Molecular Kinetics of Reviving Bacterial Spores

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Bacterial spores can remain dormant for years, yet they possess a remarkable potential to rapidly resume a vegetative life form. Here, we identified a distinct phase at the onset of spore outgrowth, designated the ripening period. This transition phase is exploited by the germinating spore for molecular reorganization toward elongation and subsequent cell division. We have previously shown that spores of different ages, kept under various temperatures, harbor dissimilar molecular reservoirs (E. Segev, Y. Smith, and S. Ben-Yehuda, *Cell* 148:139–149, 2012). Utilizing this phenomenon, we observed that the length of the ripening period can vary according to the spore molecular content. Importantly, the duration of the ripening period was found to correlate with the initial spore rRNA content and the kinetics of rRNA accumulation upon exiting dormancy. Further, the synthesis of the ribosomal protein RplA and the degradation of the spore-specific protein SspA also correlated with the duration of the ripening period. Our data suggest that the spore molecular cargo determines the extent of the ripening period, a potentially crucial phase for a germinating spore in obtaining limited resources during revival.

The Gram-positive soil bacterium *Bacillus subtilis* and its relatives respond to nutrient deprivation by executing a complex developmental process called sporulation, resulting in the formation of a highly durable spore (1–3). Once sporulation commences, the cell divides asymmetrically into two unequal-sized progenies; the smaller forespore, which develops into a spore, and the larger mother cell, which nurtures the maturing spore. Subsequently, the forespore is engulfed by the mother cell, and its cytoplasm becomes the core of the future spore (2, 3). A thick layer of peptidoglycan, called the cortex, is synthesized around the core by the mother cell, followed by the construction of multiple layers of a proteinaceous coat. These encasing shells confer unique mechanical protection to the spore, acting as a boundary between the spore and the environment (4, 5). After approximately 8 h of differentiation, the spore is liberated by lysis of the mother cell and sporulation is completed. Once nutrients become available, the spore can resume growth by converting into an actively growing cell (2, 3).

Previously held views considered the released spore to be dormant and metabolically inert, partially due to high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid) and small amounts of water and ATP (6, 7). However, increasing evidence challenges this traditional concept, as the released spore continues to display various dynamic processes. Accordingly, enzymatic reactions, occurring after spore discharge from the mother cell, have been demonstrated to be necessary to accomplish full maturation of coat proteins (8–11). Further, we have recently shown that at least 1 week postsporulation, during an adaptive period, the spore senses and responds to environmental cues. Throughout this time, the RNA content of the spore is actively shaped according to spore age and the temperature at which it is held (12).

Along with the physical defense provided by the protecting shells, the spore possesses special molecular characteristics, enabling it to face extreme conditions for many decades (13, 14). One such prominent feature is protection of the chromosome in a condensed nucleoprotein ring-like structure, achieved by the action of specialized spore-specific proteins (Ssp), known as the α/β -type SASP proteins (4, 15–18). Upon spore germination, the Ssp proteins serve as a reservoir of amino acids to support protein synthesis. At this stage, Ssp utilization is accomplished through the

activity of a sequence-specific endoprotease, termed GPR (germination protease), which facilitates Ssp degradation (19–21).

Exiting dormancy is initiated by germination, during which the spore undergoes rehydration, cortex hydrolysis, and coat disassembly. These events allow the acquisition of spore metabolism, followed by macromolecular synthesis and cell elongation, converting the germinating spore into a growing cell in a process called outgrowth (4, 22, 23). Early studies investigating the kinetics of RNA and protein synthesis indicated that these processes initiated rapidly within several minutes after induction of germination (13, 24–27). Furthermore, a more recent study monitoring the expression profile of mRNA from germinating spores detected the appearance of specific transcripts as early as 5 min following the onset of germination (28). Germination is induced by a variety of molecules, including amino acids, sugars, and cell wall muropeptides. These triggering molecules bind to receptors in the inner spore membrane, signaling the spore to revive (4, 18, 22, 29, 30). Interestingly, it has been shown that germination kinetics are influenced by sporulation conditions as well as the level of germination receptors (31–33). We have previously shown that efficiency of germination and outgrowth is influenced by spore age and incubation temperature, which determine the molecular content of the spore (12).

Here, we have characterized a distinct morphological stage early during outgrowth, extending between germination and cell elongation, termed the ripening period. We show that this period

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is dedicated to molecular reorganization, as exemplified by rRNA synthesis and protein assembly and degradation. We provide morphological and molecular characterization of the ripening period and demonstrate that it varies in length according to the initial molecular resources contained within the spore.

MATERIALS AND METHODS

Strains and general methods. *B. subtilis* strains were derivatives of the wild-type strain PY79 (34) and are listed in Tables S1 and S2 in the supplemental material. Plasmid construction is described in the supplemental material. All general methods were carried out as described previously (12, 35). Sporulation was induced by suspending cells in Schaeffer's liquid medium (DSM) (36). Germination was induced in reviving medium containing S7 minimal medium (37) supplemented with AGFK (2.5 mM L-asparagine, 5 mg/ml D-glucose, 5 mg/ml D-fructose, 50 mM KCl) and 0.01 M alanine (38).

Spore purification. *B. subtilis* strains were induced to sporulate in DSM for 24 h at 37°C and then transferred to the indicated temperatures for further incubation. Importantly, the majority of spores were released from mother cells within 24 h, as evaluated by phase-contrast microscopy. Spore cultures were purified using Histodenz (Sigma) gradients (12, 39).

RNA extraction. (i) Spore RNA extraction. RNA was extracted using a FastRNA Pro Blue kit (MP Biomedicals) as previously described (12). We quantitatively assessed the efficiency of spore rupture by plating spore samples prior to and after breakage. Our findings indicate that only 0.0001% of the spore population remains intact. RNA concentration was determined using a NanoDrop 2000C (Thermo Scientific). Samples were adjusted to 500 ng/ μ l and visualized by capillary gel electrophoresis in an Agilent 2100 Bioanalyzer.

(ii) RNA extraction from reviving spores. Sampling equivalent numbers of spores from differently treated cultures was accomplished by manual counting of bright spores under the microscope using a cytometer (Neubauer improved bright-line; Marienfeld). Samples of 12 to 15 ml were collected from each culture according to the measured spore number. Cultures were transferred to 50-ml ice-cooled tubes (Corning) and centrifuged for 5 min at 10,000 rpm at 4°C. The supernatant was discarded, and pellet-containing tubes were immediately placed in liquid nitrogen for 10 min and then stored at -70°C for a maximum of 4 days. Frozen pellets were resuspended on ice in 1 ml of phenol-containing solution (RNApro; MP Biomedicals), and RNA was extracted using a FastRNA Pro Blue kit (MP Biomedicals) as previously described (12). Samples were visualized by capillary gel electrophoresis in an Agilent 2100 Bioanalyzer.

Fluorescence microscopy. Fluorescence microscopy was carried out as described previously (12). For time-lapse germination experiments, spores were placed on 1% agarose pads made of either LB or reviving medium and incubated in a temperature-controlled chamber (Pecon-Zeiss) at 37°C. Samples were photographed using an Axio Observer Z1 (Zeiss) equipped with a CoolSnap HQII camera (Photometrics, Roper Scientific). System control and image processing were performed using MetaMorph 7.4 software (Molecular Devices). For fluorescence quantification during spore revival, cell boundaries were defined by the phase-contrast image, and the average fluorescence emanating from each cell was calculated from the corresponding fluorescence image. The average background fluorescence signal was calculated from cell-free regions within a given field. The background level was subtracted from the fluorescent values obtained for each individual cell. To determine the average GFP signal emanating from a given strain in arbitrary units (AU), the calculated net average fluorescence intensity from at least 3 different fields was averaged and the intensity of a wild-type (PY79) strain, lacking the *gfp* gene, was subtracted.

Quantification of RNA band intensities. Band intensities were quantified by comparing the total intensity of identical-sized regions using MetaMorph 7.4 software (Molecular Devices) on the electropherogram image produced by the Agilent 2100 Bioanalyzer for each electrophoresis run.

RESULTS

Reviving spores display a distinct morphological phase, termed the ripening period. In the process of exiting dormancy, two key morphological events are visible by light microscopy: the transition from a phase-bright spore to a phase-dark cell during germination, and the elongation of a new cell from the disintegrating spore shells during outgrowth (Fig. 1A). Here, the entire process of resuming a vegetative life form will be referred to as revival, while the term germination will be used to describe the earliest stage of the process, namely, the switch to a phase-dark cell. Following reviving spores using time-lapse microscopy revealed an intermediate phase taking place early during outgrowth prior to cell elongation, in which no morphological change is evident. We have termed this stage the ripening period and defined it as the phase between the loss of brightness and the very beginning of cell elongation (Fig. 1A and B).

Previously, we have shown that germination and outgrowth kinetics are vastly influenced by spore age and incubation temperature (12). Thus, we hypothesized that the duration of the ripening period is also affected by these factors. To further investigate this idea, we conducted multiple time-lapse microscopy experiments, whereby spores of different age kept at various temperatures were monitored during revival. Accordingly, cells were induced to sporulate at 37°C for 24 h, and the resultant spores were additionally incubated for 5 days at either 4, 37, or 50°C. At days 1, 2, and 6, spores from the aging cultures were collected, and their revival was monitored for 180 min. Since differently treated spores display large dissimilarities in their ability to germinate, the medium was supplemented with bona fide germinants, which largely enhance germination efficiency (38) (see Fig. S1 in the supplemental material). This approach yielded a synchronized germinating population, enabling us to focus on the later phases of ripening and elongation, within the time frame of our experiments. We could clearly detect a correlation between the length of the ripening period, spore age, and incubation temperature. Day-old spores displayed a ripening period of approximately 90 min (between 30 and 120 min during revival), while further incubation at 37°C prolonged this phase to exceed the duration of the experiment (Fig. 1C). When held at 50°C, spores exhibited an extended ripening period at day 2 of incubation (>180 min). However, at day 6 the initial length of the ripening period, 90 min, was restored (Fig. 1C). Finally, in spores held at 4°C, the ripening period was the shortest at both tested ages. These results reinforce the view that the length of the ripening period is dynamic and affected by spore age and incubation temperature. Since differently treated spores contain a dissimilar molecular cargo determining their capability to revive (12), the extent of the ripening period is also likely to be influenced by this initial molecular content.

Different kinetics of rRNA synthesis observed during spore revival. We wished to explore whether the length of the ripening period correlates with the duration of molecular processes occurring during spore revival. To monitor the initiation of essential growth-promoting processes, we monitored the kinetics of ribosome assembly during the course of spore revival by monitoring the profile of rRNA. We took advantage of the observation that spores held at high temperatures (37 and 50°C) exhibit decaying rRNA patterns over time, while spores kept at low temperature (4°C) maintain mainly intact rRNA (see Fig. S2 in the supplement-

FIG 1 Monitoring the ripening period of reviving spores. (A and B) Wild-type *B. subtilis* (PY79) cells were induced to sporulate at 37°C. Twenty-four h later, spores were incubated in reviving medium and monitored for 180 min. Shown is the sequence of events during revival as captured by phase-contrast images, taken at 30-min intervals, of a single spore (A) and a spore population (B). Germination is visualized as the loss of spore brightness (0 to 30 min; red line), the ripening period (early outgrowth) is an intermediate phase during which no morphological change is evident (30 to 120 min; orange line), and elongation (late outgrowth) is characterized by increasing cell length (120 to 180 min; green line). Inset numbers indicate measured cell length. Scale bar, 1 μm . (C) Wild-type *B. subtilis* (PY79) cells were induced to sporulate at 37°C. Twenty-four h later, spores were shifted to 4, 37, or 50°C and further incubated. Spores at the indicated age (in days) were transferred to reviving medium and monitored by time-lapse microscopy. The plots represent the ratio between revival stages (germination, ripening, and elongation) displayed by the population at each time point. Shown are data from a representative experiment (out of 2 biological repeats); $n = 100$ for each data point.

tal material) (12), thereby providing distinct sources of molecular pools to initiate the revival process.

To assay the capacity of reviving spores to synthesize rRNA, cells were induced to sporulate at 37°C, and the resultant spores were further incubated at either 4, 37, or 50°C. At days 1, 2, and 6,

similar amounts of spores from the aging cultures were collected and transferred to reviving medium. RNA was extracted at 30-min intervals during 180 min of incubation. During this period, cell division was not evident, indicating that the extracted RNA reflects predivisional processes taking place within reviving spores.

FIG 2 Kinetics of rRNA synthesis during revival. (A to C) Wild-type *B. subtilis* (PY79) cells were induced to sporulate at 37°C. Twenty-four h later, spores were shifted to 4, 37, or 50°C and further incubated. Similar amounts of spores at the indicated age (in days) were induced to revive. RNA was extracted at the indicated time points and analyzed in a microfluidic gel. Arrows designate the positions of 23S rRNA (upper) and 16S rRNA (lower). All lanes in the pseudogel are scaled to the same intensity range. Quantification of 23S and 16S rRNA band intensities (in AU) is presented below each lane (see Materials and Methods). The length of the ripening period, as determined for Fig. 1C, is designated by an orange line above each pseudogel. A dashed line indicates a ripening period longer than the duration of the experiment.

Our results demonstrate that the length of the ripening period correlates with the duration of rRNA assembly, with extended ripening periods coinciding with slower synthesis (Fig. 2). Day-old spores, with relatively high levels of intact rRNA, exhibited a gradual increase in the amount of rRNA during revival, reaching a maximal level after 120 min (Fig. 2A). However, reducing the pool of intact spore rRNA by additional incubation at 37°C led to significantly slower kinetics of rRNA assembly during revival, consistent with the observed prolonged ripening periods (Fig. 2B and C). Further, spores kept at 4°C, harboring high levels of intact rRNA, showed dynamics similar to those observed for day-old spores, in line with the short ripening periods detected at this temperature. For spores incubated at 50°C, diverse kinetics of

rRNA synthesis during revival were monitored. At day 2, spores containing partially degraded rRNA exhibited relatively slow rRNA synthesis during revival, similar to spores incubated at 37°C at this time point (Fig. 2B). A remarkable change in the rRNA profile was observed for 6-day-old spores; at time zero, the rRNA within the spores was largely undetected; however, after only 30 min it reached approximately 70% (84/120 AU) of the maximal level detected for this spore population (Fig. 2C). This rapid formation of rRNA was further examined with greater time resolution, revealing detectable rRNA levels as early as 10 min postincubation in reviving medium (Fig. 2C). The two distinct dynamics observed for spores incubated at 50°C coincide with the duration of their ripening period, being significantly longer at day 2 than at day 6.

FIG 3 rRNA patterns of spores experiencing a dual temperature shift. (A) Wild-type *B. subtilis* (PY79) cells were induced to sporulate at 37°C. Day-old spores were shifted to 50°C for 24 h and then were transferred to 4°C for an additional incubation. RNA was extracted daily from spores and analyzed in a microfluidic gel. (B) The resulting 6-day-old spores described for panel A were induced to revive. RNA was extracted at 30-min intervals and analyzed in a microfluidic gel. Arrows designate the positions of 23S rRNA (upper) and 16S rRNA (lower). All lanes in the pseudogels are scaled to the same intensity range. Quantification of 23S and 16S rRNA band intensities in panel B is presented below each lane (see Materials and Methods).

Taken together, our data demonstrate that a correlation exists between the speed of rRNA synthesis and the duration of the ripening period. Hence, the ripening period is probably a stage dedicated to molecular reorganization as a preparation toward cell elongation and division. Further, these results support the idea that rRNA synthesis during revival is affected by the initial spore RNA content. Substantially degraded rRNA (day 6 at 50°C) or plenty of intact molecules (day 6 at 4°C) provide the best reservoir for driving the process of revival forward, while a mixture of degraded and nondegraded molecules (day 6 at 37°C) is the least efficient source. This inefficiency may stem from the requirement for additional RNA processing steps prior to its utilization, resulting in an extended ripening period.

To further substantiate that the initial state of spore rRNA affects synthesis kinetics during revival, we devised an alternative way to generate spores harboring a mixed pool of intact and degraded RNA via a temperature shift. As before, cells were induced to sporulate at 37°C. Spores were shifted to 50°C for 24 h to allow RNA degradation to commence and were then transferred to 4°C for an additional 4 days in order to minimize further degradation. As predicted, following this procedure, the rRNA pattern of 6-day-old spores contained a mixed pool of intact and degraded rRNA molecules (Fig. 3A; also see Fig. S2 in the supplemental material). Similar to spores kept at 37°C, the rRNA synthesis in these spores during revival was relatively slow, reaching approximately 60% (72/120 AU) after 180 min of incubation compared to the maximal level detected in corresponding experiments (Fig. 2C). This delay in synthesis is consistent with a relatively long ripening period as well as postponed elongation (compare Fig. 3B to Fig. 2C

and data not shown). Thus, a spore possessing both intact and degraded rRNA, with neither of the species in sufficient amount, displays an extended ripening period for assembling rRNA.

Protein dynamics correlate with the extent of the ripening period. Having uncovered that the kinetics of rRNA synthesis correlate with the extent of the ripening period, we explored protein fate throughout this phase. To complement our rRNA analysis, we first examined the dynamics of RplA, an essential proteinaceous component of the ribosome (40–42). Cells harboring a functional *rplA-gfp* fusion as a sole *rplA* source (43) (see Fig. S3 in the supplemental material) were induced to sporulate at 37°C for 24 h. Spores were incubated at 4, 37, or 50°C for 5 additional days and monitored during revival by time-lapse microscopy. Day-old spores exhibited a gradual increase in their total GFP level upon germination, which was maintained throughout the ripening period. Subsequently, an additional rise in fluorescence was observed at the onset of cell elongation (Fig. 4A; also see Fig. S4A in the supplemental material). A similar yet more pronounced increase in fluorescence was seen for 6-day-old spores incubated at either 4 or 50°C (Fig. 4B and D; also see Fig. S4A). Importantly, these spores exhibit a ripening period comparable in length to that of day-old spores. Consistent with the low rate of rRNA synthesis and the extended ripening period, GFP signal from spores kept at 37°C remained low, with no evidence for RplA-GFP synthesis throughout the course of the experiment (Fig. 4C; also see Fig. S4A).

To further correlate the duration of the ripening period with protein dynamics, we monitored the ability of the reviving spore to degrade components that are required exclusively during the spore's dormant phase. For this analysis, we followed the spatial and temporal pattern of the spore-specific DNA binding protein SspA in reviving spores. In general, in spores harboring SspA-GFP, the signal appeared in a ring-like shape demarcating the nucleoid (15, 44) (see Fig. S5 in the supplemental material). A similar localization pattern was observed when SspA was fused to a monomeric GFP variant (see Fig. S5). This distinct localization was replaced by a diffusible pattern during germination (Fig. 5), consistent with the observation that SspA is dissociated from the chromosome during this stage (44). Subsequently, the fluorescence signal diminished over time, exhibiting a profile opposing that of RplA-GFP. The decrease in fluorescence occurred in a GPR-dependent manner. In the absence of GPR, SspA-GFP maintained a relatively stable fluorescence level and the typical ring-like localization pattern (see Fig. S6 in the supplemental material). These observations imply that the GFP signal reflects the *in vivo* degradation kinetics of SspA. Spores displaying short ripening periods (i.e., day-old spores and spores held at 4 or 50°C) were able to degrade SspA relatively rapidly. However, spores kept at 37°C, displaying an extended ripening period, exhibited a relatively constant fluorescent signal throughout the course of the experiment, implying a delayed degradation of spore components (Fig. 5; also see Fig. S4B).

Taken together, our results demonstrate that proteins undergo dynamic processes during spore revival. The kinetics of protein assembly, as well as degradation, are linked to the length of the ripening period affected by spore age and incubation temperature.

DISCUSSION

Spore revival provides a unique developmental process to study the conversion from cellular dormancy to extensive activity. This extraordinary transition offers an opportunity to monitor the re-

FIG 4 Kinetics of ribosomal protein expression during revival. (A to D) Cells harboring *rplA-gfp* (AR5) were induced to sporulate at 37°C. Day-old spores were shifted to 4, 37, or 50°C and further incubated. Spores at the indicated age (in days) were transferred to reviving medium and monitored during 180 min by time-lapse microscopy (see Materials and Methods). Shown are fluorescent (upper) and phase-contrast (lower) images taken every 30 min. Arrows signify the dominating revival stages of each population at a given time point (germination, red; ripening, orange; elongation, green). Revival stages were determined as designated in the legend to Fig. 1A. Scale bar, 1 μ m.

establishment of essential cellular functions. In this study, we identified the ripening period exploited by the germinating spore for molecular reorganization toward elongation and subsequent cell division. This distinct phase is fueled by the spore molecular reservoir, with a duration correlating with kinetics of ribosome construction as well as with degradation of spore-specific components. Importantly, both RNA and protein constituents show similar molecular kinetics, accelerated upon shorter ripening periods.

We have previously shown that environmental conditions shape the molecular cargo encased within the spore, with age and temperature being dominant factors. Here, we demonstrate that diverse molecular pools, which develop within spores, lead to dissimilar capacities to assemble and degrade RNA and proteins during revival. In line with this idea, spores possessing supportive molecular resources that can rapidly facilitate growth display rel-

atively short ripening periods (e.g., day 6 at 4 or 50°C). However, molecular reservoirs that require additional processing before utilization lead to extended ripening periods (e.g., day 6 at 37°C or after shifting from 50 to 4°C). These data suggest that a spore molecular content dominated by a certain RNA state, either intact or degraded, provides an efficient resource to facilitate revival.

The similar kinetic patterns observed for various macromolecules upon exiting dormancy bring about the question of whether the overall efficiency of spore revival is determined by the sum of parallel processes displaying similar kinetics. Alternatively, a rate-limiting factor, which triggers the array of downstream events, might govern the progression of the entire process. It has been previously shown that the small amount of spore ATP limits RNA and protein synthesis during the first minutes of germination. ATP is largely generated early in germination from an acid-soluble

FIG 5 Kinetics of spore-specific protein degradation during revival. (A to D) Cells harboring *sspA-gfp* (ES1) were induced to sporulate at 37°C. Day-old spores were shifted to 4, 37, or 50°C and further incubated. Spores at the indicated age (in days) were transferred to reviving medium and monitored during 180 min by time-lapse microscopy (see Materials and Methods). Shown are fluorescent (upper) and phase-contrast (lower) images taken every 30 min. Arrows signify the dominating revival stages of each population at a given time point (germination, red; ripening, orange; elongation, green). Revival stages were determined as designated in the legend to Fig. 1A. Scale bar, 1 μ m.

spore phosphorous compound named 3-phosphoglyceric acid (PGA), which might serve as a rate-limiting factor controlling revival kinetics (6, 45). It is possible that differently treated spores contain dissimilar levels of PGA, accounting for the diverse kinetics exhibited during resuscitation. Additionally, differences in the abundance of components required for ATP production may contribute to diverse revival phenotypes.

Interestingly, in a population of reviving spores, a minority of spores was shown to germinate very poorly. These superdormant spores were shown to be genetically identical to their normally germinating siblings, and their delayed response was found to originate from low levels of specific germination receptors (32, 46). In the present work, after minimizing dissimilarities in germination efficiency, we were still able to observe diverse revival kinetics among populations of differently treated spores. There-

fore, the speed of the revival process as a whole is also controlled by events subsequent to germination, occurring during the ripening period.

As *B. subtilis* is a common soil bacterium, it resides in diverse natural niches. Different characteristics of each habitat are likely to produce spores that harbor dissimilar molecular pools, impacting revival kinetics. In nature, the speed of exiting dormancy might be crucial, as most environmental resources supporting bacterial growth are restricted. Thus, the duration of the ripening period might play a key role in the ability of a reviving spore to utilize limited nutrients and resume an active life form.

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