cytokinesis. From cytokinesis until the cells physically separate, growth occurs predominantly at the old pole, which generates two daughter cells of unequal sizes. In this model, septation and the initiation of constriction were not specifically measured (cells shown in grey). **b** | In the second model[10], in which the initiation of constriction during the physical separation of daughter cells is used to define cell division, the poles elongate at different rates throughout the cell cycle, and the old pole elongates faster than the new pole at all stages of the process. This model did not specifically measure cytokinesis or septation (cells shown in grey).

Despite discrepancies in the timing of asymmetric growth, both models show that mycobacterial growth generates daughter cells of unequal sizes.

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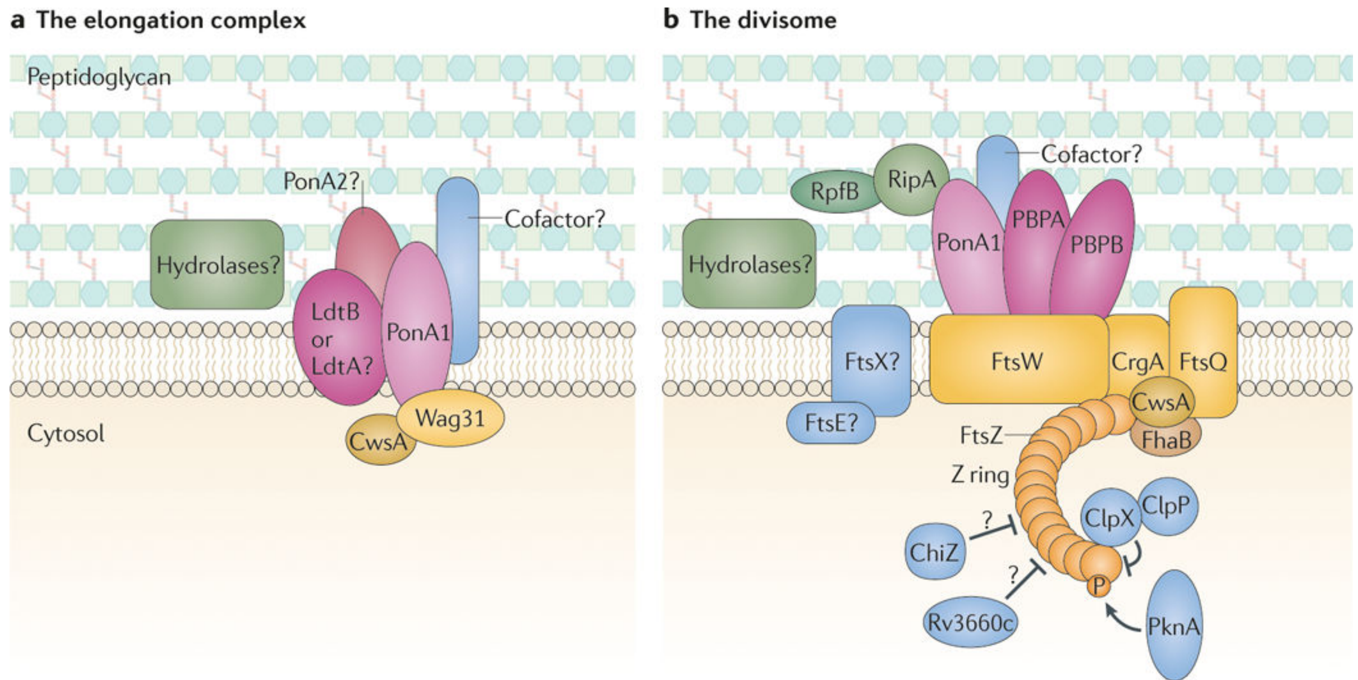

Figure 2 |. Protein machineries direct cell growth and division.

Large macromolecular complexes that are comprised of peptidoglycan synthases (pink), peptidoglycan hydrolases (green), structural proteins (yellow) and regulatory factors (blue) coordinate cell elongation and cell division. **a** | The multiprotein elongation complex drives polar elongation and is anchored at the poles by Wag31, which is stabilized by cell wall synthesis protein A (CwsA). The peptidoglycan synthase PonAl incorporates new peptidoglycan subunits into the spaces that are created by the hydrolases. PonA2 and the non-classical transpeptidases LdtA and/or LdtB might coordinate peptidoglycan synthesis with PonAl, which would generate the two different types of crosslinks (4–3 and 3–3 crosslinks) that are observed in mycobacterial peptidoglycan. Protein cofactors may coordinate this activity or activate the peptidoglycan synthases. Peptidoglycan hydrolases are required to open the mesh-like peptidoglycan wall for the insertion of new material; however, the identity of these hydrolases is currently unclear. **b** | Another large macromolecular machine, known as the divisome, is responsible for cell division. Septation is initiated by polymerization of FtsZ, and the subcellular placement of the Z ring is regulated by several factors, including ClpX, ChiZ and Rv3660c, as well as phosphorylation of FtsZ by PknA. FhaB stabilizes the Z ring, and then the structural proteins FtsW, FtsQ, CrgA and CwsA assemble to form the divisome. The peptidoglycan synthases penicillin-binding protein B (PBPB), PBPA and PonAl synthesize the septum, and the hydrolases RipA and RpfB (and possibly other hydrolases) cleave the septum to separate the daughter cells. Septal hydrolysis might also be facilitated by FtsE and FtsX, but this remains to be confirmed.

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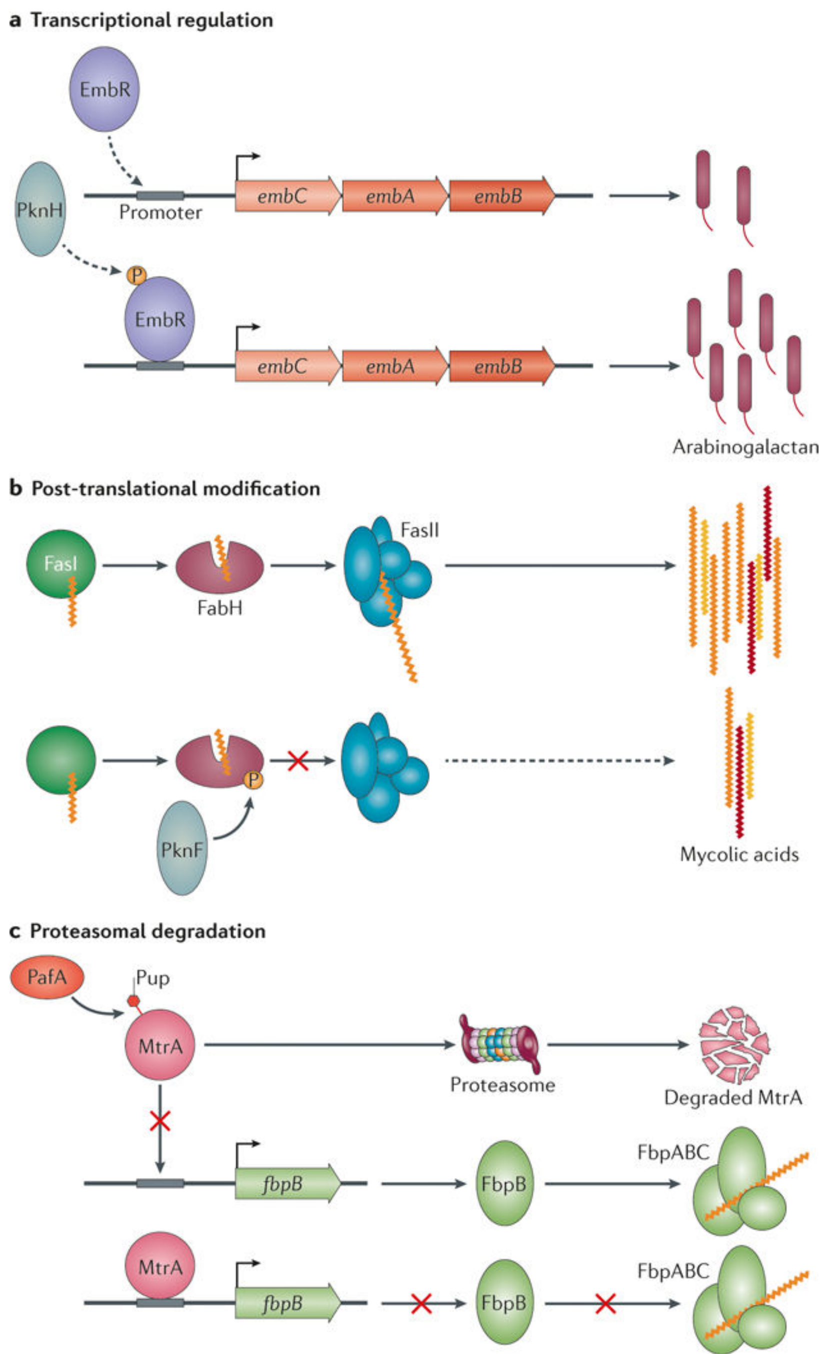

Figure 3 |. Regulatory mechanisms that control synthesis of the mycobacterial cell wall.
Mycobacteria use several mechanisms to regulate cell wall synthesis and cell division. **a** | At the transcriptional level, the transcription factor EmbR activates the expression of the *embCAB* operon, which encodes EmbA, EmbB and EmbC. The two arabinosyltransferases EmbA and EmbB are involved in arabinogalactan synthesis and increase the production of arabinogalactan. Phosphorylation of EmbR by the kinase PknH stimulates promoter binding, which leads to an increase in arabinogalactan synthesis. **b** | Post-translational modifications of cell wall precursor synthases, such as the phosphorylation of FabH, also occurs. FabH

shuttles mycolic acids from FASI to FASII for acyl chain extension, but its activity is inhibited by PknF-mediated phosphorylation, which thereby decreases the production of mycolic acids. **c** | Mycobacteria encode a unique pupylation system that targets proteins for proteasomal degradation, including proteins that are involved in cell wall synthesis. For example, the response regulator MtrA is pupylated by proteasome accessory factor A (PafA), which promotes its proteasomal degradation. This relieves MtrA-mediated repression of *fbpB* (which encodes a member of the mycolic acid transferase complex FbpABC, thereby increasing the incorporation of mycolic acids into the cell wall.

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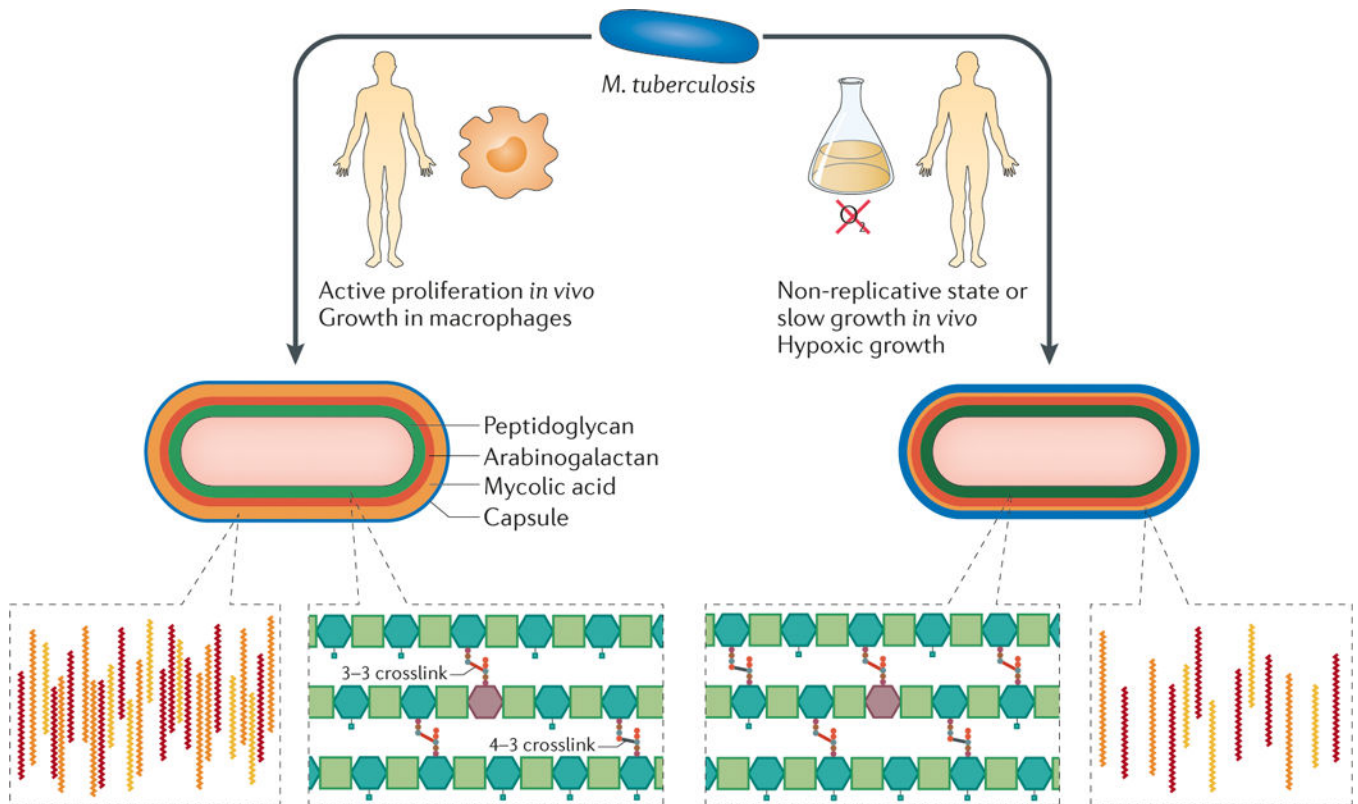

Figure 4 |. Cell wall remodelling may promote survival during infection.

During growth *in vivo,* substantial changes occur in the cell wall of *Mycobacterium tuberculosis.* Active proliferation during infection andthe use of host carbon sources during growth in macrophages lead to an increase in the production of mycolic acids and cell wall lipids, such as phthiocerol dimycocerosate (PDIM), which may promote survival in the host environment. In addition, during growth *in vivo*, peptidoglycan is remodelled and the crucial 3–3 crosslinks that promote chronic infection and antibiotic resistance are maintained. During slow growth and in non-replicative states, the cell wall becomes thicker, probably owing to an increase in the synthesis of multiple cell wall layers. During slow growth and non-replicative states, as well as in hypoxic environments, *M. tuberculosis* downregulates the abundance of immunostimulatory mycolic acids to potentially avoid immune recognition, and crosslinking of peptidoglycan may be increased to promote cell wall rigidity and bacterial survival under stress. These cell wall changes, combined with the inherent plasticity of cell wall synthesis, generate a population of unique single cells that may have variable fitness during infection.