Do mycobacteria produce endospores?

Bjorn A. Traag[a], Adam Driks[b], Patrick Stragier[c], Wilbert Bitter[d], Gregory Broussard[e], Graham Hatfull[e], Frances Chu[f], Kristin N. Adams[f], Lalita Ramakrishnan[f], and Richard Losick[a,1]

[a]The Biological Laboratories, Harvard University, Cambridge, MA 02138; [b]Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL 60153; [c]Institut de Biologie Physico-Chimique, Centre National de la Recherche Scientifique-Unité Propre de Recherche R 9073 Université Paris-Diderot, 75005 Paris, France; [d]Department of Medical Microbiology and Infection Control, VU University Medical Centre, 081 BT Amsterdam, The Netherlands; [e]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; and [f]Departments of Microbiology, Medicine, and Immunology, University of Washington, Seattle, WA 98195

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The genus *Mycobacterium*, which is a member of the high G+C group of Gram-positive bacteria, includes important pathogens, such as *M. tuberculosis* and *M. leprae*. A recent publication in PNAS reported that *M. marinum* and *M. bovis* bacillus Calmette–Guérin produce a type of spore known as an endospore, which had been observed only in the low G+C group of Gram-positive bacteria. Evidence was presented that the spores were similar to endospores in ultrastructure, in heat resistance and in the presence of dipicolinic acid. Here, we report that the genomes of *Mycobacterium* species and those of other high G+C Gram-positive bacteria lack orthologs of many, if not all, highly conserved genes diagnostic of endospore formation in the genomes of low G+C Gram-positive bacteria. We also failed to detect the presence of endospores by light microscopy or by testing for heat-resistant colony-forming units in aged cultures of *M. marinum*. Finally, we failed to recover heat-resistant colony-forming units from frogs chronically infected with *M. marinum*. We conclude that it is unlikely that *Mycobacterium* is capable of endospore formation.

sporulation | tuberculosis

The pathogen *Mycobacterium tuberculosis* is the leading cause of death worldwide by a single bacterial pathogen (1). An insidious feature of *M. tuberculosis* is the mysterious phenomenon of latency in which the pathogen is able to persist in asymptomatic individuals, only to emerge and cause disease many years later (1). Recently, Ghosh et al. (2) reported that the species *M. marinum* and *M. bovis* bacillus Calmette–Guérin, a species of the *M. tuberculosis* complex, produce a type of spore known as an endospore. This discovery, if true, is potentially of great medical significance because it could help explain latency.

Endospores are unique among bacterial spores in that they are produced inside of another cell (the mother cell) and, upon maturation, are released as free spores by lysis of the mother cell (3, 4). They are readily recognized under phase-contrast microscopy by their phase bright (refractile) appearance. They also exhibit diagnostic features under electron microscopy, such as a protein shell consisting of an inner coat and an electron dense, outer coat (5, 6). Endospores are composed of numerous molecules found, thus far, only in bacterial endospores. These molecules include most of the proteins that encase the spore in a protective shell (called the coat), a family of DNA-protective proteins known as SASP that are bound to the chromosome, and a unique small molecule, dipicolinic acid. All previously known examples of endospore-forming bacteria are members of the low G+C group of Gram-positive bacteria (Firmicutes) belonging either to *Bacilli* or to *Clostridia*, and in all cases in which a genome sequence is available, orthologs of genes involved in endospore formation are readily seen. The *Mycobacterium* genus is a member of the high G+C group of Gram-positive bacteria (Actinobacteria) for which there are no prior claims of endospore formation. Certain members of the group, such as *Streptomyces*, do produce spores, but spores of a fundamentally different kind that are not produced inside a mother cell (7).

Because of the potentially high significance of the discovery of Ghosh et al. (2) for the treatment of tuberculosis, we investigated their claims by carrying out genome sequence analysis and by testing for the production of endospores and for heat-resistant colony forming units by *Mycobacterium marinum* in vitro and in a frog model.

Results and Discussion

***Mycobacterium* and *Streptomyces* Genomes Lack Orthologs of Highly Conserved Endospore Genes.** We carried out genome sequence analysis by using BLAST and Psi-BLAST of the 15 *Mycobacterium* genomes (including those of *M. marinum* and *M. bovis*) and the 18 *Streptomyces* genomes present in the National Center for Biotechnology Information database of microbial genomes. The analysis revealed no orthologs of any of the signature genes for endospore formation (4). Examples are the absence of genes for the above mentioned SASP family, *spoIVA*, which encodes a highly conserved morphogenetic protein required for coat assembly, *spoIIR* and *spoIIGA*, which mediate the activation of a mother-cell-specific transcription factor, *spoIID*, which governs the process by which the forespore is engulfed by the mother cell, *spoIIIAE*, a critically important membrane protein produced in the mother cell, and the *spoVF* operon, which encodes a dipicolinic acid synthetase. [Certain clostridia do, however, lack *spoVF* and generate dipicolinic acid via an electron transfer flavoprotein that is widely distributed among both endospore-forming and nonendospore-forming bacteria (D. Popham, personal communication).] These genes encode proteins that are almost identical among *Bacillus* species (E values close to zero), including species that are distantly related to each other, such as *B. subtilis* and *B. anthracis*. In contrast, no reliable homologies were detected against predicted proteins from *Mycobacterium* genomes. Another example is *sigG*, which encodes the forespore-specific transcription factor σ^G. The σ^G protein is related to a family of regulatory proteins found in nonendospore forming bacteria but σ^G itself has residues that distinguish the sporulation transcription factor from other members of the family.

The authors cite examples of *M. marinum* sporulation genes but they are not in fact diagnostic of endospore formation, as shown on the related pages of the GTOP database (http://spock.genes.nig.ac.jp/~genome/search.html). For example, *spo0J* (CAB16133.1) encodes a member of the ParB family of proteins involved in DNA segregation (8), *spoIIIE* (CAB13553.1) encodes a member of a family of DNA translocases (9), and *spoVE* (CAB13394.1) is a homolog of *mrdB*, encoding a rod-shape determining membrane protein (10, 11). The *spoVK* (CAB13626.1) homolog cited by the authors corresponds to the 3′ half of a gene

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[1]To whom correspondence should be addressed. E-mail: losick@mcb.harvard.edu.

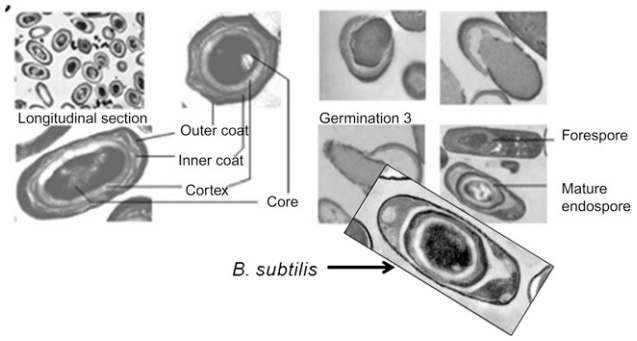

Fig. 1. Shown is a reproduction of figure 2*B* from Ghosh et al. (2) with an inset of a published electron micrograph of a *B. subtilis* endospore inside of sporangium [Reproduced with permission from ref. 27 (Copyright 2001, American Society for Microbiology).].

orthologous to Rv0282 from *M. tuberculosis*, which encodes part of ESX-3 (type VII secretion machinery) (12, 13). The *Bacillus* gene *jag* (CAB16140.1), for which the authors identified an ortholog [table S1 of Ghosh et al. (2)], forms a bicistronic operon with *spoIIIJ* (CAB16141.1), which encodes a member of the YidC family of membrane protein insertases, which are widespread among bacteria including mycobacteria. However, whereas *spoIIIJ* was found to be necessary for sporulation, mutants of *jag* sporulate normally (14). All of the genes proposed to support sporulation in *M. marinum* (2) are present in many nonendospore-forming species, including outside of the Firmicutes and Actinobacteria classes, as opposed to the true signature *spo* genes, such as *spoIIR* (CAB15714.1) or *spoIVA* (CAB14196.1).

The Endospores of Ghosh et al. Look Remarkably Similar to Endospores of *B. subtilis*. Fig. 1 overlays a published electron micrograph of a mature *B. subtilis* endospore contained within a mother cell next to the corresponding micrograph [figure 2*B* of Ghosh et al. (2)]. It can be seen that the spores in the two images are strikingly similar. Indeed, Ghosh et al. indicate the presence of the outer and inner coat features characteristic of endospores. Yet, the *M. marinum* genome lacks orthologs of coat proteins, such as CotE, CotJ, CotH, CotVWXYZ, and the above-mentioned SpoIVA, which plays a pivotal role in formation of the coat's distinctive layered architecture (3, 15, 16).

It is noteworthy that even among members of the genus *Bacillus*, endospore ultrastructure can differ dramatically from species to species (17). The spore coats of some species have spikes or more elaborate appendages, and many species possess an additional outer structure encasing the coat (called the exosporium) (Fig. 2). It seems unlikely that an otherwise distantly related bacterium that lacks orthologs of the signature genes for endospore formation would nonetheless produce endospores of such high similarity to those of the species *B. subilis*.

Failure to Detect Endospores in Aged Cultures of *M. marinum*. Four of our laboratories (those of B.A.T. and R.L.; W.B.; F.C., K.N. A., and L.R.; and G.B. and G.H.) attempted, and failed to observe, the appearance of endospores by light microscopy over time periods extending up to 12 weeks under the growth conditions described by the authors. Endospores were also not seen when the spore isolation procedure described by Ghosh et al. (2) was followed. This work was carried out by using *M. marinum* T CCUG 20998 (ATCC 927) and M ATCC BAA-535, including in the case of work by B.A.T. and R.L. strain *Mm* T CCUG 20998 (ATCC 927), kindly provided by the authors (Fig. 3). To summarize our collective observations, after several weeks of growth, phase-contrast light microscopy sometimes revealed phase-gray distensions of various shapes but few, if any, phase-bright bodies that looked convincingly like nascent endospores. Very occasionally we saw free, phase-bright objects but rarely objects that might be interpreted as endospores. Certainly, we did not see fields with multiple phase-bright, rod-shaped, free endospores as in figure 1B of Ghosh et al.

Finally, we examined laboratory stocks of eight different liquid cultures that had been incubated for periods of 4 weeks to ≈8.5 months at 33 °C (but in the case of the older cultures with periods at 4 °C). Again, we failed to detect phase bright endospores, either free or within cells (Fig. 4*A*). To ensure that we would have been

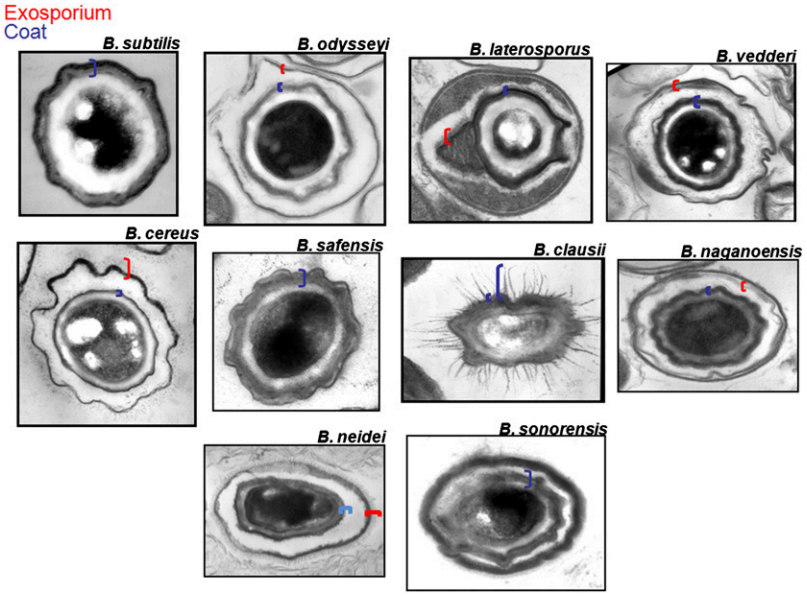

Fig. 2. Shown is a gallery of thin-section electron micrographs of endospores from the indicated *Bacillus* species. Samples were sporulated and prepared for electron microscopy as described (28). The image of *B. subtilis* (*Top Left*) was published previously [Reproduced with permission from ref. 29 (Copyright 2004, American Society for Microbiology).].

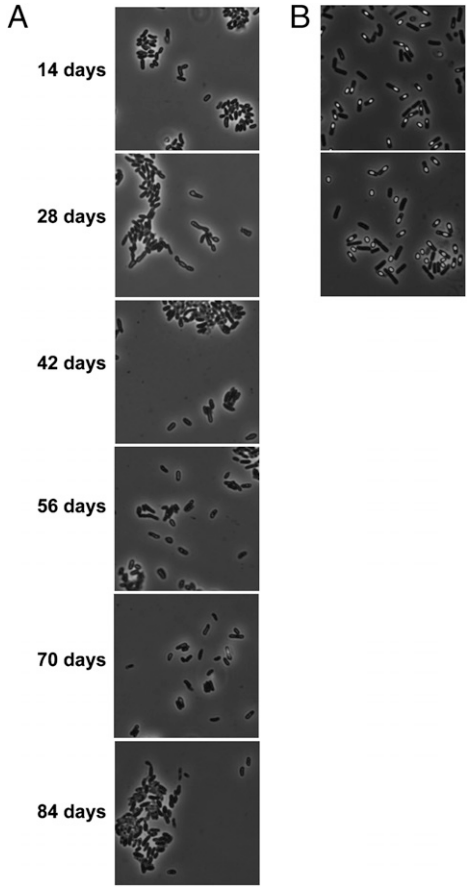

Fig. 3. Light microscopy images of a *M. marinum* culture upon prolonged incubation. (*A*) Strain *Mm* T CCUG 20998 (ATCC 927) (a kind gift from L. Kirsebom, Uppsala University, Uppsala, Sweden) was streaked for single colonies on 7H10 agar plates supplemented with 0.5% glycerol, 10% acid-albumin-dextrose complex, and cycloheximide [as in Ghosh et al. (2)]. After 1 week, four individual single colonies were streaked on fresh agar plates and followed by phase-contrast light microscopy every week for 12 weeks. Samples were fixed with 70% EtOH (2) or 4% paraformaldehyde in PBS with essentially the same results. (*B*) A field of sporulating *Bacillus subtilis* cells, including sporangia with endospores and free spores.

able to detect endospores if they had been present, we spiked *B. subtilis* endospores into samples of three cultures at concentrations of 1%, 5%, or 10% by volume. This was a "blind" experiment in which the microscopist did not know which samples had been spiked before examination. Phase-bright endospores could be detected in all three of the spiked cultures (Fig. 4*B*).

Failure to Detect Heat-Resistant Colony-Forming Units in Aged Cultures of *M. marinum*. We attempted, and failed to observe the reported appearance of heat-resistant colony-forming units in 2-week-old cultures of *M. marinum* (Fig. 5). Whereas Ghosh et al. (2) reported that 40% of the cells survived an incubation of 15 min at 65 °C, we (W.B.) found that the same conditions essentially killed all *M. marinum* cells from a 2-week old culture of the same strain (Fig. 5). Similar results of essentially complete killing were obtained for 5-, 10-, and 12-week-old cultures after incubation for 15 or 30 min at 65 °C (B.A.T. and R.L.).

We also note that the heat sensitivity of *M. bovis* has been known for a long time; indeed the invention of the pasteurization of milk represents one of the most notable advances in the prevention of infectious disease (18, 19). It stopped the transmission of bovine tuberculosis to humans, eradicating this previously endemic disease.

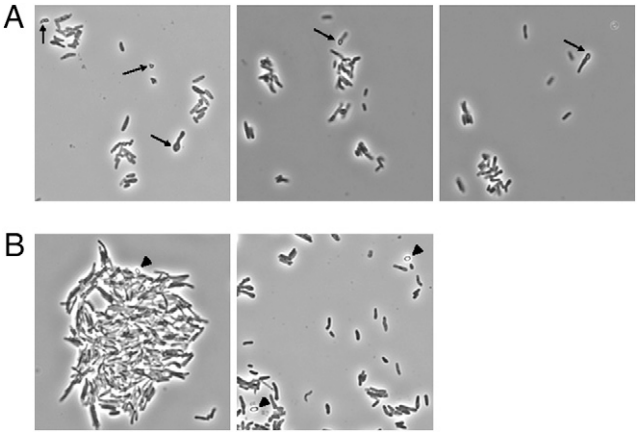

Fig. 4. Phase-contrast light microscopy images of *M. marinum* cultures. (*A*) Laboratory stock cultures that had been grown in liquid for ≈8.5 months at 33°C but with intervals at 4 °C. Arrows indicate phase-gray structures. (*B*) *M. marinum* cultures that had been grown in liquid culture for either 4 weeks (*Left*) or 7.5 months (*Right*) and spiked with the addition 1% or 5% vol/vol of *B. subtilis* endospores, respectively. Arrowheads indicate *B. subtilis* phase-bright endospores. Cultures were spiked by a different individual (K.N.A.) than the individual (F.C.) who did the microscopy, who did not know in advance which cultures had been spiked and which not.

Failure to Detect Heat-Resistant Colony-Forming Units in Frogs Chronically Infected with *M. marinum*. Ghosh et al. propose that endospore formation is a mechanism for the phenomenon of latency. To investigate this hypothesis, one of our laboratories (that of F.C., K.N.A., and L.R.) used leopard frogs chronically infected with *M. marinum* (20). *M. marinum* infection in leopard frogs results in an asymptomatic infection, reminiscent of humans infected with latent *M. tuberculosis*. The livers from nine frogs infected for 2 weeks, three frogs infected for 10 weeks, and nine frogs infected for 18 weeks were harvested and tested for heat resistant colony-forming units after treating homogenates of the livers at 70 °C for 20 min. To ensure we could detect heat-resistant colony-forming units using our protocol, *B. subtilis* spores were spiked into the liver homogenate of a frog infected with *M. marinum* for 2 weeks. We observed an 82% recovery of colony-forming units from the spiked homogenate. No heat-resistant colony-forming units were detected for 20 of the infected frogs. In the case of one of three frogs infected for 10 weeks, colony-forming units were recovered from the most concentrated sample of liver homogenate (2.0×10^3 heat-resistant colony-forming units/g liver from a total of 2.7×10^7 colony forming units at the time of harvest). However, no heat-resistant colony-

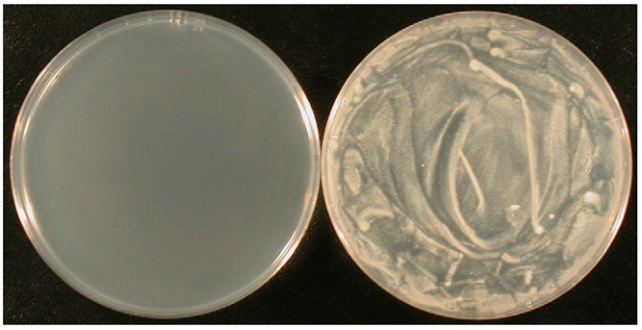

Fig. 5. Approximately 1,000 colony forming units of *M. marinum* cells from a 2-week-old culture were plated with (*Left*) or without (*Right*) 15 min of heat treatment at 65 °C.

forming units were recovered from other dilutions from the same liver homogenate nor from the two other frogs infected for 10 weeks. In summary, our results provide little or no support for the hypothesis that latency by *M. marinum* is mediated by the production of heat-resistant spores.

The report of Ghosh et al. (2) is not the first or only claim of spore formation by *Mycobacterium* (21, 22), which goes all the way back to the original paper of Robert Koch (23). However, these spore-like inclusions turned out to be neutral lipid bodies (24, 25) and in another case probably a contamination (26). Still, we cannot rule out the possibility that *M. marinum* produces spores of some kind under conditions that could not be replicated in our laboratories. What remains most difficult to accept about the work of Ghosh et al. (2) is the representation that *Mycobacterium* produces bona fide endospores of striking similarity to those of a particular species, *B. subtilis*. If this were true, it would be an extraordinary case of convergent evolution in which a completely distinct mechanism leads to the same outcome or an equally extraordinary case of divergence in which the orthologs of signature genes can no longer be detected. A more likely explanation is that the endospores, and those of Fig. 2*B* in particular, are not of *M. marinum* but rather of a low G+C, Gram-positive bacterium, such as *B. subtilis*.

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