Mycobacterial Growth

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In this work, we review progress made in understanding the molecular underpinnings of growth and division in mycobacteria, concentrating on work published since the last comprehensive review (Hett and Rubin 2008). We have focused on exciting work making use of new time-lapse imaging technologies coupled with reporter-gene fusions and antimicrobial treatment to generate insights into how mycobacteria grow and divide in a heterogeneous manner. We try to reconcile the different observations reported, providing a model of how they might fit together. We also review the topic of mycobacterial spores, which has generated considerable discussion during the last few years. Resuscitation promoting factors, and regulation of growth and division, have also been actively researched, and we summarize progress in these areas.

The answers to many biological questions are facilitated by technological advances that allow us to observe and interrogate objects easier, faster, and more accurately than before. Time-lapse observations of bacteria have been around for many years, even for more difficult to grow prokaryotes, such as mycobacteria. Elegant studies by Brieger and Glauert more than 60 yr ago show what can be performed with ingenuity and patience (Brieger and Glauert 1952, 1956; Brieger et al. 1953, and references therein). We now have autofocus microscopes with motorized stages, digital cameras with more and more pixels, and software to control it all. Historically, the basics of growing bacteria for imaging used bacterial agar; recently, however, microfluidics arrived on the microscope stage, which allowed us to grow bacteria in suspension and to control growth conditions with relative

ease. These systems make it possible to change growth medium in a controlled manner, introduce antimicrobials, and image what happens, as frequently as the bacteria will tolerate exposure to the high-intensity light needed to excite the fluorophores they express. The availability of commercial systems means that investigations of bacterial growth and division and the response to environmental changes are once again only constrained by our ingenuity.

MYCOBACTERIAL IMAGING

Technological advances in micro- and nanotechnology permit the study of microorganisms at the single-cell level. Electron microscopy provided insights into mycobacterial cell division (Dahl 2004), showing *Mycobacterium tuberculosis* forming branching structures with

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V-shaped cells undergoing a “snapping” mechanism of cell division common to actinomycetes. Electron micrographs allow high cellular detail to be visualized, but tell nothing about the dynamics of cell division and chromosome segregation; this requires time-resolved images that represent separate growth stages to visualize the process in its entirety. Live-cell time-lapse imaging allows single cells to be imaged periodically, so that the movement of a protein during cell growth and division can be observed directly and quantified to assess cell-to-cell variability (Locke and Elowitz 2009). Advances in microscopy, fluorescent proteins, and dye technologies have allowed multiple protein and cellular structures to be viewed simultaneously, which, coupled with time-lapse imaging, is emerging as a powerful tool to investigate mycobacterial cell division.

Live-Cell Time-Lapse Imaging

To date, only a few studies of cell division in mycobacteria have been reported using this technology (Joyce et al. 2011, 2012; Aldridge et al. 2012; Golchin et al. 2012; Santi et al. 2013; Wakamoto et al. 2013). The method is not without challenges; mycobacteria grow slowly and tend to divide to form disorganized microcolonies, which impacts on the imaging protocol to ensure the microscopy conditions remain permissive for growth and capture in-focus images. Fluorescence microscopy requires an excitation source (laser, xenon, or helium lamp) to produce a detectable emission from the fluorophore, but prolonged high-intensity light causes phototoxicity, affecting cell viability. Two methods of growing mycobacteria on thin agar pads or in microfluidic devices, both housed in temperature controlled chambers and imaged by bright-field or confocal microscopy, have been reported. Growth on thin agar pads on a microscope slide has been used to study fundamental processes such as bacterial growth patterns, protein localization dynamics, antibiotic action, and bacterial persistence (Fiebig et al. 2006; Thanbichler and Shapiro 2006; Letek et al. 2008). However, the environmental conditions are difficult to manipulate, and it is

challenging to prevent desiccation of the agar pad and maintain levels of oxygen and nutrients for the longer experiments needed for slow-growing mycobacteria. A modified agar pad method for mycobacteria was developed (Joyce et al. 2011) using glass-bottomed dishes and an agar pad, with the bacteria sandwiched between coverslip and agar, and imaged with an inverted microscope. This method allowed imaging over longer times (>65 h); can be used with fluorescence microscopy and permits some manipulation of environmental conditions, by diffusion through the thin agar pad during imaging (Joyce et al. 2012). The availability of multiwell glass-bottomed plates and automated microscope stages means that this method could be used for higher-throughput analyses. However, more complex alterations of environmental conditions, such as changing the medium composition or removal of antibiotics, are impractical.

Microfluidic devices are more sophisticated and expensive, but they are a rapidly advancing technology, with commercial platforms now available as an alternative to in-house devices. This technology has been used to study mycobacterial cell division (Aldridge et al. 2012; Golchin et al. 2012; Santi et al. 2013; Wakamoto et al. 2013). Typical custom-built microfluidic devices use photolithographic methods to create a positive template of microchannels which is used to cast negative-aspect replicas in polydimethylsiloxane (PDMS). The PDMS structures are bonded to glass slides, and holes are added to make input and output channels for the addition and removal of growth medium using syringe pumps (Aldridge et al. 2012; Golchin et al. 2012; Santi et al. 2013; Wakamoto et al. 2013). Continuous perfusion of the cells ensures a suitable growth environment for long-term experiments and permits manipulation of environmental conditions, such as the addition or removal of antibiotics. Such devices have the advantage that they can restrict movement of the cells in the Z-plane, keeping them in focus, although it is unclear as yet whether this is detrimental to mycobacterial growth. To date, only imaging of the fast-growing *Mycobacterium smegmatis* has been reported using microfluidics. Recent advances include the development

of microfluidic plates and automated perfusion systems (e.g., the CellASIC ONIX Microfluidic Perfusion Platform [Lee et al. 2009]) permitting higher-throughput and automated methods of single-cell tracking. Although not without drawbacks, live-cell imaging using microfluidics is arguably now the method of choice, and it will be interesting to see if super-resolution microscopy can be incorporated into such systems.

ASYMMETRIC GROWTH

Several groups have studied cell elongation and septum placement in mycobacteria, reaching different conclusions about the asymmetry or symmetry of these processes. However, some of the disagreements may be attributed to the use of different experimental conditions and analysis methods (Table 1 and Fig. 1), as will be discussed below.

Unipolar/Bipolar Growth

The absence from actinomycete genomes of the actin homolog MreB, which directs lateral wall biogenesis (Carballido-López 2006), together with the localization of nascent peptidoglycan synthesis to the cell tips of mycobacterial and corynebacterial cells, supports polar growth in these bacteria (Daniel and Errington, 2003; Thanky et al. 2007; Hett and Rubin 2008). Additional work showed a role for Wag31—homolog of the Gram-positive cell-division protein DivIVA—in the maintenance of cell morphology (Kang et al. 2008).

Polar growth has been the topic of several recent studies. Aldridge et al. (2012) proposed that mycobacterial growth is unipolar and mainly from the old cell pole. However, exclusively unipolar growth was not observed by other authors, although some did notice a different growth rate between poles without reference to pole age (Joyce et al. 2012; Singh et al. 2013). Another study showed the same rate of mycobacterial growth from both poles (Santi et al. 2013). According to the Aldridge model, after division, one daughter cell inherits the growing (old) pole, whereas its sibling creates a new

growth pole, leading to a slower elongation rate. As the growth pole matures, cells elongate faster and birth length increases, assuming that cell cycle is dependent on time, not cell size (Aldridge et al. 2012). The results and conclusions reached by Santi et al. (2013) are nonetheless quite different. In their model, old and new poles elongate at the same rate. However, they quantified exponential growth rate, considering elongation velocity to be size-dependent (larger cells growing faster), and their data show that the cells inheriting the old pole tend to be larger at division and so grow faster than the new pole cells (Santi et al. 2013). The elongation rate remains nevertheless the same in sibling cells, as described by Wakamoto et al. (2013). Thus, this model supports the exponential growth of single cells, as suggested for other bacteria (Godin et al. 2010; Mir et al. 2011), and is in agreement with a size-control mechanism that would prevent cells from dividing before reaching a minimum size; once achieved, the division would proceed in a random fashion (Santi et al. 2013).

Antibiotic Susceptibility/Persistence

Supporters of unipolar growth suggested related consequences for antibiotic susceptibility (Aldridge et al. 2012). The cells inheriting the growing pole were more sensitive to several peptidoglycan synthesis inhibitors, as expected, and also to isoniazid, targeting mycolic acid synthesis, but more resistant to the RNA polymerase inhibitor, rifampicin (Aldridge et al. 2012). Thus, variability in the susceptibility of these two populations of cells to antibiotics was proposed (Aldridge et al. 2012). The association between growth rates and sensitivity to isoniazid was later challenged, as other investigators did not find a correlation (Wakamoto et al. 2013). In contrast, a weak positive association was described between survival in the presence of isoniazid and the inheritance of the old pole (Wakamoto et al. 2013). Santi et al. (2013) also did not find any difference between old pole and new pole cells regarding sensitivity to the antibiotics tested by Aldridge et al. (2012), although the possibility that the different techniques used

Table 1. Summary of reported live cell time-lapse imaging studies of mycobacterial cell division

Study	Growth conditions	Size-dependent cell division	Septum marker	Septum placement	Asymmetric growth	Asymmetric division	Growth rate estimation method
Joyce et al. 2011, 2012	Agar pad	No	PBP1a-mCherry (mid)	Toward midcell (87% within central 20th percentile) n.d.	Yes Bipolar, but unequal velocities Yes Unipolar	Yes	Exponential
Aldridge et al. 2012	Microfluidics	No (time-dependent)	None	n.d.		Yes	Linear
Singh et al. 2013	Agar pad	No	FtsZ-mCherry (early)	Random	Yes Bipolar, but unequal and unipolar	Yes	n.d.
Santi et al. 2013	Microfluidics	No (or time-dependent)	Wag31-GFP (late)	Toward midcell but slight bias toward new cell pole	Yes Bipolar, but equal velocities	Yes	Exponential

Summary of results reported for the recently published studies, highlighting the marker used and results obtained.
n.d., not determined.

Figure 1. Cartoon summarizing the results published to date on mycobacterial cell division using time-lapse imaging. A timeline has been constructed to try to reconcile the different results reported for polar and asymmetric growth. PG, peptidoglycan.

could affect the results cannot be discarded (Santi et al. 2013). Survival in the presence of isoniazid was attributed to single-cell dynamics of KatG, the isoniazid-activating enzyme; cells expressing KatG in stochastic pulses would be more sensitive to the drug (Wakamoto et al. 2013). As the survival of sibling cells was positively correlated, it was suggested that epigenetics contribute to this phenomenon. This result supports the opinion that persistent cells may continue to grow in the presence of antibiotics and that the stochastic expression of any factor which promotes or inhibits the activity of the antibiotic could be key in the survival of single cells (Wakamoto et al. 2013). That the persistent state of *M. tuberculosis* in chronic infection may be attributable to on-going bacterial replication is in opposition to models that defend a non-replicating state (Gill et al. 2009). Other work on *M. smegmatis* showed that some cells exit dormancy stochastically and independently of time or environmental factors, perhaps through the stochastic expression or repression of a master regulatory gene (Buerger et al. 2012). These results suggest that slowing of growth may not be

the only common strategy for microorganisms to achieve persistence (Buerger et al. 2012).

Septum Positioning

The apparently asymmetric septum placement observed could be a consequence of the asymmetric polar growth reported (Aldridge et al. 2012; Joyce et al. 2012; Singh et al. 2013). Indeed, this is the conclusion of Joyce et al. (2012), using penicillin-binding protein 1a (PBP1a) and Van-BODIPY as markers of poles and septum, respectively. They found that septum placement is less accurate in mycobacteria than in corynebacteria, but a mid-cell position was favored. However, this was altered by subsequent unequal polar growth, leading to asymmetric division. Septum placement was independent of cell length (Joyce et al. 2012); other investigators state that cells need to reach a critical size before division (Santi et al. 2013; Singh et al. 2013). Singh et al. (2013) showed that FtsZ ring positioning was nearly random, but when using membrane (FM4-64) or cell wall (FL-Vanco) markers, the septum position was found

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to be more symmetric. Finally, when using the late-division protein Wag31 as a septum marker, it was biased toward the new pole (Santi et al. 2013). These discrepancies might be because of the use of different markers; FtsZ is probably the earliest component in septum formation, with membranes, PBP1a, and cell wall components recruited at later stages. Alternative explanations taking this into account may bring these apparently disparate observations together. If the FtsZ ring is placed nonsymmetrically, then subsequent unequal polar growth may lead to an apparent mid-cell positioning of later septum components. As growth continues, the septum is again found in an asymmetric position before cell division, when Wag31 is located there (Joyce et al. 2012; Santi et al. 2013; Singh et al. 2013). Asymmetric division sites have been reported over the nucleoids, requiring a DNA translocase to transfer the DNA through the septum before it closes (Singh et al. 2013); however, this has not been reported by others. More observations are required to account for the heterogeneity reported in mycobacterial growth and division, and investigations using consensus cell division markers in the different experimental systems would further help clarify the situation.

Asymmetric growth and division is widespread in bacteria and may have evolved multiple times (Kysela et al. 2013), because it influences population aging and produces phenotypic heterogeneity. This could provide fitness advantages for the population through a bet-hedging strategy (Kussell and Leibler 2005). The recent work in mycobacteria suggests that phenotypic heterogeneity could arise at multiple levels through asymmetric polar growth and inaccurate cell division. The ultimate importance of the specific forms of growth asymmetries in host–pathogen interactions is a key area for further investigations.

Drug-Resistant Strains: Growth and Division

It is interesting to note the characteristic morphology observed in resistant *M. tuberculosis* strains (Velayati et al. 2009a,b; Farnia et al. 2010). During exponential growth, multidrug-resistant (MDR) and susceptible isolates are

mainly rod-shaped, whereas oval, rounded, or branching cells are seen in extensively drug-resistant (XDR) isolates (Farnia et al. 2010). XDR subpopulations also displayed an extremely thick wall (Velayati et al. 2009b). Symmetrical, asymmetrical, and branching division were observed in all isolates, but XDR isolates displayed a novel division type in which a rod-shaped mother cell produced a small round bacillus (Farnia et al. 2010). These changes in morphology may help these strains escape immune responses and adapt to the host environment (Champion and Mitragotri 2006; Farnia et al. 2010).

CHROMOSOME SEGREGATION

Before cell division, the replicated chromosomes must be partitioned into the daughter cells. The presence of chromosomal homologs of the genes needed to segregate low-copy-number plasmids during cell division raises the possibility that chromosomes are segregated in a similar way. Both plasmid and chromosome encoded *par* loci consist of an ATPase (ParA), a site-specific DNA-binding protein (ParB) that interacts with ParA, and a DNA sequence motif (*parS*) to which ParB binds. The type I plasmid-encoded *par* loci encode a Walker A Cytoskeletal ATPase and are the ones also found on bacterial chromosomes (Ebersbach and Gerdes 2005).

The chromosomal ParAB system has been studied in several bacteria including *Mycobacterium spp.* (Jakimowicz et al. 2007; Casart et al. 2008; Maloney et al. 2009; Nisa et al. 2010; Chaudhuri and Dean 2011; Ginda et al. 2013). In *Caulobacter crescentus* (Ptacin and Shapiro 2010), the only species in which *parA* and *parB* are essential, a model was proposed in which ParA dimerizes when bound to ATP and forms linear polymers. These polymers are dissociated from the ends, stimulated by the binding of the ParB-*parS* partition complex. As ParA depolymerizes, the partition complex moves toward the new pole during chromosome segregation.

Chromosomal *par* loci are located proximal to and upstream of *oriC* (Lin and Grossman 1998). Genes homologous to *parA* and *parB* have been identified in all mycobacteria se-

quenced so far (Jakimowicz et al. 2007). Two *parS* copies, identical to those in *Streptomyces coelicolor* (Jakimowicz et al. 2002), have been identified in *M. smegmatis*, *Mycobacterium bovis* BCG (Bacillus Calmette–Guérin) and *M. tuberculosis* (Jakimowicz et al. 2007; Casart et al. 2008), and a third *parS* copy with one mismatch to the consensus is also located close to *oriC*. These *parS* sequences interact with ParB in *M. smegmatis* (Jakimowicz et al. 2007). The *par* genes are arranged in an operon, where they cluster with another six conserved genes (including *gidB*), upstream and divergent to the *dnaA* gene (Casart et al. 2008). One *parS* sequence is located in the *gidB* promoter, and the other is in the coding region of *parA*, suggesting that *parB* could regulate both *gidB* and *parA* expression and control the levels of Par proteins (Casart et al. 2008). Indeed, the amino-terminal domain of ParB does interact with *parS* (Chaudhuri and Dean 2011). The mycobacterial ParB–*parS* partition complex involves ParB–ParB interactions (Ginda et al. 2013). On binding to *parS* sequences, it has been proposed that in *M. tuberculosis*, ParB dimers undergo a drastic compaction, which primes ParB spreading on the *parS*-adjacent DNA scaffold to form the type of higher-order partition assembly (Chaudhuri and Dean 2011) described for other bacteria (Murray et al. 2006; Breier and Grossman 2007).

The *parA* and *parB* genes are annotated as essential in *M. tuberculosis* based on transposon mutagenesis (Sasseti et al. 2003; Zhang et al. 2012). However, they are nonessential in *M. smegmatis* (Jakimowicz et al. 2007; Ginda et al. 2013), in which both locate to the poles of the cell (Maloney et al. 2009; Ginda et al. 2013). However, this localization pattern was only studied with static microscopy, and it may be dynamic in live cells, as seen in *C. crescentus* (Ptacin and Shapiro 2010). In fact, a gradient of ParA from one pole toward the other has been observed in *M. smegmatis* using in vivo time-lapse microscopy (G Joyce, KJ Williams, and BD Robertson, unpubl.). In contrast in *Corynebacterium glutamicum*, ParB remains stably attached to the cell poles during time-lapse experiments (Donovan et al. 2010). The bipolar

localization of ParB observed in static microscopy is altered when *parA* is deleted; however, the deletion of *parB* does not seem to alter ParA localization, suggesting that mislocalized partition complexes have a more negative influence on chromosome segregation than their complete absence (Ginda et al. 2013).

Overproduction or deletion of ParA or ParB causes growth inhibition and severe chromosome segregation defects (Jakimowicz et al. 2007; Maloney et al. 2009; Ginda et al. 2013). Deletion of either *parA* or *parB* leads to a high percentage of anuclear cells, three times higher in the *parA* deletion strain (Ginda et al. 2013). Surprisingly, the *parAB* mutants have milder growth defects and fewer anucleate cells, suggesting that a specific ParA:ParB ratio is crucial for correct operation. ParA colocalizes and interacts with Wag31, and aberrations in cell length (elongated or shortened cells) were observed in all three mutants (Ginda et al. 2013). Thus, the ParAB system could coordinate chromosome segregation with other cell cycle processes through interaction with a polar determinant, as has been proposed in *C. crescentus* and *C. glutamicum* (Ptacin and Shapiro 2010; Donovan et al. 2012).

To achieve a mechanistic understanding of chromosome segregation, septum placement, asymmetric growth, and their interdependence, mathematical modeling of the spatiotemporal dynamics of the key players will be essential. Similar modeling approaches have provided insight into the Min system in *Escherichia coli* (Kruse et al. 2007) and ParAB-mediated plasmid partitioning (Ringgaard et al. 2009). Quantitative data can be used to test the validity of different mechanisms, for example, theory sets physical limits to the positional information and accuracy provided by protein gradients (Tostevin et al. 2007). Modeling can also shed light on fitness advantages of observed asymmetries and heterogeneities in growth and division (Kussell and Leibler 2005).

Regulation of Growth and Cell Division

Bacterial eukaryotic-type serine/threonine protein kinases (STPKs) transmit signals from the

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extracellular environment via the reversible phosphorylation of proteins, permitting bacteria to adapt to external changes. In mycobacteria, these kinases are thought to be involved in several processes, including cell shape, growth, and cell division. *M. tuberculosis* possesses 11 STPKs (PknA-PknL, except C), whose biochemical activity and substrates have been widely studied and reviewed (Hett and Rubin 2008; Molle and Kremer 2010). More recently, PknA and PknB have been implicated in the phosphorylation of the lone Ser/Thr phosphatase (PstP) in *M. tuberculosis*, suggesting a signaling regulation of STPKs/PstP through mutually dependent mechanisms (Sajid et al. 2011). PknB—which localizes at mid-cell and poles (Mir et al. 2011)—phosphorylates the peptidoglycan biosynthetic protein MviNP, leading to the recruitment of FhaA, which modulates peptidoglycan synthesis at the cell poles and septum (Gee et al. 2012; Warner and Mizrahi 2012). Osmotic stress stimulates a signaling pathway in *M. tuberculosis* regulated by PknD (Hatzios et al. 2013). PknE kinase has been implicated in the suppression of apoptosis during nitrate stress and an adaptive response regulating cellular integrity and survival (Kumar and Narayanan 2012; Kumar et al. 2013). PknI and PknK are thought to be involved in slowing the growth of *M. tuberculosis* during infection (Gopalaswamy et al. 2009; Malhotra et al. 2010), whereas PknJ phosphorylation of pyruvate kinase A (mtPykA) affects central metabolism (Arora et al. 2012) and PknL may modulate apoptosis (Lakshminarayan et al. 2008).

Two-component systems (TCSs) are formed by a sensor kinase that autophosphorylates when receiving signals from the environment, transferring a phosphoryl group to a response regulator, which then activates specific regulons for adaptation to the signal sensed. *M. tuberculosis* encodes 11 TCSs, and the MtrAB system has been implicated in the maintenance of cell wall integrity (Moker et al. 2004; Cangelosi et al. 2006; Nguyen et al. 2010; Plocinska et al. 2012). Expression of the MtrA regulon is necessary for regulated cell division, and one of these genes encodes the essential cell wall hydrolase RipA (Plocinska et al. 2012). MtrB localizes at the

cell poles, membranes, and septa in an FtsZ-dependent manner, and MtrA regulon expression is compromised under conditions that interfere with MtrB septal localization, suggesting that MtrB sensor kinase activation takes place at the septum (Plocinska et al. 2012).

Rpfs

The first resuscitation promoting factor protein (Rpf) was described in *Micrococcus luteus* (Mukamolova et al. 2002). Its name indicates its capacity to promote the resuscitation of dormant bacteria. This secreted protein has homologs among other high G + C Gram-positive bacteria, including *Mycobacterium* spp. *M. tuberculosis* contains five Rpf homologs, RpfA-E, scattered throughout the genome, that can resuscitate dormant BCG or *M. tuberculosis* (for review, see Kana and Mizrahi 2010). All of them contain putative signal sequences at the amino terminus, suggesting that they are active extracellularly (Kana and Mizrahi 2010). Rpfs share a conserved 70-amino-acid region, the Rpf domain, responsible for biological activity (Mukamolova et al. 2002). This domain is similar to C-type lysozyme and the *E. coli*-soluble lytic transglycosylase 70 (Cohen-Gonsaud et al. 2005), both involved in the degradation of peptidoglycan; the muralytic activity of this family of proteins has been shown (Mukamolova et al. 2006; Telkov et al. 2006). Peptidoglycan hydrolysis could produce muropeptides that might modulate innate immune responses or activate the cell resuscitation pathway (Jo 2008; Kana and Mizrahi 2010; Nikitushkin et al. 2013). Recently, a novel class of 2-nitrophenylthiocyanates has been found to inhibit the muralytic activity of Rpfs and prevent the resuscitation of dormant *M. smegmatis* and *M. tuberculosis*, which makes them interesting candidate compounds against non-replicating mycobacteria (Demina et al. 2009). The crystal structure of RpfB catalytic domain in complex with the lysozyme inhibitor NAG3 suggests that Glu-292 is the sole residue essential for catalysis in this protein (Squeglia et al. 2013). The use of the structure of RpfB to model the catalytic domains of the other four Rpfs

shows a high degree of similarity but also differences among these five proteins (Squeglia et al. 2013). The expression of all five *rpf*s varies in the function of the growth phase and under different stress conditions, but all of them are expressed in the early exponential phase, and the relative expression of all five is enhanced in resuscitation phase (Gupta et al. 2010). Differential expression of the five *rpf* genes in *M. tuberculosis* appears to be related to differential regulation. *rpfA* has been shown to be regulated by the cAMP receptor protein, and *rpfC* by the alternative σ factor SigD and the site two protease homolog, Rv2869c (Raman et al. 2004; Makinoshima and Glickman 2005; Rickman et al. 2005).

The five *M. tuberculosis* *rpf* genes are not essential for growth in vitro and in vivo, individually or collectively (Downing et al. 2004; Tufariello et al. 2004; Kana et al. 2008), but triple, quadruple, and quintuple mutants show impaired spontaneous resuscitation in vitro and are attenuated in vivo (Downing et al. 2005; Kana et al. 2008). The quintuple mutants show increased sensitivity to inhibitors of late peptidoglycan biosynthesis steps, probably owing to increased cell envelope permeability (Kana et al. 2010; Wivagg and Hung 2012). The in vivo attenuation of the multiple mutants led to their consideration as novel vaccine candidates. Both quadruple *rpf* deletion mutants, Δ ACBD and Δ ACDE, induced protection in the mouse model of tuberculosis infection (Kondratieva et al. 2011). RpfB and RpfE are sufficient for normal growth, suggesting a functional hierarchy (Kana et al. 2008), and both interact with the cell wall hydrolase RipA, whereas RpfB and RipA colocalize in the septum of dividing cells, suggesting that the RpfB–RipA interactions could be involved in the separation of daughter cells during reactivation (Hett et al. 2007, 2008). RipA also interacts with the PBP1, a peptidoglycan-synthesizing enzyme, and a model was proposed in which PBP1 competes with RpfB for binding to RipA, thus limiting RipA–RpfB peptidoglycan hydrolyzing activity (Hett et al. 2010). RpfB is considered a promising candidate for inclusion in novel tuberculosis vaccines (Romano et al. 2012).

THE CONTROVERSY OF MYCOBACTERIAL SPORULATION

The ability of *M. tuberculosis* to cause long-term subclinical infections that later develop into clinically active disease is one of the main obstacles to tuberculosis control. The production of spores by mycobacteria could be a bacterial strategy to survive during this long subclinical phase, but this possibility has not been widely accepted. Studies from the 1950s suggested the existence of spores in old mycobacterial cultures (e.g., see Brieger and Glauert 1956), but they were not reproducible and often declared to be caused by contamination or artifacts (for references, see Traag et al. 2010 and Singh et al. 2010).

More recently, Ghosh et al. (2009) reported the existence of endospores in old cultures of *M. marinum* on solid agar medium and suggested their existence in *M. bovis* BCG. To ensure the absence of contaminants, the germination of individual endospores was tracked, as was the return to sporulation in late stationary phase. An endospore-like structure was visualized using transmission electron microscopy, and the *M. marinum* endospores tested positive for spore-specific stains, resistance to heat and chemical stress, and the presence of dipicolinic acid (Ghosh et al. 2009). These investigators also suggested that the asymmetric placement of the septum during cell division observed in mycobacteria could be in agreement with their ability to form spores (Singh et al. 2010).

These results (Ghosh et al. 2009) were disputed by Traag et al. (2010), who failed to detect the presence of spores in *M. marinum* cultures after reproducing the experimental procedures of Gosh and colleagues. They were also unable to recover spores from frogs chronically infected by *M. marinum*. The observation that the endospores found were strikingly similar to those of *Bacillus subtilis* (Ghosh et al. 2009) suggested a possible contamination of the samples. In a follow-up paper from the Kirsebom laboratory, differences in the appearance between these two kinds of spores were reported (Singh et al. 2010). Traag et al. (2010) showed that mycobacteria lack orthologs of key genes involved in

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endospore formation in low G + C Gram-positive bacteria, perhaps unsurprising for high G + C mycobacterium species. It has also been noted that although rare, some species of *Streptomyces* can form endospores under specific conditions (Stastná et al. 1992) and that the absence of homologous sporulation genes does not prove the absence of proteins with similar function (Singh et al. 2010).

Although it remains possible that spores might be produced by *M. marinum* under certain conditions, Traag et al. (2010) were unable to reproduce this. More recently, spore-like structures were reported in a 1-yr-old liquid culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Lamont et al. 2012). These spores were resistant to heat and chemical stresses and positive for MAP 16sRNA and dipicolinate, but not for gene elements of well-known endospore-forming strains. Although these resemble endospores, the authors note that they are also reminiscent of *Streptomyces* spp. aerial hyphae spores. The fact that MAP has been found as a contaminant in pasteurized food supports the existence of spores in this species (Lamont et al. 2012). The existence of spores in mycobacterium species is still controversial, and the exact conditions leading to their formation need to be more widely studied to produce sufficient data to clarify the situation.

CONCLUSIONS

This is an exciting time for research on bacteria at the single-cell level, and we now have the tools to address questions about phenotypic variation within genetically clonal populations, and how this impacts on cell survival in the face of environmental stress.

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