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Growth, cell division and sporulation in mycobacteria

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Abstract Bacteria have the ability to adapt to different growth conditions and to survive in various environments. They have also the capacity to enter into dormant states and some bacteria form spores when exposed to stresses such as starvation and oxygen deprivation. Sporulation has been demonstrated in a number of different bacteria but *Mycobacterium* spp. have been considered to be non-sporulating bacteria. We recently provided evidence that *Mycobacterium marinum* and likely also *Mycobacterium bovis* bacillus Calmette–Guérin can form spores. Mycobacterial spores were detected in old cultures and our findings suggest that sporulation might be an adaptation of lifestyle for mycobacteria under stress. Here we will discuss our current understanding of growth, cell division, and sporulation in mycobacteria.

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Introduction

Mycobacterium

Bacteria of the genus *Mycobacterium* are acid fast, show a great resilience against environmental insults and they inhabit various environmental reservoirs e.g. ground and tap water, soil, animals and humans. They are grouped among the actinomycetes and include nonpathogens as well as highly successful pathogens e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans*, the etiological agents of tuberculosis (TB; 10 million new cases per year develop active disease), leprosy and Buruli ulcer (the third most common mycobacterium infection), respectively (see e.g. Sasaki et al. 2001; Russell 2007; Demangel et al. 2009). While *M. tuberculosis* is human-specific, the closely related *Mycobacterium bovis* can infect both humans and animals and lead to TB regardless of host. Another *Mycobacterium* is the multihost chronic enteric pathogen *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) that causes chronic diarrhea among ruminants (Johne's disease). It can be present in food products derived from cattle (Chacon et al. 2004) and some data suggest that *MAP* is the causative agent of Crohn's disease in humans

(see e.g. Greenstein 2003; Uzoigwe et al. 2007). Mycobacteria are also commonly present in water, e.g. *Mycobacterium avium* and *M. ulcerans*, and hence they can constitute a threat to both animals and humans. Interestingly it appears that mycobacteria have a growth advantage in water that contains disinfecting agents e.g. chlorine (Vaerewijck et al. 2005; Primm et al. 2004). Moreover, *Mycobacterium marinum* causes a systemic TB-like infection and disease in ectothermic hosts e.g. fish and frogs. In comparison to *M. tuberculosis*, *M. marinum* grows with a relatively short doubling time and growth is limited at higher temperatures, making *M. marinum* easier to handle than e.g. *M. tuberculosis* and study in the laboratory. *M. marinum* has therefore become a model system to identify and study factors important for mycobacterial infections, disease development and its persistence (Ramakrishnan 2004).

Mycobacteria can grow in fluid environments, they can form aggregates, form biofilms and they are invasive i.e. they can grow and multiply within cells such as macrophages. Inside the macrophage they reside within a membrane-bound cytoplasmic vacuole referred to as the *Mycobacterium* phagosome. Pathogenic mycobacteria such as *M. tuberculosis* avoid phagolysosomal degradation by: (i) blocking acidification of the phagosome, (ii) altering the protein content of the vacuole and (iii) preventing interactions with other endosomal compartments. This ensures survival of the bacteria within the macrophage and subsequent spread of the pathogen to other cells e.g. dendritic cells and neutrophiles (Wayne and Sohaskey 2001; Glickman and Jacobs Jr 2001; Flynn and Chan 2003, 2005; Deretic et al. 2004; Nguyen and Pieters 2005; Vergne et al. 2004; Ojha et al. 2005; Carter et al. 2003).

Mycobacterium tuberculosis and other mycobacteria can also establish long-term infections that lead to acute or chronic stages of disease or even a stage that is clinically asymptomatic. In the latter situation the bacteria have the potential to resume growth, resulting in active disease, after several decades of dormancy resulting in active disease. This asymptomatic infection or latent stage is a long-term hidden threat to the host. Moreover, in the persistent stage mycobacteria reside in granulomas, which are organized collections of differentiated macrophages and other cell types. One important question is how mycobacteria survive within the granulomas for such a long time. It has been hypothesized that the bacteria remain in a dormant nonreplicative (or low level of replication) state at the centre of the granuloma or that the load of bacteria is kept constant through a dynamic equilibrium between killing by the immune system and bacterial replication/growth (Monack et al. 2004; Russell 2007).

Transition into dormancy and maintenance of the passive state until an appropriate signal(s) activates the vegetative, virulent state is one of the remarkable attributes of mycobacterial pathogens. As this entails temporary cessation of growth and cell division and their restart, the controls of bacterial growth, chromosome duplication, segregation and cell division must be clearly understood at the basic level for any meaningful insight into mycobacterial dormancy and virulence. It was during our investigations into the stages of growth, the basic cell cycle mechanisms and their controls in *M. marinum*, that we came upon the spore-forming ability of mycobacterial strains (Ghosh et al. 2009). We have continued those studies into the assembly and execution mechanisms of the cell division machinery in *M. marinum* and more recently also in *M. smegmatis*. In the present article, we will first briefly describe our current understanding of the division site selection process in bacterial model organisms. In view of our discovery that mycobacteria are capable of forming spores, we will then examine how mycobacteria might modify the division-site selection process to suit their own structural and proliferation requirements. We will also discuss the recently published criticism of our finding (Traag et al. 2010).

Growth and cell division

Most rod-shaped bacteria we know about grow along their axis and divide by binary fission. Cell division occurs by formation of a division septum at the midcell region after chromosome replication has been completed and the two daughter chromosomes have segregated into two halves of the cell (Harry 2001; Errington et al. 2003; Trevors 2004). The precise placement of the division site in coordination with growth, replication and segregation is critically important for propagation and all bacteria must have evolved elaborate division site selection systems appropriate for the growth and division schedule specific for their species (Goehring and Beckwith

2005). Most of our understanding of division-site selection systems has been acquired from investigations into the growth and division mechanisms of the model gram-negative and gram-positive organisms *Escherichia coli* and *Bacillus subtilis*, respectively (Harry 2001; Errington et al. 2003; Lutkenhaus 2007; Adams and Errington 2009). The midcell placement of the FtsZ ring, the nucleating site for the division machinery (or divisome) in these cells is ensured by a combination of negative control systems. The MinCDE (or MinCDDivIVA) proteins prevent the Z-ring formation and subsequent divisome assembly at the cell poles. While the nuclear occlusion factors Noc and Slm prevent polymerization of FtsZ over the nucleoids; the nucleoid-free midcell region is thus the only zone available for FtsZ ring formation, the nucleation site for divisome assembly (Margolin 2001; Lutkenhaus 2007; Errington et al. 2003; Rothfield et al. 2005; Bernhardt and de Boer 2005; Wu and Errington 2004; Wu et al. 2009). Mycobacteria, however, possess uniquely complex cell walls that offer additional challenges to cell division requiring specialized mechanisms in addition to the general problems of coordination with growth and chromosome segregation (Hett and Rubin 2008). Also, as will be discussed below, mycobacteria apparently lack some key cell division proteins such as MinCDE, ZipA and ZapA that are involved in division site selection.

Symmetric cell division

Both in *E. coli* and *B. subtilis*, the cell division is orchestrated by FtsZ, a small prokaryotic cytoskeletal protein analog of eukaryotic tubulin, which polymerizes as a ring at a preselected site acting as a scaffold for assembly of the division machinery (Dajkovic and Lutkenhaus 2006; Barák and Wilkinson 2007). The Z-ring recruits all the downstream components of the divisome and participates in the complete division process. FtsZ is almost universally conserved and has also been identified in *M. tuberculosis* as one of the major cytoskeletal organizers of the mycobacterial divisome (Hett and Rubin 2008; Dziadek et al. 2003; Chen et al. 2007).

The spatial regulation of Z-ring formation at the midcell in *E. coli* and *B. subtilis* is enforced by two negative regulators: the Min and the nucleoid occlusion systems. In *E. coli* the Min system comprises the MinC and MinD proteins that are responsible for elimination of the possibility of division at the cell pole. MinC inhibits FtsZ ring formation while MinD, a membrane-associated ATPase, functions as an activator for making the FtsZ ring. Visualization of the interior of live cells using fluorescence-tagged proteins has revealed that the MinCD complex undergoes pole-to-pole oscillation with a periodicity of 10–20 s along a spiral trajectory distinct from the spiral of the cytoskeletal shape-determinant MreB protein (Rothfield et al. 2005; Lutkenhaus 2007). The remarkable oscillation of the MinCD complex is driven by the cyclic association of MinD-ATP with the membrane, ATP hydrolysis by MinE and release of MinD-ADP and MinE into the cytoplasm. The FtsZ-inhibitor MinC forms a complex with MinD and co-oscillates with it such that its time-integrated concentration is the lowest at the midcell region and highest at the cell poles (see Margolin 2001; Barák and Wilkinson 2007; Lutkenhaus 2007). On the other hand, *B. subtilis* lacks MinE and does not exhibit MinCD oscillation. DivIVA, a protein without any similarity to MinE, controls MinCD by preventing FtsZ ring formation at the cell poles. DivIVA, localized at the cell poles, recruits MinCD by binding directly with MinD creating a pole-to-midcell concentration gradient of MinC. It has been demonstrated recently that the affinity of DivIVA for the poles is dictated by curvature of the membrane at the poles alone. It is not affected by any of the known cytoskeletal proteins (Lenarcic et al. 2009; Ramamurthi and Losick 2009). The primary role of MinCD in vegetatively growing *B. subtilis* is the prevention of Z-ring formation at the poles; nuclear occlusion ensures the midcell positioning of the division site.

Asymmetric cell division

There are special circumstances that drive *B. subtilis* and some *Actinomycetes* into sporulation as a survival strategy under extreme stress. In the case of *B. subtilis* the first morphological step after commitment to sporulation is formation of polar septa eventually leading to asymmetric cell division (Errington 2003; Errington et al. 2003; Hilbert and Piggot 2004). The division site is shifted from midcell to a position close to the cell poles. The septum is still formed by recruiting divisome components with a Z-ring as the nucleation center but its ultrastructure is altered in

preparation of the downstream events that include conjugational uptake of one of the chromosomes after segregation and engulfment into mother cell. The genetics and physiology of this ordered series of complex events are only recently being unravelled. Starvation and crowding have emerged as the main but not the only stimulus for sporulation. The transcriptional regulator Spo0A is considered to be the master regulator for the switch from vegetative growth to sporulation controlling about 10% of all *B. subtilis* genes involved in the formation of spores. Spo0A expression is transcriptionally controlled and its activity is regulated by phosphorylation through several kinases in response to environmental stimuli and/or changes. Another component of the regulatory switch for sporulation is the stationary-phase sigma factor σ^H that combines with RNA polymerase to control the expression of more than 87 genes through 49 promoters (Britton et al. 2002). In addition to these positive transcription factors there are several negative regulators that keep sporulation suppressors under control (Ireton et al. 1994). Once the cell is committed to sporulation, a combination of increased FtsZ level and the expression of SpoIIIE cause disassembly of the medial Z-ring which now spirals out towards the poles (Ben-Yehuda et al. 2003). It is not clear how this combination of events overcomes the blockage of Z-ring formation near the pole caused by the Min system nor how the nuclear occlusion is bypassed.

Division site selection in mycobacteria

Unlike other gram-positive bacteria mycobacterial cell walls are not composed of a simple peptidoglycan network surrounding the membrane layer. Instead they comprise complex outer layers of arabinogalactan surrounding the peptidoglycan sacculus; the arabinogalactan layer itself acts as a scaffold-anchoring long-chain mycolic acids. This complex layered cell wall profoundly influences growth, survival under stress, pathogenicity and antibiotic resistance of mycobacterial species. Unlike other rod-shaped bacteria such as *B. subtilis* whose growth occurs by lateral intercalation of newly synthesized peptidoglycan along the helical MreB cytoskeleton, mycobacterial species of similar shape display a more flexible mode of apical growth where nascent peptidoglycan precursors are added exclusively at cell poles (Letek et al. 2008a, b; Thanky et al. 2007). (Mycobacteria appears to lack the helical MreB cytoskeleton since no MreB or MreB-analog have yet been identified.) Furthermore, when the cells' growth by polar extension, indicated by the presence of nascent peptidoglycan, has reached its limit of growth, building of the cell wall is shifted to the potential division site already marked by the FtsZ ring leading to the formation of the typical V-shape of dividing mycobacteria (Thanky et al. 2007; Singh et al. unpublished data; see also Lack and Tanner 1953; Dahl 2004). The mechanisms of division-site selection for formation of the Z-ring and subsequent recruitment of divisome components in mycobacteria remains to be elucidated. Neither the Min system nor the nucleoid occlusion mechanism are present in mycobacterial genome (no homologue systems have yet been identified). However, in the actinomycete *Corynebacterium glutamicum*, which also lacks MreB and MinCD, it was demonstrated that the FtsZ ring could assemble overlapping nucleoids even before they segregated, thereby confirming the absence of any nucleoid occlusion activity (Ramos et al. 2005).

In the absence of the spatial regulation by the Min system, DivIVA (or its analog Wag31) loses its role in septal site determination as in *B. subtilis*. Instead it operates more as a growth marker at the poles and is active in recruiting the cell wall synthesis machinery (Flärdh 2003a, b; Kang et al. 2005). Wag31 has also been found to bind PBP3 (FtsI), a transpeptidase required for peptidoglycan synthesis at the septum of dividing rod-shaped bacteria such as mycobacteria (Mukherjee et al. 2009). Thus recruitment of PBP3 might be needed for cell wall synthesis at growing poles as well as at the division site in mycobacteria (Datta et al. 2006; Scherr and Nguyen 2009). DivIVA forms higher-order homologs through its coiled coil domains. These have an affinity for curved membrane structures such that cell poles and division sites provide the topology that attracts DivIVA or Wag31 (Datta et al. 2006; Scherr and Nguyen 2009). Two serine/threonine protein kinases (PknA and PknB) are involved in Wag31 regulation in mycobacteria and they have also been implicated in controlling mycobacterial growth and shape (Kang et al. 2005). Overexpression of Wag31 is known to cause local outgrowth converting the rod-shaped cells into enlarged, bulging bowling pins (Nguyen et al. 2007), an indication that there has been a loss of regulation of growth and shape.

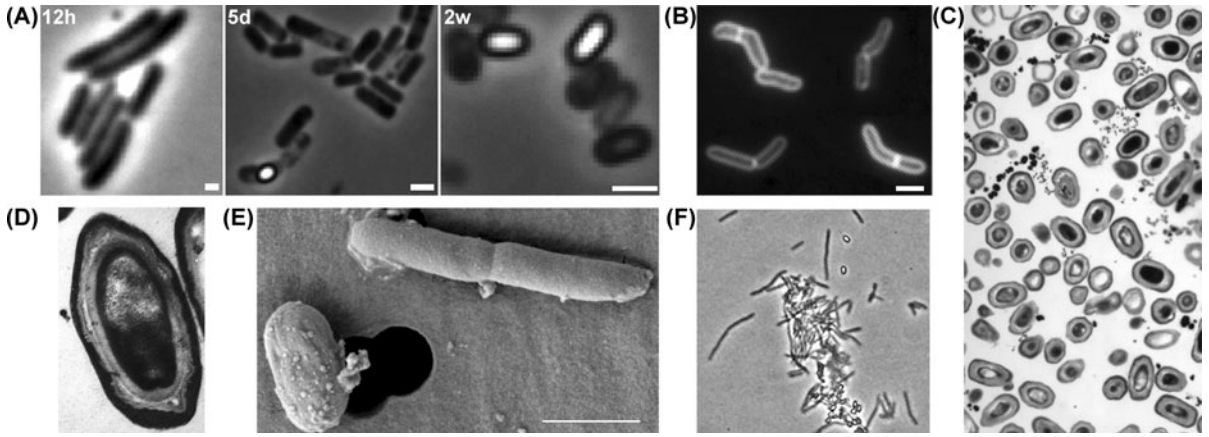

Fig. 1 *Mycobacterium marinum* spores. **a** Phase contrast micrograph of vegetative cells, 12 h; endospore, 5d; and spores, 2w. **b** FM4-64 (membrane dye) stained vegetative cells showing asymmetrical cell division. **c** Transmission electron micrograph showing the morphological diversity of spores; magnification ×2,000. **d** Transmission electron micrograph (TEM) of a mature spore; magnification ×60,000. **e** Scanning electron micrograph (SEM) showing mature spore and vegetative cell. **f** Malachite green staining for spores, spores appears green. (Space bar = 1 μm). For further details see Ghosh et al. (2009)

Analysis of a large number of *M. marinum* and *M. smegmatis* cells undergoing division during steady growth showed that the division-site placement in mycobacteria is not necessarily at midcell (Fig. 1b). This is consistent with the absence of spatial regulation by Min system in mycobacteria. However, the FtsZ ring is still the determinant in establishing division site as previously reported (Dziadek et al. 2003; Chen et al. 2007). Despite either the asymmetric or random selection of the division site, the completion of division of mycobacteria produces predominantly normal sized cells with an intact genome content (in the absence of any nucleoid-occlusion function). Thus mycobacteria must have some, as yet undiscovered, compensatory mechanism for correcting the lack of spatial regulation in division site placement such that newborn cells are not too unequal (Singh et al. in preparation). Recently, asymmetric/polar division was shown to occur in *M. tuberculosis* cells when partition protein ParB was overexpressed from an acetamide inducible promoter (Maloney et al. 2009) yielding nucleoid-free cells. This was different from the randomized division site during normal divisions in wild type *M. marinum* and *M. smegmatis* where production of nucleoid-free cells were rare possibly due to compensatory asymmetric growth at poles and chromosome translocations through the division site prior to cell division (Singh et al. in preparation). In view of endosporulation we emphasize that asymmetric septum formation is in keeping with the possibility that mycobacteria could enter a sporulation pathway if and/or when necessary (Ghosh et al. 2009) without any major switch in division site selection.

Sporulation in mycobacteria

We recently reported that spore like particles appeared in old cultures of *M. marinum* which disappeared during exponential growth and reappeared in the same culture late into the stationary phase (Ghosh et al. 2009). These particles possessed physical, biochemical, morphological and cell biological properties associated with spores and retained the genomic/genetic identity of *M. marinum*. For example we could demonstrate the presence of dipicolinic acid in old cultures, a biochemical hallmark of bacterial endospores (Church and Halvorson 1959; Paidhungat et al. 2000). When subjected to a classical, differential spore-staining protocol, the spore particles retained the malachite stain and appeared green while vegetative cells did not. Moreover, transmission electron microscopic (TEM) images showed that the structure of the *M. marinum* spore particles are morphologically similar to *Bacillus* spp. endospore (Fig. 1). Using bioinformatics to compare the mycobacterial genome with those of *B. subtilis* and *Streptomyces coelicolor*, we were also

Table 1 Examples of signature sporulation genes from *B. subtilis* and their poor orthologs in *M. marinum* along with their general functions in non-sporulating bacteria

Orthologue	Sporulation function in *B. subtilis*	General function
SpoVK, SpoIIIE	Maturation of spores and DNA translocase	Widespread ATPases involved in many other functions in non-sporulating bacteria
CotSA	Spore coat associated protein	Glycosyltransferase
SpoVE, Soj	Spore cortex synthesis, centromere-like function	Universal cell division proteins
SigF	Sigma factor controlling sporulation genes	Sigma factor

able to identify genes encoding putative orthologs (some are shown in Table 1) involved in mycobacterial sporulation. The expression profiles of some of these genes seem to suggest that they play a role in the sporulation pathway i.e. they increase their expression when the cells grow older (Ghosh et al. 2009). However, putative orthologues for some of the genes that play key roles in *B. subtilis* sporulation (Hilbert and Piggot 2004), e.g. *spoIVA* and *spoIIIG* (encoding the σ^G sigma factor), have not yet been identified (see also next section).

We also found that old cultures of *M. bovis* bacillus Calmette–Guérin seemed to form particles similar to *M. marinum* spores. Hence, we concluded that sporulation is not unique for *M. marinum*. Rather it is likely that sporulation is a more general feature and survival strategy among mycobacteria (Ghosh et al. 2009).

Consistent with their ability to form spores, mycobacteria might be expected to establish divisomes with a certain degree of randomness yielding daughter cells of unequal size as they do not have any mechanisms for division site selection around midcell (see above) with any degree of precision. Examination of a large number of cells in the process of undergoing division showed frequent establishment of the FtsZ-ring and/or peptidoglycan wall formed off-center and away from the midcell position (Singh et al. manuscript in preparation). Conceivably a small fraction of cells with asymmetric FtsZ-ring formation can proceed to form spores under as yet unknown, special circumstances.

Do mycobacteria sporulate?

Our finding that mycobacterial species can form spores faced extensive scrutiny by reviewers already before its acceptance for publication (Ghosh et al. 2009) and has met with considerable skepticism from several experts in the fields of sporulation and mycobacterial genetics/physiology after it was published. One of the main concerns was the absence of reliable reports of mycobacterial spores' existence despite the long history of extensive research on this organism. In fact, presence of spore-like particles in old mycobacterial cultures has been reported in the past but most of them could not be confirmed by other laboratories and were often declared to be artifacts commonly seen in the slow growing cultures of mycobacteria (see e.g. Lack and Tanner 1953; Brieger et al. 1954; Brieger and Glaubert 1956; Csillag 1961, 1963, 1964, 1970; Hilson 1965; and references in these). Another reasoning has been that endospore formation requires a unique set of proteins—synthesis of which is regulated by environmental and metabolic signals. Such an elaborately specialized phenomenon involving 150–200 gene functions orchestrated in highly precise spatio-temporal coordination would have to be conserved through evolution after its emergence; consequently, all endosporulating organisms would be expected to share a large number of orthologs or homologs in their sporulation pathway as the gram positive bacteria with low GC-content genomes classified as firmicutes do.

Recently, Traag et al. (2010) have published a paper challenging the validity and interpretation of our data showing that ageing cultures of *M. marinum* grown on plates at 30°C form endospores (Ghosh et al. 2009). Their critique rests on three key arguments that briefly can be summarized as follows. First, their independent blast and psi-blast searches against 15 mycobacterial and 18 streptomyces genomes failed to reveal any ortholog of the signature genes for endospore formation. Traag et al. (2010) also criticized the choice of the orthologs cited by Ghosh et al. (2009) to be diagnostic for endospore formation since these are also present in many non-endospore forming bacteria. Significant in their

absence were the genes of the small, acid-soluble spore proteins (SASP) family of proteins dedicated to protecting the integrity of DNA within spores and the SpoVF operon, essential for dipicolinic acid synthesis (see e.g. Paidhungat et al. 2000; Setlow 2007; and references therein). Second, the spore and endospore images in Ghosh et al. (2009), in particular the transmission electron microscopic images, were remarkably similar to those of *B. subtilis* which is highly unlikely unless due to contamination with *B. subtilis* (Traag et al. 2010). Third, Traag et al. (2010) failed to detect the presence of spores or evidence of endosporulation either by microscopy or through heat resistance in laboratory cultures or from infected frogs chronically infected with *M. marinum.* Moreover, no spores were seen even when they used our published protocol for growing the cells obtained from our laboratory.

In summary, they took our results to be one of the several unfortunate cases of contamination of the slow growing mycobacteria with *B. subtilis* and wondered about the reproducibility of the sporulation data from a fresh culture in the hands of other workers. Below we respond to their three key arguments; but before that let us assert that several members of our laboratory have since reproduced the presence of spore-like particles independently in several different isolates of *M. marinum* strains.

As far as the bioinformatic criticism is concerned, it should be pointed out that our conclusion about endospore formation was neither derived from, nor did it depend on the discovery of orthologs of sporulation genes in mycobacterial genome—but relied on direct empirical observations of spores and establishment of spore-like properties which stand with or without bioinformatic support. Search for orthologs was prompted by the observational discovery itself and the ones found were published along with the "Forward Expect Values" that indicated their homology (see Table S1 in Ghosh et al. 2009) without assigning any function to these putative orthologs. These data were presented as intriguing information but our claim for sporulation was never dependent upon them. In Table 1 we provide examples of signature sporulation genes from *B. subtilis* and their orthologs in *M. marinum* along with their general functions in non-sporulating bacteria. As noted none of these proteins are specific for sporulating bacteria while sporulation specific genes, such as Spo0A are notably absent from mycobacterial genomes although a weak Spo0A candidate was discussed. However, the question arises whether absence of homology guarantees the absence of proteins with similar structure and function. Partition genes *mukB* and *smc*; or nucleoid occlusion genes *slm* and *noc*, though almost identical in structure and perform similar functions in *E. coli* and *B. subtilis*, respectively, could not be identified by blast searches. Thus, structurally similar proteins performing identical functions in bacteria do not have to be bioinformatically matching orthologs of each other. Furthermore, many "sporulation" proteins show widespread phylogenic distribution, and perform alternative functions in non-sporulating bacteria (Rigden and Galperin 2008). It is therefore conceivable that apparently "non-sporulating" proteins involved in division site selection, septum formation, chromosome translocation, etc. might be activated to participate in sporulation triggered by appropriate metabolic or environmental signal(s). There are examples of sporulating bacteria differing significantly in essential sporulation pathways (Stephenson and Lewis 2005). Alternatively there are phototrophic *Firmicutes* (Heliobacteria) that contain the full complement of genes for sporulation pathway in their genomes but show ambiguous sporulation patterns; some species of Heliobacteria have never been observed to sporulate under laboratory conditions (Kimble-long and Madigan 2001; Sattley et al. 2008). Regarding the question: How reliable are bioinformatic arguments for denying the presence, or guaranteeing the absence, of a biological phenomenon or pathway in an evolutionary context? The answer from Dr M. Galperin (personal communication) was: "Establishing the absence of homology can be done when respective proteins have known three-dimensional structures but becomes a very difficult task when their structures are not known."

Regarding similarity with *B. subtilis* spores we emphasize that *M. marinum* spores show variation in appearance (Fig. 1c).

The failure to reproduce spores from our samples following our published protocol is by far the most serious and worrisome point raised by Traag et al. (2010); but even in our laboratory spore production has not been uniformly efficient and numerous. We believe this is because we have not yet identified the metabolic or environmental signal that switches on sporulation in mycobacteria. Work is in progress

addressing this issue and we have preliminary data that show increasing sporulation frequency by changing growth conditions (manuscript in preparation). We emphasize that we have produced spore like particles from the cultures that was sent to Traag et al. (2010) to work with. Moreover, Fig. 2 shows spore particles derived from old cultures of a *M. marinum* derivative carrying the GFP gene integrated into the chromosome at the L5 *attB* site using an integrative suicide plasmid and that had been grown in the presence of kanamycin. The spore particles from 1 to 2 months old culture showed GFP fluorescence and we did not detect any contamination.

Finally, let us point out some of our reasoning why we rejected the arguments of contamination in the *M. marinum* cultures from some sporulating bacterial strains such as *B. subtilis* in the original paper (Ghosh et al. 2009). (i) The kinetics of appearance of spore-like particles were more consistent with life cycle and generation times of the relatively slower growing *M. marinum* (168 h after inoculation; see Fig. 1 in Ghosh et al. 2009) than *B. subtilis* where spores in the latter case appear at a very high concentration in an overnight culture. (ii) The spore-like particles in the old cultures disappeared in the fresh inoculum and reappeared in the same culture late into stationary phase (Supplementary Fig. S1 in Ghosh et al. 2009). (iii) DNA content of the spore-like particles as estimated from the fluorescence profiles of flow cytometric histograms was consistent with the genome size of *M. marinum*, which is significantly larger than e.g. the *B. subtilis* genome (*M. marinum* $\approx$6.6 Mbp vs. $\approx$4.2 Mbp for *B. subtilis*; Kunst et al. 1997; Stinear et al. 2008). (iv) The presence of DPA in old cultures but not in fresh inocula of *M. marinum* provided a strong support to the idea of sporulation in mycobacteria. In addition to this our recent data where we have observed continuity of fluorescence due the presence of the *gfp* gene and associated antibiotic resistance integrated into the mycobacterial genome (see Fig. 2) identifies the origin of spore particle as *M. marinum.* Combining all these together, we consider mycobacterial sporulation to have a strong empirical foundation despite poor bioinformatic support. In conclusion, our published and unpublished data suggest that *M. marinum* and likely also the *M. bovis* BCG strain form spores.

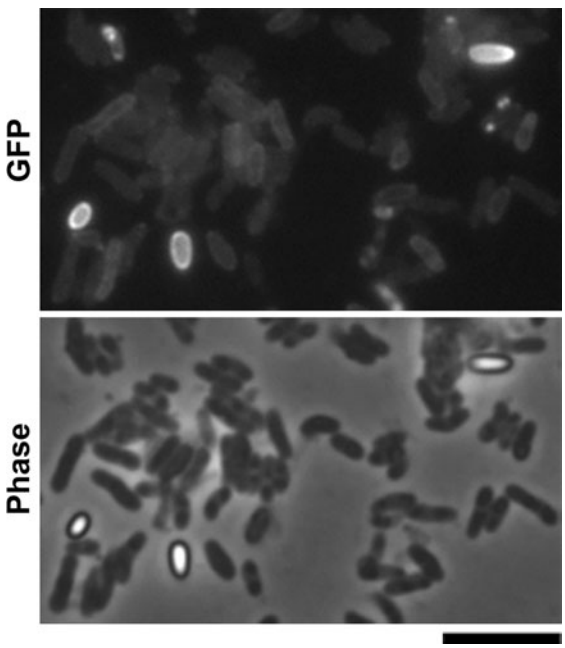

Fig. 2 Spores from an old culture (40 days) of the *Mycobacterium marinum* (strain T CCUG 20998 corresponding to ATCC 927) derivative *M. marinum attB*::*gfp*-Kan^R (GFP, green fluorescent protein gene linked to the Kan^R gene integrated into the genome at the L5 attB site, the plasmid was kindly provided by Dr DG Ennis and Ms A. Mallick, Univ of Louisiana, Lafayette, USA) grown in the presence of kanamycin. The two images show phase contrast and fluorescence of the same field as indicated (space bar = 5 μm)

Sporulation in mycobacteria and spore formation in other bacteria

Sporulation has been extensively studied in *Bacillus* spp. (for reviews see e.g. Errington 2003; Hilbert and Piggot 2004). *Bacillus* spp. are low GC content Gram positive bacteria that belong to the *Firmicutes*. *B. subtilis* can also form cell types other than spores, for example motile, matrix-producing and competent cells. The underlying regulatory networks/factors that are involved in generating the different cell types have recently been reviewed (Lopez et al. 2009). Other *Firmicutes* such as *Clostridia* spp. (Paredes et al. 2005), some phototropic heliobacteria (Kimble-Long and Madigan 2001) and the hydrogenogen *Carboxydothermus hydrogenoformans* Z-2901 (Wu et al. 2005) also form spores.

The *Firmicutes* form spores via an endosporulation pathway and sporulation depends on an array of events that depend on the expression and function of specific gene products. Analysis of the

C. hydrogenoformans Z-2901 genome revealed that this organism appears to lack many of the genes involved in sporulation in e.g. *B. subtilis* (Wu et al. 2005). Moreover, in the case of the phototrophic heliobacteria the data suggest that these bacteria form spores at a low frequency (Kimble-Long and Madigan 2001; see also above).

The filamentous bacterium *Streptomyces*, which is an actinomycete, also produces spores. However, *Streptomyces* spp. produce spores on aerial hyphae. Compared to, for example *B. subtilis* spores, those of *Streptomyces* are less resistant (Flärdh and Buttner 2009). In a recent review Scherr and Nguyen (2009) discussed the similarities and differences between *Mycobacterium* and *Streptomyces* (Scherr and Nguyen 2009). Other actinomycetes bacteria produce non-motile spores without forming aerial hyphaes (Asano and Kawamoto 1986; Ara and Kudo 2006). For example *Salinispora* gen. nov., which belongs to a marine actinomycetes produce substrate hyphae carrying either single spores or cluster of spores. The diameter of these spores is in the range of 0.8–3.8 μm (Maldonado et al. 2005). Another actinomycetes, the *Nocardia* spp., also appear to form spores (Bradley 1959; Brown-Elliott et al. 2006) while the actinomycetes *Rhodococcus* spp. show variations in cell morphology when grown under different growth conditions (Larkin et al. 2006 and references therein). However, none of the *Rhodococcus* spp. have been demonstrated to form spores and hence it would perhaps be of interest to investigate whether this is the case or not. In this context it is interesting to note that *Mycobacterium* and some *Nocardia* spp., e.g. strains of *N. farcinica*, as well as *Rhodococcus* spp. have properties in common (Goodfellow and Minnikin 1981; Zhi et al. 2009).

Among the Gram negative δ-proteobacteria myxobacteria, where *Myxobacteria xanthus* is the best studied, starvation for phosphate or carbon can initiate formation of fruiting bodies (for recent reviews see Kroos 2007; Kaiser 2006). Within these bodies spores are subsequently generated. Comparing the regulatory networks leading to the production of spores in *M. xanthus* and *B. subtilis* reveals that they have different sporulation strategies. In the latter, a cascade of different sigma factors are involved, whereas this does not appear to be the case for *M. xanthus*. In *M. xanthus* the regulatory network appears to encompass a cascade of transcription factors that become phosphorylated, most likely in response to different signals and/or by binding to signal molecules (Kroos 2007). Interestingly, a natural isolate of *B. subtilis* has been demonstrated to form fruiting-body like structures containing spores, although this has not yet ever been observed in laboratory cultures of *B. subtilis* (Branda et al. 2001). This raises the question which genes are involved in generating fruiting bodies in *B. subtilis*. Perhaps they have been lost or "silenced" upon cultivation in the laboratory under controlled conditions.

Recently Girija et al. (2009) described a new *Rhodobacter* species, *Rhodobacter johrii* sp. nov. Interestingly this Gram-negative bacteria forms spores apparently via an endosporulation pathway. This is in contrast to what is known for this group of bacteria and it is important to identify the genes that are involved in spore formation in *R. johrii* sp. nov. The controversial observation that spore particles, spore-like bodies or mycococci form (a coccoid form in mycobacterial isolates) exist in *Mycobacterium* cultures has, as emphasized above, been discussed extensively in the early literature (see e.g. Lack and Tanner 1953; Brieger et al. 1954; Brieger and Glaubert 1956; Csillag 1961, 1963, 1964, 1970; Hilson 1965; and references in these). Our finding that mycobacteria indeed can form spores therefore seems to confirm these previously reported observations. In general bacteria have the capacity to adapt to various environmental conditions and can differentiate into different cell types (see e.g. Lopez et al. 2009). Together with the discussion above, it is therefore worth emphasizing that frequent subcultivation under laboratory conditions might select for specific types of growth that suppress formation of spores. Hence, with respect to mycobacterial growth, it is important to study the way they adapt to different environments. This is of particular importance if we are to find new strategies to detect, treat and prevent infections caused by the mycobacteria. Perhaps the adopted and standardized cultivation conditions in the laboratory has lead to a bias in our understanding of mycobacteria.

Sporulation in mycobacteria and possible clinical implications

Our finding, that *M. marinum* and seemingly also *M. bovis* BCG sporulate in late stationary phase

(Ghosh et al. 2009), raises the interesting possibility that their ability to persist for a long time inside the host, may be due to the ability of mycobacteria to sporulate or enter into a spore-like stage. Thus, this hitherto uncharacterized phase of mycobacterial life style could be of critical importance for their pathogenicity, persistence and transmission. Important future questions are therefore to study whether other mycobacteria e.g. *M. tuberculosis*, also sporulate. Assuming that this is the case it is not unreasonable that the spores are subjected to phagocytosis by macrophages, as are *Bacillus anthracis* spores. Hence, another important question is whether mycobacteria spores enter macrophages and are degraded or if they reside in a dormant non-replicative state in the phagosome or elsewhere in the host. In the case of *B. anthracis*, spore germination in phagocytic cells is a key step in the pathogenic cycle (Henriques and Moran 2007). A third question is whether mycobacterial spore particles are involved in the transmission of pathogenic mycobacteria. For example, if *M. ulcerans* the causative agent of Buruli ulcer and a close relative of *M. marinum* (Stinear et al. 2000; Chemal et al. 2002), form spores, then these particles could be instrumental for the transmission of the bacteria. Likewise, mycobacterial spore particles might also be transmitted via our food in spite of sterilization protocols. In this context heat inactivation studies suggested that *M. avium* subsp. *paratuberculosis* can survive pasteurization if present in raw milk (Foddai et al. 2010). Hence the question of whether other mycobacteria form spores is an important one that warrants continued study.

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