Supporting information

Ghosh et al. 10.1073/pnas.0904104106

SI Text

Materials and Methods

Flow Cytometry. Cells were harvested at different growth times, fixed, stained, and run in the flow cytometer as described previously (1). Briefly, the cells were fixed in 70% (final concentration) ice-cold ethanol and stored at 4°C. For flow cytometry, the fixed cells were washed with particle-free (passed through 0.2 μm nitrocellulose filters) 10 mM Tris-HCl (pH 7.9) buffer supplemented with 10 mM MgCl2. The cells were subsequently resuspended into 0.1 × vol of the same buffer. Washed cells were stained with ethidium bromide (0.4 μg/mL, final concentration) and mithramycin A (16 μg/mL, final concentration) and kept on ice for 30 min before flow cytometry (Compact A440 analyser Unit, Apogee Flow Systems and BioRad Bryte HS). At least 20,000 cells were counted for each flow histogram.

Serial Dilution of Pure Spores to Isolate Single Spore Particle. The idea of serial dilution to isolate a single spore was based on a previous report (2). A batch of isolated spores was split into 2 fractions; one was plated freshly, and after a week of growth the colonies were counted to estimate the number of viable spore particles in the spore preparation. This number was ~107/mL. The other fraction was serially diluted 104-fold, such that there would be no more than 1 viable spore in each tube. The so-obtained spore solutions were plated on 7H10 plates with necessary supplements and the plates were incubated at 30°C.

To address the possibility that the spores were contaminations, we also extracted DNA from vegetative M. marinum (Mm) cells, spores, and Bacillus subtilis (Bs) (or from independent clones originating from isolated single-spore particles) (Fig. S1B) by boiling in water for 5 min followed by centrifugation for 5 min at 11,000 × g in an Eppendorf centrifuge. The DNA was used as templates in PCR amplification reactions of regions of the 16S rRNA gene, rnpA (which encodes the RNase P protein C5) and rnpB (which encodes the RNase P RNA). The results are shown in Fig. S1. The choice of using rnpB is based on that it has previously been used to differentiate closely related bacteria (3–8).

Detailed Description of Extraction of Total RNA from Mm and RNA Dot Blot Analysis. Cells were broken in a 1-mL solution of TRIzol (Invitrogen) and 0.3 mL of chloroform with 0.1 mm silica beads (Q-Biogene, Lysing matrix B) using a BIO101 Savant FastPrep FP120 machine (6.5 speed, 30 sec pulse, 4 cycles). After 10 min of centrifugation at 11,000 × g, the aqueous phase was reextracted with 300 μL of chloroform and transferred to a tube containing 250 μL of isopropanol. Total RNA was precipitated overnight at 4°C and washed with 1 mL of a 75% ethanol before resuspension in RNase free water. Contaminating DNA was removed by digestion with RNase-free DNase (Ambion) following the manufacturer’s instructions.

Dot blot and probing of RNA were done as described previously (9). DNase-treated RNA samples prepared from 1-, 3-, 5-, and 7-day-old Mm cultures were denatured at 90°C for 5 min and then spotted (10 μg in each spot) on Hybond-n + nylon membranes (Amersham Biosciences) and air dried. The RNA was immobilized by UV cross-linking by using a Bio-Rad cross-linker, followed by hybridization with specific 32P-labeled oligonucleotides at 42°C. The membranes were washed as described previously (9). After probing for the mRNAs, the membranes were stripped with hot 0.1% SDS, and then rehybridized with 5S rRNA probes and washed as above. Hybridization signals were analyzed using a Phosphor Imager (Image-Quant, Molecular Dynamics). Escherichia coli K12 stationary phase RNA samples were also spotted on the same membranes and probed with the same oligonucleotides to calculate the background signals of each dot arising from unspecified binding, which were very low in our hybridization conditions. The background values were subtracted from the corresponding values of the mRNA and 5S rRNA signals. Oligonucleotide probes were labeled at their 5’-ends with γ[32]ATP using T4 polynucleotide kinase (PNK) according to standard procedures. The nucleotide sequences of the oligonucleotide probes are listed in Table S6 (PDF). To probe for Mm 5S rRNA, the oligonucleotide 5’-GCTGACAGGCTTAGCTTCCG was used.

Dipicolinic Acid (DPA) Assay. Mm cultures were harvested after 1 day (vegetative cells), 7 weeks, and at the second month (both are expected to contain a mixture of vegetative cells and spores). The cells and purified sample of spores from the 2-month-old culture (2.0–10.0 OD600) were resuspended in physiological saline and broken by grinding with glass beads followed by autoclaving at 15 lb/in2 for 15 min. The lysate was clarified by centrifugation after treatment with acetic acid (0.1 mL of 1N acetic acid added to the lysate) for 1 h at room temperature. This was followed by the addition of freshly prepared ferrous ammonium sulfate hexahydrate and the color was measured at 440 nm (10). A calibration curve, plotted by using standard solutions of pure DPA (Sigma-Aldrich) in 0.15 M NaCl, was used to calculate the amount of DPA in the clear lysates.

Percoll-Sucrose Gradient Centrifugation Analysis of Fresh and Old Mm Cultures. We used a modified version of Percoll-sucrose density gradient (11, 12) in an attempt to separate putative spore particles from vegetative cells in Mm cultures. A mycobacterial expression vector carrying the gene for GFP expression, pG13 (13), was used to follow the Mm strain through gradient fractionation and subsequent treatments with GFP-fluorescence tag. Briefly, equal numbers of cells (with similar OD600 values) from 3- and 30-day-old plate cultures were resuspended into PBS and 1 mL of the fresh and old Mm (with the pG13 plasmid) suspensions were layered on top of 25 mL preformed Percoll-sucrose gradients (70% Percoll with 7.8% sucrose in 30 mM phosphate buffer supplemented with 10 mM Tris-HCl (pH 7.9)) and centrifuged for 60 min at 60,000 × g. The fractions were collected from bottom and GFP fluorescence tag. The relative fluorescence intensities were plotted as shown in Fig. S3A. To detect the presence of spore particles, the fractions were individually wet-heat-treated at 65°C for 30 min and then spread on 7H10 plates supplemented with 25 μg/mL kanamycin. This eliminated all vegetative cells (3-day cells) as well as the background acquired during the centrifugation and fractionation process. As shown in Fig. S3B, the 3-day-old cells did not contain any survivors whereas in the gradient for 30-day-old culture, a peak of heat-resistant survivors appeared in a band at a slightly heavier density (fractions 12 and 13) than the main peak. The colonies from the heat-resistant fractions all emitted green fluorescence due to the expression of GFP, confirming their identity as Mm (carrying the pG13 plasmid).

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Sequence Data. Protein sequences from Bs were downloaded from the SubtilList web server (14–16). Sporulation-associated proteins were obtained according to the SubtilList functional category 1.8. In addition, protein sequences for sporulation-related factors SigE, SigF, SigG, Spo0A (TrcR in Fig. 5), and SpoVT were also downloaded. Amino acid sequences of identified ORFs from Mycobacterium tuberculosis CDC1551, M. tuberculosis H37Rv, and Streptomyces coelicolor (Sc) A3 (2) were downloaded from the Comprehensive Microbial Resource (17–20). Amino acid sequences of the initial gene predictions for Mm were downloaded from the Sanger Institute FTP server (21). Proteins involved in Sc sporulation were obtained by querying the StreptDB database (26) for genes with annotations containing any of the words “sporulation,” “whiA,” “whiB,” “whiD,” “whiE,” “whiG,” “whiH,” “whiI,” or “whiJ,” as well as genes with gene names matching “whi*” (‘*’ corresponding to any letter). In addition, the Sc gene for sigF was included.

Reciprocal Best-Hit Ortholog Search. The Washington University BLAST (release date 10-May-2005; ref. 22) was used in a reciprocal best hit approach (RBH) (23) to identify putative orthologs for sporulation-associated Bs and Sc proteins in the mycobacteria species listed above. We consider a pair of genes, a and b from genomes A and B, respectively, as putative orthologs if a search with a against B identifies b as the most significant match and a search with b against A results in a as the most significant match. The blastp program was used with default parameters and an expect cutoff of 0.001. Putative orthologs are listed in Tables S1–S4. In total, 169 sporulation-associated Bs proteins were used as queries and 25, 21, 23, and 28 putative orthologs were identified in Mm, Mtb CDC1551, Mtb H37Rv, and Sc, respectively, corresponding to 35 unique Bs sporulation-associated genes. Similarly, of 63 Sc sporulation-associated genes, 16, 14, 14, and 10 putative orthologs were identified in Mm, Mtb CDC1551, Mtb H37Rv, and Bs, respectively, corresponding to 22 unique Sc genes. In addition, top-scoring hits for the Bs transcription factors Spo0A, SpoIIAB, and SigF were included. These hits did not return the initial Bs query protein as the top-scoring hit in a reciprocal search and are therefore considered as weak hits.

Fig. S1. Purified spores’ germination into vegetative *M. marinum* cultures and formation of spores at late stationary phase. (A) Life cycle of *Mm* from purified spores by microscopy. 0h, Purified spore suspension; 6h, germinating spores; 8h, germinated cells; 1d, germinated cells; 2d and 3d, same as 1d; 5d-1 and 5d-2, appearance of endospores; 1w, appearance of mature spore; 2w, mature spores with cell debris. Note that spores, germinating spores, and endospores appear as bright reflecting particles. (Scale bars: 5 μm.) (B) Life cycle of *Mm* from purified spores by flow cytometry. An *Mm* culture was started from a purified spore suspension and followed through germination, exponential growth to sporulation in late stationary phase by flow cytometry. The left and right columns show DNA content (fluorescence calibrated in chromosome numbers) and cell-size (light scattering in arbitrary units) distributions, respectively. The top frames refer to stationary phase LB cultures of *E. coli* used as calibration standards for DNA content and cell size. The 9 lower frames correspond to *Mm* samples taken at 0, 6, 8, 24, 48, 72, 120, 168, and 336 h after suspension of the purified spores into the growth medium. Fixation and staining procedures were as described in Materials and Methods, and in Fig. 1A legend. The narrow size distribution and the shift of the peak as a whole with little widening implied synchronous transition of spores into vegetative cells. At late stationary stage (after 120 h of growth), the cell-size distribution implies emergence of mature spores from the narrowing of the peak and its shift toward smaller size (right column: 168h and 336h). The fluorescence profiles show the onset of DNA synthesis by 6 h after addition of growth medium; replication continues for 72 h, after which nonreplicative cell division is followed by sporulation.
GTCCTG-3 region 272–291). Set 2, complementary to the Mm rnpA outlined above. The so-obtained DNA preparations were used as templates in PCR amplification reactions using the following primers. Set 1, complementary to the parts of the 16S rRNA gene, DNA extracted from buffer. Lanes 1, 6, 11, 16, and 21, 100 base pair DNA ladder; lanes 2–5, no template added; lanes 7–10, DNA extracted from vegetative amplification, the samples were kept at 72° C for 7 min. The PCR-amplified products were run on 2% agarose gels in 50 mM Tris-Borate, pH 8.3, 1.2 mM EDTA For PCR amplification, we used the following settings: denaturation for 3 min at 95° C, 30 cycles at 95° C (15 sec), 60° C (15 sec), and 72° C (30 sec). After amplification, the samples were kept at 72° C for 7 min. The PCR-amplified products were run on 2% agarose gels in 50 mM Tris-Borate, pH 8.3, 1.2 mM EDTA buffer. Lanes 1, 6, 11, 16, and 21, 100 base pair DNA ladder; lanes 2–5, no template added; lanes 7–10, DNA extracted from vegetative Mm cells; lanes 12–15, DNA extracted from Mm spores; lanes 17–20, DNA extracted from Bs culture. The following primer sets (see above) were used: lanes 2, 7, 12, and 17, set 1 (rnpB Bs; product in lane 17); lanes 3, 8, 13, and 18, set 2 (rnpA Mm; products in lanes 8 and 13; however, we also detected products in lane 18 which is most likely due to the fact that the rnpA primers used are not specific); lanes 4, 9, 14, and 19, set 3 (16S rRNA gene Mm; products in lanes 9 and 14); lanes 5, 10, 15, and 20, set 4 (rnpB Mm; products in lanes 10 and 15). On the basis that Sc is an exosporulating bacteria (27), and that Sc spores are morphologically different compared with mycobacteria spores, we can also conclude that our Mm cultures were not contaminated with Sc.
Fig. S3. Fractionation of spore particles from fresh (3 days) and old (30 days) *Mm* (carrying the pG13 plasmid) cultures on Percoll-sucrose gradients. (A) Relative fluorescence intensity of each fraction in arbitrary units are plotted; open and solid symbols represent 3- and 30-day-old cultures, respectively. All fractions were subjected to wet-heat treatment at 65°C for 30 min and plated on 7H10 medium supplemented with 25 μg/mL kanamycin and incubated at 30°C. (B) Fractions that contained survivors after wet-heat treatment. Only the 30-day-old culture shows the presence of heat-resistant survivors: fractions 12 (97 colonies), 13 (255 colonies), 15 (5 colonies), and 20 (3 colonies). Note that the position of the band that corresponds to the wet-heat-resistant particles shows a slightly heavier density compared with the main peak. The colonies were analyzed by using a Nikon SMZ1500 stereomicroscope with Epi-fluorescence attachment. An exposure time of 400 ms was used, and images were captured by Nikon NIS Elements D3.0 software. This allowed us to scan for the presence or absence of fluorescence originating from the expression of GFP in the entire colony, as shown in C and D. A 4-week-old *Mm* culture without pG13 was used as a negative control. (C) Colonies after wet-heat treatment of fraction 12. (D) One colony after wet-heat treatment. Together, C and D confirm the identity as the GFP plasmid-containing *Mm* strain. Note that all wet-heat-resistant colonies from fractions 12, 13, 15, and 20 showed green fluorescence.