RNA Dynamics in Aging Bacterial Spores

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SUMMARY

Upon starvation, the bacterium Bacillus subtilis enters the process of sporulation, lasting several hours and culminating in formation of a spore, the most resilient cell type known. We show that a few days following sporulation, the RNA profile of spores is highly dynamic. In aging spores incubated at high temperatures, RNA content is globally decreased by degradation over several days. This degradation might be a strategy utilized by the spore to facilitate dormancy. However, spores kept at low temperature exhibit a different RNA profile with evidence supporting transcription. Further, we demonstrate that germination is affected by spore age, incubation temperature, and RNA state, implying that spores can acquire dissimilar characteristics at a time they are considered dormant. We propose that, in contrast to current thinking, entering dormancy lasts a few days, during which spores are affected by the environment and undergo corresponding molecular changes influencing their emergence from quiescence.

INTRODUCTION

In response to nutrient limitation, the Gram-positive bacterium Bacillus subtilis (B. subtilis) and its relatives enter a process of cellular differentiation called sporulation, culminating in the formation of a highly resilient spore. Bacterial spores can survive long periods of time and withstand extremes of heat, radiation, and chemical assault via mechanisms that are not yet fully understood (Errington, 2003; Piggot and Hilbert, 2004; Stragier and Losick, 1996). During the course of sporulation, a polar septum forms, dividing the cell into two unequal-sized progenies: a smaller forespore compartment that becomes the mature spore and a larger mother cell, which nurtures the developing spore. Subsequently, the forespore is engulfed by the mother cell, and its cytoplasm becomes the spore core (Piggot and Hilbert, 2004; Stragier and Losick, 1996). A thick cell wall material called the cortex is synthesized around the core, followed by a dense multilayered proteinaceous coat that is assembled by the mother cell onto the forespore surface. Recently, a new additional outermost layer of the spore, named the crust, was identified (McKenney et al., 2010). These outer layers of cortex, coat, and crust provide the spore with mechanical protection while excluding most molecules (Driks, 2002; Setlow, 2003). Eventually, after approximately 8 hr of development, when sporulation is complete, the mature spore is liberated by lysis of the mother cell (Piggot and Hilbert, 2004; Stragier and Losick, 1996).

The spore can convert within minutes to an actively growing cell via a process called germination, which is induced by a variety of molecules including amino acids, sugars, and cell wall muropeptides. These germinating molecules bind to receptors in the inner spore membrane, signaling the spore to resume a vegetative life form (Dworkin and Shah, 2010; Moir, 2006; Setlow, 2003; Shah et al., 2008). During germination, the spore undergoes rehydration, cortex hydrolysis, and coat disassembly. In addition, many of the spore-specific proteins are hydrolyzed to amino acids, probably in order to provide precursors for protein synthesis (Setlow, 1995). Following germination, in a process called outgrowth, the new cell starts to emerge from the disintegrating coat, elongates, synthesizes macromolecules, and progresses toward cell division (Moir, 2006; Santo and Doi, 1974; Setlow, 2003).

Despite significant advances in our understanding of spore formation (Errington, 2003; Piggot and Hilbert, 2004; Stragier and Losick, 1996), little is known about the characteristics and complexity of the dormant spore. The spore is protected by thick layers and has unusual biochemical and biophysical properties that make it a challenging biological system to investigate (Setlow, 1995; Driks, 2002). Spores are considered metabolically inert, as they contain high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid) and a low amount of water (Gerhardt and Marquis, 1989). It is believed that spore dehydration results in the formation of an insolubly gelled-core with reversible bonds between macromolecules (Black and Gerhardt, 1962; Carstensen et al., 1971; Cowan et al., 2003; Driks, 2002). Such interactions could form an immobile matrix that keeps the spore stable and metabolically inert (Black and Gerhardt, 1962; Carstensen et al., 1971; Cowan et al., 2003; Nakashio and Gerhardt, 1985).

It is still disputed whether the spore is entirely dormant or whether it maintains some metabolic processes. In fact, it has been shown that bacterial spores do contain certain enzymatic activities; full maturation of coat proteins was found to be dependent on enzymatic reactions taking place subsequently to release of the spore from the mother cell (Zilhão et al., 2005; Kuwana et al., 2006; Ragkousi and Setlow, 2004; Sanchez-Salas et al., 2011). In addition, spores of Bacillus species isolated from...
coastal marine sediments have been shown to enzymatically oxidize manganese, an activity that was found to be associated with the spore outer layers (Francis and Tebo, 2002). Interestingly, some evidence for molecular dynamics within the spore core have also been obtained; spores of Clostridium botulinum were found to repair single-strand DNA breaks during their dormant state (Durban et al., 1974), and nuclear magnetic resonance (NMR) studies of B. subtilis spores revealed molecular mobility of phosphorous compounds within the spore core (Leuschner and Lilford, 2000).

Here we show that B. subtilis spores display changes in their RNA content a few days post-sporulation, at a time they are considered dormant. In mature spores, incubated at high temperatures, the RNA underwent global degradation, but when kept at low temperature, transcription was evident. Our data indicate that entering quiescence is a process that lasts a few days. During this period spores are responsive to environmental cues and undergo molecular changes affecting their exit from dormancy.

RESULTS

rRNA Is Degraded in Aging Spores

To explore whether spore macromolecules are subjected to dynamic changes, we surveyed the content of spore RNA for several days post-sporulation. For this purpose, we established dynamic changes, we surveyed the content of spore RNA for a few days. During this period spores were responsive to environmental cues and undergo molecular changes affecting their exit from dormancy.

mRNAs are targeted to the future destination of their encoded proteins (Nevo-Dinur et al., 2011). Alternatively, the coat transcripts could have been actively translocated from the mother cell into the forespore via the recently identified “feeding tube” connecting the two chambers (Camp and Losick, 2009; Meisner et al., 2008). Alternatively, the coat transcripts could have been trapped in the coat during its construction, a possibility that is supported by the recent observation that bacterial mRNAs are targeted to the future destination of their encoded proteins (Nevo-Dinur et al., 2011). In the latter case, however, these coat transcripts may not be accessible for processes occurring in the core. Taken together, a day-old spore encases mRNAs transcribed during sporulation, including mother cell-specific molecules, along with transcripts required for fundamental cellular activities.

mRNA Levels Are Decreased in Aging Spores

Having discovered that the rRNA reservoir is influenced by spore age, we examined whether the spore mRNA also exhibits dynamic behavior. We first determined the transcriptome of a day-old spore and then followed the fate of the identified transcripts for several days. Microarray analysis revealed the spore to contain 369 transcripts encoded by genes of various functional groups (Table S2). Previously, only 25 transcripts were identified in B. subtilis dormant spores (Keijser et al., 2007), 17 of which were detected here. The spore transcripts are encoded by genes mainly involved in basic cellular processes such as DNA metabolism (6 genes), transcription (20 genes), translation (28 genes), and several metabolic pathways (48 genes). The spore also harbors mRNA from genes required for sporulation and germination (70 genes), those required for adaptation to atypical conditions (20 genes), and a large group of genes with unknown function (143 genes). Unexpectedly, the sporulation group included many mother cell-specific genes particularly involved in coat assembly (40 out of 68 genes). The detection of coat transcripts in the spore was confirmed with fluorescence in situ hybridization (FISH) (Figure S3A). These transcripts may have been actively translocated from the mother cell into the forespore via the recently identified “feeding tube” connecting the two chambers (Camp and Losick, 2009; Meisner et al., 2008). Alternatively, the coat transcripts could have been trapped in the coat during its construction, a possibility that is supported by the recent observation that bacterial mRNAs are targeted to the future destination of their encoded proteins (Nevo-Dinur et al., 2011). In the latter case, however, these coat transcripts may not be accessible for processes occurring in the core. Taken together, a day-old spore encases mRNAs transcribed during sporulation, including mother cell-specific molecules, along with transcripts required for fundamental cellular activities.
Next, we followed the destiny of the identified mRNAs for a period of 1 week post-sporulation. As observed for rRNAs, the majority of spore transcripts exhibited decreasing mRNA levels over time. This drop took place mainly between days 1 and 5, whereas relatively little variation was observed between days 5 and 8 (Figures 3A and S3B; Table S2), suggesting that the spore reaches a steady state or halts its activity. qRT-PCR examination of several transcripts was consistent with this degradative pattern (Figures 3D and S3E). In addition, a few transcripts that exhibited a nonsignificant decrease in the microarray analysis showed decreasing profiles using the more sensitive qRT-PCR method (e.g., ribT in Figures 3A and 3D). The degradation profile was largely dependent on RNaseY, as the level of several examined transcripts was nearly constant in the absence of this protein (Figure 2C; data not shown). The global degradation of spore RNA might be a strategy to facilitate spore dormancy by shutting off essential cellular activities.

Figure 1. rRNA Dynamics in Aging Spores

(A–C) RNA was extracted from decocked B. subtilis (PY79) spores at the indicated age and temperature and analyzed in a microfluidic gel (see Experimental Procedures). Arrows designate the positions of 23S rRNA (upper) and 16S rRNA (lower). All lanes in the pseudogel are scaled to the same intensity range. Quantifications of 23S and 16S rRNA band intensities are presented below each lane (see Extended Experimental Procedures).

(D) qRT-PCR analysis was performed on cDNA derived from aging B. subtilis (PY79) spores incubated at the indicated temperatures (see Experimental Procedures). Similar results were obtained with five different primer sets covering the full length of the 23S rRNA (data not shown).

(E) Bioanalyzer pseudogel of RNA extracted from decocked spores and vegetative cells of the WT strain (PY79) (see Experimental Procedures). RNA was extracted from aging spores with either externally added vegetative RNA or vegetative cells, and rRNA patterns were visualized. Importantly, only spore cultures were treated with a decocking solution prior to mixing. Shown are the following: (1) pre-extracted RNA of vegetative cells grown at 37°C and harvested at optical density (OD) 600 = 0.8; (2) RNA extracted from vegetative cells grown at 37°C and harvested at OD 600 = 0.8; (3) RNA extracted from 5-day-old spores incubated at 37°C; (4) RNA extracted from a mixture of 5-day-old spores incubated at 37°C and pre-extracted vegetative RNA; (5) RNA extracted from a mixture of 5-day-old spores incubated at 37°C and vegetative cells in a volume ratio of 3:7 cells to spores. Arrows indicate the positions of 23S rRNA (upper) and 16S rRNA (lower). All lanes in the pseudogel are scaled to the same intensity range. See also Figure S1.

RNA Undergoes Accelerated Degradation in Spores Held at High Temperature

The detection of active RNA degradation in aging spores prompted us to explore whether environmental conditions, such as temperature, can influence this process. We therefore induced cells to sporulate at 37°C for 24 hr and then shifted the day-old spores to 50°C for an additional week of incubation. RNA was extracted from aging spores, and samples were analyzed. The RNA obtained from spores incubated at 50°C evidenced similar rRNA patterns to those observed for 37°C-incubated spores, yet degradation occurred more rapidly (Figures 1B, 1D, and S1C). We confirmed again the in vivo origin of the observed degradation profiles and found them to be genuine (Figure S1C). Microarray and qRT-PCR analyses showed that the mRNA levels of 50°C-incubated spores dropped mainly between days 1 and 3 and then reached a steady state (Figures 3B, 3D, S3C, and S3E; Table S2). These transcriptome dynamics preceded those observed at 37°C and resembled the accelerated rate of rRNA degradation observed at 50°C. Our results suggest that spores incubated at high temperatures speed up their progression toward dormancy.
RNA Has Diverse Fates in Aging Spores Kept at Low Temperature

Next, we wished to examine the effect of low temperature on spore RNA dynamics. As before, cells were induced to sporulate at 37°C for 24 hr and then shifted to 4°C for an additional week of incubation. Markedly, the spores maintained a relatively constant level of rRNA molecules with almost no visible degradation in the course of 8 days (Figures 1C, 1D, and S1D). To follow the mRNA fate at this temperature, we monitored the levels of the identified transcripts present in a day-old spore (Table S2) during additional incubation at 4°C. At day 3 a significant portion (35%) of the examined mRNAs exhibited decreased levels, similar to the pattern observed in 37°C- and 50°C-kept spores (Figures 3C, 4A, S3D, and S3F). However, a large fraction of the examined transcripts maintained constant levels (53%), whereas the remaining transcripts (12%) surprisingly exhibited increased levels (Figures 3C and 4A). Thus, spore RNA experiences various fates at 4°C, unlike the unidirectional decreasing trend observed at high temperatures.

Further investigation of the upregulated spore transcripts revealed a group of 250 mRNAs, poorly represented in a day-old spore, displaying a sharp increase in their level between days 1 and 3 (Figure 4B; Table S3). Increasing profiles of several mRNA molecules were confirmed by qRT-PCR, corroborating these unexpected results (Figure 4C; data not shown). The observed upregulated transcripts are encoded by diverse genes, including genes required for different metabolic pathways, motility and chemotaxis, transport, and transcription regulation as well as a large group of genes with unknown function. Moreover, genes belonging to the same operon were frequently detected, suggestive of a potential transcriptional coregulation. Taken together, these results indicate that spores incubated at low temperature are subjected to a delayed entry into dormancy, possibly involving de novo transcription within the mature spore.

Transcription Is Evidenced in Aging Spores

The phenomenon of increasing transcripts in spores held at low temperature may indicate the occurrence of transcription and
raises questions regarding the potential function of the corresponding proteins. It is possible that at least some of these proteins are required for coping with cold, either during quiescence or upon germination. To examine these possibilities, several genes encoding upregulated transcripts were fused to gfp, and the expression patterns of the resultant proteins were followed (Table S4). Interestingly, one of the investigated proteins, YozG, a hypothetical transcriptional regulator, displayed increased expression in vegetative cells shifted to low temperature (Figure 5A), suggesting that some of the monitored upregulated transcripts are indeed cold induced. Further, the fusion to YraN, another putative transcription factor, displayed a distinctive expression pattern when assayed during germination: 1-day-old spores exhibited no detectable signal; however, when spore cultures were further incubated at 4°C, transiently expressed GFP was detected in germinating spores (Figure 5B). Importantly, no GFP signal was seen in spores incubated at either 37°C or 50°C at all tested time points (Figure S4). Therefore, spore incubation at 4°C leads to accumulation of the yraN transcript, which in turn enables production of the corresponding protein upon germination. Hence, it seems that some of the upregulated transcripts are synthesized within the mature

**Figure 3. mRNA Profiles of Spores Held at Different Temperatures**

(A–C) Microarray profiles of aging WT (PY79) spores incubated at 37°C (A), 50°C (B), and 4°C (C). Listed are representative genes encoding mRNAs highly represented in a day-old spore (Table S2). Transcript levels at each spore age (in days) are relative to their level in the first day. Green shades designate decreasing levels, and red shades increasing levels. The color intensity denotes the fold change in a log2 scale, as indicated by the scale bar. Only values exceeding a ±0.7 log2 ratio were considered statistically significant (see Experimental Procedures).

(D) qRT-PCR analysis of select transcripts (underlined in Figure 3A) in aging WT (PY79) spores at 37°C and 50°C (see Experimental Procedures). See also Figure S3.
Spores Develop Diverse Capabilities to Germinate and Outgrow

So far we have established that mature spores can display distinct RNA contents, implying that they can acquire dissimilar molecular features in a time- and temperature-dependent manner. Because different aged spores incubated at the various temperatures were equally able to form colonies (data not shown), we explored whether molecular differences can lead to the formation of spores with diverse germination and propagation kinetics. Therefore, spore germination and outgrowth were assayed under challenging conditions in minimal medium, whereby differences in molecular content may result in phenotypic variations. As before, cells were induced to sporulate at 37°C for 24 hr, and spores were then transferred to 4°C, 37°C, or 50°C. Germination kinetics of different aged spores (1, 3, 5, and 8 days) was monitored for 5 hr at 37°C with time-lapse microscopy. We found that spores exhibit diverse kinetics of germination and outgrowth (Figures 6A and 6B). Spores kept at 37°C displayed the lowest germination and outgrowth abilities, with less than 5% of the spore population exiting dormancy in the course of the experiment, irrespective of age. On the other hand, germination efficiency of spores incubated at 50°C or 4°C was increased, reaching approximately 15%, with improved outgrowth exhibited by older spores. Similar results were obtained when spores were heated (15 min, 60°C) prior to germination (data not shown). Importantly, a prolonged incubation of 48 hr revealed that all assayed spores were equally able to germinate and form colonies (data not shown), implying that they differ mainly in kinetics during the initial steps of the process, at the time when their pre-existing reservoir is significant. To further elucidate these differences, we repeated the above experiments while supplementing the medium with bona fide germinants to improve germination efficiency. Although germination was enhanced under these conditions, the differences between the spores kept under the various temperatures were maintained, implying that they originate from intrinsic dissimilarities in spore properties (Figure S5).

To strengthen the correlation between the kinetics of exiting dormancy and the spore RNA state, we followed germination and outgrowth of spores depleted for RNaseY by time-lapse microscopy. Our results indicate that the depleted spores, incubated at 37°C, were deficient in their ability to exit dormancy in comparison to WT and nondepleted spores (Figure 6C). Interestingly, the nondepleted spores, in which RNaseY is produced...
from an inducible promoter, showed improved germination and outgrowth capabilities when compared with WT spores (Figure 6C), most likely due to higher RNaseY levels. Thus, we have established that the extent of RNA degradation affects the ability of the spore to resume growth.

**DISCUSSION**

Here we demonstrate that molecular changes are taking place within mature spores several days after sporulation. Hence, entry into dormancy appears to be a longer process than previously appreciated, involving RNA degradation along with RNA synthesis. The time between the release of the mature spore from the mother cell and the achievement of dormancy can be defined as an “adaptive period,” during which the spore still senses and responds to environmental cues and modifies its molecular content accordingly (Figure 7A). The occurrence of this “adaptive period” evokes that spores residing in diverse natural habitats harbor different molecular reservoirs that are likely to affect their capabilities to germinate and outgrow.

At least part of the acquisition of different characteristics by spores kept under various conditions can be attributed to the RNA state. According to our findings, we propose that spore RNA can be classified into two major species: intact molecules ready to be utilized and degradation products readily available for de novo synthesis. The balance between these two RNA species may have a key influence on spore emergence from quiescence (Figure 7B). Accordingly, at 50°C when the RNA pool comprises mostly degradation products, exiting dormancy is efficient due to rapid synthesis of required RNAs utilizing the available precursors. A similar outcome is achieved at 4°C, when the majority of the RNA is intact. However, a mixed pool containing both intact and degraded RNA, with neither of the species sufficiently available, leads to delayed germination as displayed at 37°C. Consistent with this idea, depletion of RNaseY perturbed outgrowth, whereas induction of RNaseY resulted in an improvement of both germination and outgrowth (Figure 6C). Possibly additional cellular macromolecules, such as proteins, display molecular dynamics similar to those observed for RNA. Consequently, the ability to germinate and propagate is determined by the overall composition of the molecular pool of the spore.

Having shown that global degradation of spore RNA occurs at high temperatures, we propose that this process is a mechanism utilized by the spore to achieve quiescence. Reducing the levels of rRNA required for ribosome construction, as well as transcripts, could be a strategy to minimize cellular activity. Whether spores preserve a minimal amount of rRNA molecules,
or whether these molecules are entirely degraded and resynthesized upon germination, is yet undetermined. In this regard, spores of Clostridium novyi were found to contain fragmented species of rRNA (Bettegowda et al., 2006), indicating that spore RNA degradation is a common mechanism for achieving dormancy among spore formers. Interestingly, ribosome degradation was described to be exploited by E. coli during transition to the stationary phase (Piir et al., 2011), suggesting that ribosome degradation is a general feature of bacterial growth deceleration. Unlike the massive RNA degradation observed at high temperatures, spores kept at low temperatures displayed significantly reduced RNA degradation. This pattern was consistent when spores were examined as late as 1 month post-sporulation (data not shown), suggesting that strategies other than RNA degradation are employed to achieve dormancy. This finding is consistent with previous observations demonstrating that the rRNA content of B. megaterium day-old spores held at −20°C maintained a similar pattern to that of vegetative cells (Chambon et al., 1968a, 1968b). Furthermore, transcription was evident in our experiments during the “adaptive period” of spores incubated at low temperature. The differences in the RNA fate between low and high temperatures may emanate from dissimilarities in the spore water content. At low temperature, the spore core might be more hydrated in a manner significant for transcription. Interestingly, higher sporulation temperatures were shown to lead to the production of spores with a less hydrated core (Melly et al., 2002). Nevertheless, it is possible that at high temperatures, transcription occurs within...
The energetic resources that allow transcription to take place within the spore, but most of the produced RNA is continuously degraded. Reaching dormancy at low temperatures may be mediated by progressive dehydration as well as exhaustion of energy sources.

The energetic resources that allow transcription to take place during the spore adaptive period are still unknown. Spores have been shown to contain very low levels of ATP (Setlow and Kornberg, 1970) along with high levels of the acid-soluble phosphorous compound 3-phosphoglyceric acid (PGA) (Nelson and Kornberg, 1970a). It has been proposed that PGA serves as an energy reservoir required for early metabolism during germination (Nelson and Kornberg, 1970b). It is therefore possible that PGA or similar compounds provide a source of energy for executing transcription within the spore.

B. subtilis, being a prevalent soil bacterium, is exposed to a wide range of temperatures, depending on its geographic location and depth in the soil. Therefore, spores in diverse habitats will potentially possess different molecular features. Diverse organisms including plant seeds and a variety of bacteria such as Mycobacterium tuberculosis display a quiescent life form. It is tempting to speculate that, similar to Bacilli spores, these organisms would yield quiescent cells with different molecular compositions and properties when entering dormancy under various environmental conditions.

Our observations revealed little variation in spore RNA levels between 5 and 8 days post-sporulation, at all temperatures. This plateau may represent the accomplishment of spore dormancy; alternatively, the lack of variation might reflect a certain steady state of cellular activities. It is yet unclear whether spore metabolism entirely ceases during quiescence, or whether a basal metabolic level persists, possibly in slower rates than within the vegetative bacterium. Although bacterial spores have been documented from the early days of microbiology by Robert Koch and Ferdinand Cohn (Cohn, 1877; Koch, 1876), the molecular characterization of the mature spore is still partial, and basic questions concerning spore quiescence remain to be explored.

**EXPERIMENTAL PROCEDURES**

**Strains and General Methods**

B. subtilis strains were derivatives of the WT strain PY79 (Youngman et al., 1984) and are listed in Tables S5 and S6. Plasmids construction is described in Extended Experimental Procedures. All general methods were carried out as described previously (Harwood and Cutting, 1990). Sporulation was induced by suspending cells in Schaeffer’s liquid medium (DSM) (Schaeffer et al., 1965). For strains harboring essential genes under inducible promoters, cells were grown on inducer containing plates (0.5% xylose or 0.5 mM IPTG, as indicated) and transferred to liquid DSM with or without inducer. Germination was induced in S7 minimal medium (Vasantha and Freese, 1980). When indicated, S7 was supplemented with AGFK (2.5 mM L-asparagine, 5 mg/ml D-glucose, 5 mg/ml D-fructose, 50 mM KCl) and 0.01M alanine (Pelczar et al., 2007).

**Spore RNA Extraction**

B. subtilis strains were induced to sporulate in DSM for 24 hr at 37°C, then transferred to the indicated temperatures for further incubation. Importantly, the majority of spores were released from the mother cell within 24 hr, as evaluated by phase-contrast microscopy. Prior to RNA extraction, spore cultures were treated with a modified freshly made decoating solution (0.5 M EDTA, 1 M Tris, 8 M Urea, 50 mM dithiothreitol, 10% sodium dodecyl sulfate) (Riesenman and Nicholson, 2000) for 1 hr at 70°C. The treatment eliminated cells and cell debris but had no significant influence on spore viability, as determined by colony formation of heat-treated spores (85°C, 30 min; data not shown) (Moeller et al., 2006). After decoating, spores were washed three times with PBS × 1, and RNA was extracted using FastRNA Pro Blue kit (MP Biomedicals) with the following modifications: 15 ml of spore culture was processed three times with the FastPrep-24 (MP Biomedicals) in setting 6.5 m/s, for 45 s each time, with 2 min of ice cooling between cycles. We

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**Figure 7. A Model Describing Spore RNA Dynamics**

(A) A scheme showing timeline and fate of RNA within mature spores. Following spore formation, the spore is released from the mother cell and undergoes an “adaptive period” that lasts a few days. During this time, environmental conditions, such as temperature, influence the RNA content of the spore. High temperature leads to global RNA degradation, whereas low temperature enables transcription. Consequently, the spore can develop different molecular reservoirs. Afterward, the spore reaches the phase of dormancy or steady state. Shown are early spore RNA molecules (black) and molecules synthesized during the adaptive period (blue).

(B) A scheme describing the correlation between temperature, spore RNA content, and germination and outgrowth capabilities. See Discussion for details.
quantitatively assessed the efficiency of spore rupture by plating spore samples prior to and post breakage. Our findings indicate that only 0.0001% of the spore population remains intact. Finally, RNA concentration was determined using NanoDrop 2000C (Thermo Scientific), and RNA samples adjusted to 500 ng/μl were visualized by capillary gel electrophoresis in the Agilent 2100 Bioanalyzer.

To substantiate that RNA is derived only from intact spores, we additionally purified spores prior to RNA extraction with an alternative strategy using Histodenz (Sigma) gradient (Abel-Santos and Dodatko, 2007). Briefly, a Histodenz gradient of 48% to 64% was layered in a 15 ml plastic tube (Corning), and each layer was frozen at ~70 °C for at least 30 min. A 10 ml spore culture was resuspended in 1 ml of 20% Histodenz solution for 30 min on ice. Spores were then placed on top of the frozen gradient and centrifuged for 1 hr at 10,000 RPM at 23 °C. After centrifugation, three distinct layers were visible; the lowest contained a 99% pure spore population, as evaluated by phase-contrast microscopy. RNA was extracted from pure spore populations by FastRNA Pro Blue kit (MP Biomedical) as described above. RNA concentration was determined using NanoDrop 2000C (Thermo Scientific). Samples were adjusted to 500 ng/μl and visualized by capillary gel electrophoresis in the Agilent 2100 Bioanalyzer (Figure S1D) and further used as templates for qRT-PCR analyses. All qRT-PCR analyses presented in this work were repeated using RNA derived from gradient-purified spores; the data obtained by either decoating or gradient purification were vastly similar (data not shown).

qRT-PCR
RNA (2 μg) extracted from decoated spores was treated with RQ1 DNase (Promega) and subjected to cDNA synthesis using SuperScriptIII reverse transcriptase (Molecular Probes, Invitrogen), according to the manufacturer’s protocol. Standard qRT-PCR reactions were conducted using SYBR-green mix (LightCycler 480 SYBER Green I Master, Roche), and fluorescence detection was performed using Rotor-Gene 3000A (Corbett Life Science) according to manufacturer’s instructions. qRT-PCR primers (Table S6) were designed using Primer3 software (v. 0.4.0, available online). For RNA extracted from 4°C-incubated spores, yoxA was used to normalize expression data, as its expression was unmodified throughout the time of incubation, as indicated by both microarray and qRT-PCR analyses. For RNA from 37°C- or 50°C-incubated spores, because global degradation of RNA was detected, a normalizing gene was not available. Thus, a known amount of an exogenous linear DNA carrying the gfp gene was added to the concentrated fraction of each cDNA used as template, prior to serial dilutions. The threshold cycle (Ct) of gfp was used to ensure efficient amplification reactions in the presence of highly degraded templates and to normalize dilution variations. Hence, for 37°C- and 50°C-incubated spores, results are presented in arbitrary units (AU) reflecting the differences in Ct. Each assay was performed in duplicates with at least two RNA templates prepared from independent biological repeats. Data were analyzed according to the standard curve method (Rotor-gene analysis software 6.0).

Microarray Analysis
cDNA Preparation and Array Hybridization
RNA was extracted from decoated spores and subjected to cDNA synthesis, fragmentation, and end terminus biotin labeling according to the standard Affymetrix prokaryotic GeneChip expression protocol with the following modifications: the initial RNA amount was 20 μg, cDNA synthesis was preformed with SuperScriptIII reverse transcriptase (Molecular Probes, Invitrogen), and cDNA reactions were incubated overnight at 42°C. Finally, labeled cDNA samples were hybridized to Affymetrix GeneChip B. subtilis arrays. Hybridized arrays were washed and scanned using the Affymetrix fluidic station 450. After staining, arrays were scanned with a GC3000 scanner.

Statistics
RNA normalization was performed on CELL files using the Partek Genomic Suite 6.5. Additional statistical analysis was carried out using the Spotfire software package (Somerville, MA, USA) and custom Matlab (R2010B) routines. Histogram plot of the log2 ratio resembled a normal shape distribution with values of ±0.7 being the first standard deviation (SD) of the curve; therefore values exceeding a ±0.7 log2 ratio were considered statistically significant.

Additional experimental procedures: Fluorescence microscopy, FISH, and quantification of RNA band intensities are described in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2011.11.059.

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