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Heat Activation of *Bacillus Anthracis* Persists Over a Seven Day Period

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**HEAT ACTIVATION OF *BACILLUS ANTHRACIS*
PERSISTS OVER A SEVEN DAY PERIOD**

by

Nathan T. Ross, B. S. Biology

A Thesis Presented in Partial Fulfillment
of the Requirements of the Degree
Master of Science

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ABSTRACT

Bacteria of the *Bacillus* genera are capable of forming dormant and resilient cells called spores in response to starvation. Spores can break dormancy in the presence of nutrients in a process called germination. Historically, spores are exposed to sublethal heat treatments to increase the extent and rate of germination. This process is known as heat activation (HA). Previous studies on *Bacillus* species indicate the effects of HA are reversible after 72 hours. After this time period, the spores must be reactivated. However, spores of *Bacillus anthracis*, a member of the *Bacillus* family, have not been tested to see how long they remain activated.

In this study, *B. anthracis* spores were heat activated and germination was measured for seven days to see if they remained activated. *B. anthracis* spores were prepared by the exhaustion method in Difco Sporulation Media and extensively water washed. Each sporulation was split into three samples: no heat treatment (UH), heat activated on day one of the experiment (HA), and activation on the particular day relative to day one (Hn). Germination was initiated with 1mM L-alanine and 1mM inosine and was measured by the loss of optical density (OD) at 580nm. The assays were performed on days one, three, five, and seven.

As expected, heat activation had a positive impact on spore germination. On day one, HA and H1 spores had a final OD loss of 57.24% and 57.88%, respectively, while the UH spores had an OD loss of 34.69%. On day seven, HA spores had an OD loss of

54.16%, H7 had a 52.66% OD loss, and UH had a 27.49% OD loss. Although all reactions showed a decrease in germination on day seven compared to day one, a two-way ANOVA test showed this decrease to be statistically insignificant. The heated samples (HA, Hn) were shown to be significantly different from the UH sample on both day one and day seven. Interestingly, there was no significant difference in germination for HA spores between day one and day seven, suggesting that HA spores can remain activated over seven days. No significant difference was seen between HA and Hn for either day.

This study shows that HA impacts *B. anthracis* spores for seven days, much longer than originally expected. This changes our understanding of heat activation in *B. anthracis*. Further studies will help define the time it takes for spores to be inactivated as well as test the effects of storage conditions on activation.

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CHAPTER 1

INTRODUCTION

Bacteria of the *Bacillus* genera are capable of forming a specialized dormant cell type called a spore in response to nutrient limitation and unfavorable environmental conditions (1 - 4). Spores are multi-layered structures that are highly resistant to environmental stresses such as temperature changes, pH, and chemical damage (2 - 6). Spores have been known to remain dormant for decades before favorable conditions return and allow them to enter a vegetative growth state that begins with the process of germination (1, 2, 7, 8).

Germination is the process through which spore dormancy is broken. Germination in *Bacillus anthracis* begins when the spore encounters certain amino acids and/or nucleosides called germinants (1, 9, 10). These germinants bind to specific receptors within the inner membrane of the spore. Once activated, these germinant receptors initiate the multi-step process of germination (8 - 10).

Prior to experiments, spores in a lab setting are often exposed to sublethal heat treatment to increase the extent and rate of germination (8, 9, 11). This treatment, which is known as heat activation (HA), has been utilized for well over 50 years (11, 12). HA aids in germination by reducing T_{lag} - the time from the spore's exposure to adequate germinant to its release of calcium ions and its commitment to germinate (8, 13, 14).

Many published papers state the effects of HA on *Bacillus* endospores are reversible (9, 11, 15). A past study showed the effects of HA on the germination of *Bacillus cereus* spores stored at 4°C and 28°C began to reverse after just one day. All spores showed a germination rate of 2.3% per minute on the day of heat activation. After 24 hours, spores stored at 4°C had a germination rate of 2.0% per minute while those at 28°C dropped to 1.2% per minute. At 72 hours, the germination rate of spores stored at 4°C dropped to 1.5% per minute, while those stored at 28°C had a decrease in germination rate to 0.5% per minute (11). Storage temperature clearly was a factor in the return to an inactive state.

It is common knowledge that the effects of HA on spores begin to revert over a period of time (4, 9). Recent studies in our lab have shown the effects of HA on *Bacillus subtilis* and *B. anthracis* might not reverse over a 72-hour period. We wondered if the effects of HA on the germination of *Bacillus* spores would persist after 72 hours. Data gathered during portions of these recent experiments suggested the effects of HA on *B. anthracis* may last seven to 14 days.

B. anthracis is a rod shaped, gram-negative, aerobic bacteria of the *Bacillus* genera (16, 17). This bacterium is the infectious agent of the disease anthrax (16, 17, 18) and has the potential to be weaponized (17, 19 - 21). Upon entry into a host, the environmental conditions become favorable to the spore, allowing it to revert to a metabolically active bacteria in a process that begins with germination (17, 18, 20).

It is well recognized that germinated spores are easier to kill than dormant spores (1, 12, 22, 23). HA is known to increase the rate and extent of germination. A better understanding of the length of time *B. anthracis* spores maintain the effects of HA can

impact decontamination procedures which include germination of the spores. Therefore *B. anthracis* was the species chosen for this experiment.

B. cereus was used in many previous experiments on heat activation (4, 8, 11, 24). *B. anthracis* and *B. cereus* have similar structures and similar, but not identical, responses to germinants. Therefore, it is possible *B. anthracis* will have a different reaction to HA than *B. cereus*.

The purpose of this study was to measure the time *B. anthracis* spores remain active after a single heat treatment by measuring germination kinetics over a period of several days. My hypothesis is that the effects of HA on *B. anthracis* will last for at least seven days.

CHAPTER 2

BACKGROUND

2.1 Spore Structure

Through the process of sporulation, the *Bacillus* genera are capable of forming a resilient cell type known as a spore in response to harmful environmental stress or starvation. The spore's structure and composition factor prominently in its dormancy and resiliency (8, 18). Most spores from the *Bacillus* genus share a common design (9, 25) as illustrated in Figure 2-1. Innermost is the spore core which contains the DNA and protective DNA-binding proteins known as small, acid-soluble proteins (SASP). The SASP bind to DNA and provide protection from UV radiation and depurination, as well as heat and oxidizing agents (26, 27). Immediately adjacent to the core is the inner membrane. The inner membrane is associated with most of the proteins, including germinant receptors, necessary for germination (2, 8). The inner membrane is surrounded by the cell wall which, in turn, is encompassed by the cortex. The cortex is a specialized peptidoglycan layer that exerts pressure which limits the movement of water into the core (7, 27). This protects the spore's DNA while helping to maintain its dormant state (27). An outer membrane, which may or may not be another permeability barrier, overlays the cortex. The outer membrane is then encased in a multilayered shell called the spore coat (21, 28). The spore coat provides protection, facilitates germination, and mediates interactions with the environment (21, 25). Studies in *B. subtilis* have

shown the spore coat plays an important role in the spore's resistance to chemicals, enzymes, and predation (7, 29).

Certain *Bacillus* species, such as *B. anthracis* and *B. cereus*, possess an additional layer called an exosporium. The exosporium is separated from the spore coat by a region called the interspace (21, 28). Although its exact role is unknown, the exosporium is the primary contact surface between the spore and the environment (30, 31) and contains spore antigens (30 - 32). This suggests a role in host/cell interaction (31, 32). Some data suggest the exosporium plays a role in germination, such as the assembly of alanine racemase (Alr) which neutralizes the germinant L-alanine by converting it to D-alanine (33).

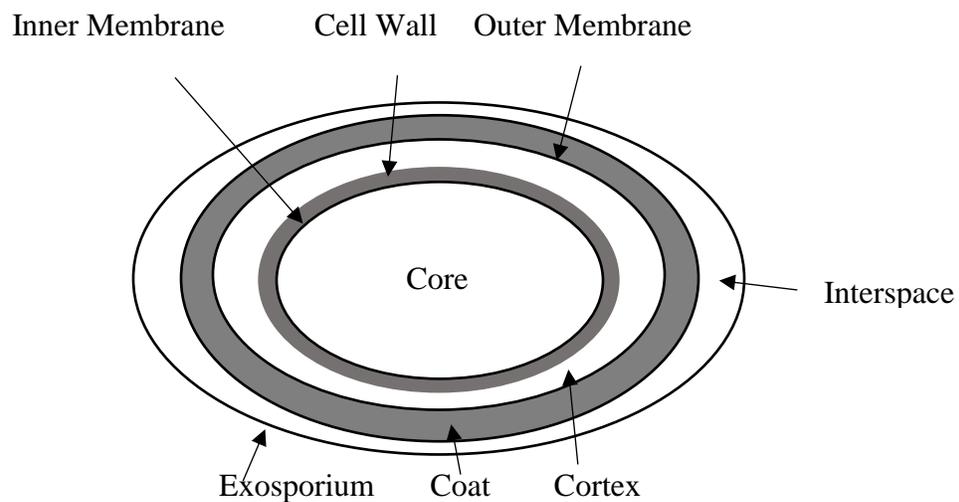


Figure 2-1. Generalized cross section of a *Bacillus anthracis* spore with spore layers labeled.

2.2 Germination

Though a spore is mostly inert in its dormant state, it is able to monitor its environment (4, 12). When the spore detects favorable environmental conditions, the spore has the potential to break its dormancy (4, 12). This process begins with the initiation of

germination and, if successful, will ultimately result in returning the spore to a metabolically active bacterium (4, 12). Normally, germination is initiated by introducing certain amino acids or nucleosides called germinants. In nature, a spore that encounters environmental conditions with the correct type and adequate amount of germinant, can break its dormant state (4, 12, 34).

The spore contains germinant receptors in its inner membrane. The binding of germinants to the appropriate receptor initiates germination (2, 9, 34, 35). There is a period of time between the introduction of nutrient germinants to the spore and the beginning of germination. This time period is known as T_{lag} (1, 8, 12). The length of T_{lag} for individual spores of a population is highly variable (9, 10). Although little is known about what occurs during T_{lag} , the time period can be reduced by HA (7, 8).

Signals from a germinating spore influence germination in neighboring spores. The consequence of this communication is that adjacent spores will have a higher chance of synchronized germination, especially under high density. The exact signaling mechanism creating this influence is not known (1). The effect, if any, of HA on this synchronicity is unknown. However, because HA reduces the T_{lag} in germination, it is not a stretch to hypothesize that HA might also affect this synchronicity as well.

At some point, the binding of specific germinants to their appropriate germinant receptors results in the spore's commitment to germination. Upon commitment, stores of dipicolinic acid and calcium ions are released allowing water to flow back into the cortex (2, 34). This causes an expansion that fractures the outermost layers, allowing for the

hydration of the core and full reactivation of bacterial metabolism (2, 7, 34). Once hydrated, RNA, protein, and DNA synthesis resume in the outgrowth phase, returning the spore to its vegetative state (7, 22, 29).

Hydration is a critical activity in spore germination. During hydration, the cortex hydrolyzes allowing water to flood to the core. This rehydration allows metabolism and macromolecular synthesis to begin (36, 37). Hydration reduces the spore's refractivity which is indicated by a reduction of optical density (OD). A drop in the optical density demonstrates that a spore has germinated, and the rate and percentage of germination can then be calculated (37, 47).

Germination in *B. anthracis* involves the processes described above. It is dependent upon appropriate germinant/germinant receptor interactions. *B. anthracis* has five germinant receptors: *gerH*, *gerK*, *gerL*, *gerS*, and *gerX*, with each stereospecific to a certain germinant (2, 38). L-alanine and inosine, cooperatively, serve as the primary germinants (2, 38). L-alanine can initiate germination by itself, but only at very high concentrations, and works best with a co-germinant. Inosine alone is unable to initiate germination, so it is often used as a co-germinant (2, 38).

2.3 Heat Activation

Heat activation (HA) has long been used in laboratory settings to improve the rate and extent of germination (11, 12, 34). HA does this by reducing the T_{lag} - the amount of time it takes from the introduction of a germinant to the spores' release of calcium ions and dipicolinic acid, resulting in the commitment to germination (8, 13, 14). The mechanism by which HA reduces the T_{lag} is unknown (8, 9). Some theories suggest that the exposure to heat softens the outer layer of the spore which allows quicker access of the germinants

to the receptors (4, 9). Another theory hypothesized that the heat denatures specific spore proteins and releases calcium ions, dipicolinic acid, and free amino acids into the surrounding environment, making the spores easier to break down during germination (4, 8).

Although its specific processes are unknown, the effects of HA on spores appear to mimic the effects that occur as a spore ages (11). HA appears to only affect nutrient driven germination (8, 9). Germination through non-germinant receptor pathways, such as high pressure, pH, or chemicals (8, 11), does not appear to be affected by HA (8, 9).

Many researchers state the effects of HA are reversible (4, 9). An early paper cited a 39% drop in germination rate in *B. cereus* spores stored at 4°C over three days. Spores stored at 28°C showed a 79% drop in germination rate after three days (11).

Some subsequent studies on *B. cereus* and *B. subtilis* have shown some effects of HA are not reversible. Hashimoto and Conti stated activation was irreversible when striations appear in the spore coat (24). Zhang, Setlow, and Li indicated some structural changes to the spore components of *B. cereus* and *B. subtilis* did not reverse after cooling (15).

Recent data by our lab have shown the effects of heat activation on the germination of *B. anthracis* and *B. subtilis* might be maintained beyond 72 hours. The heat activation portions of these experiments were conducted in a manner similar to those used in this research. My experiment was an attempt to prove the effects of HA on *B. anthracis* spores will last longer than three days.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Handling

3.1.1 *Bacillus anthracis* Bacteria

The strain of bacteria used in this experiment was *B. anthracis* from Sterne wild type 34F2. The strain is devoid of the capsule plasmid pXO2 rendering the bacteria avirulent (39 - 43). Bacteria were spread across a Luria Bertani (LB) plate (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, 15g/L agar) with a wooden stick using the quadrant streak method and then incubated overnight at 37°C.

3.1.2 Spore Preparation

Sporulation of *B. anthracis* was performed via the exhaustion method (44). A single colony of bacteria from the LB plate was suspended in 100µL of Difco Sporulation Medium (DSM). This DSM-bacteria solution was then spread across a DSM plate using glass beads. The plate was incubated at 37°C for five hours and the bacteria were washed off with 4mL of DSM. A portion of the bacterial wash (0.5mL) was then added to 25mL DSM. Bacteria were exhausted while shaking at 250rpm at 37°C for 24 hours.

After incubation, the DSM solution was examined using a phase contrast microscope at 1,000X magnification to determine that the bacteria had sporulated and

there was no contamination. If 90% of bacteria had released spores and no contamination was seen, the solution would be transferred to a 50mL centrifuge tube for washing. The spores were pelleted at 4,000rpm for 10 minutes and the DSM siphoned off. The pelleted spores were resuspended in 25mL of ice cold deionized, distilled water (ddH₂O) and centrifuged again using the above settings to water wash. The water wash was conducted three times. After washing, the spore suspension was placed on ice and rocked for four hours to lyse any remaining bacteria or sporangia. The spores were then washed three more times in the same manner as described above. The spores were left to rest in 25mL water at 4°C overnight.

The spores were then centrifuged, resuspended in 4.5mL of ddH₂O, and aliquoted into three microfuge tubes with 1.5mL per tube. The tubes were designated as follows: 1) UH – an unheated control group; 2) HA – spores that will all be heat activated only on day one of the experiment; and 3) Hn – spores that will be activated on a particular day relative to day one. Afterward, all groups were washed an additional three times at 15,000rpm for 10 minutes using ice cold ddH₂O. After washing, each group (UH, HA, and Hn) was suspended in 1mL ddH₂O and divided into five 200μL samples – one for each day an assay was performed during the experiment. Assays were performed on days one, three, five, seven, and 14 after washing. Spores were stored at 4°C overnight before performing the first assay. Spores were stored in water at 4°C when not in use.

3.2 Germination Assay

3.2.1 Heat Activation

Germination assays using loss of OD were performed on days one, three, five, seven, and 14 after final washing. The five 200μL UH samples were never heat

activated. All five 200 μ L HA samples were heat activated on day one only. One 200 μ L Hn sample was heat activated each time an assay was performed. (H1 was activated on day one, H3 on day 3, etc). Heat activation was accomplished by heating the appropriate spore sample for thirty minutes at 65°C. All spore samples for the particular day of assay were then placed in an ice bath for fifteen minutes to cool.

3.2.2 Germination Assay by Optical Density

An initial optical density at a light wavelength of 580nm (OD₅₈₀) was taken using 495 μ L ddH₂O and 5 μ L spores to determine the concentration of spores needed from each group to perform the assay. The initial OD₅₈₀ was set to 0.5. The calculated volume of spore suspension was taken from each sample and aliquoted to 1.5mL Eppendorf tubes.

The Eppendorf tubes for all three groups (UH, HA, Hn) were spun down at 15,000rpm for 10 minutes, the water siphoned off, and all the pellets resuspended in 20mM Tris-HCl buffer at a pH of 7.5. Each assay was performed in a 1cm pathlength cuvette with a 1mL volume containing spores, buffer, and germinant at an initial OD₅₈₀ of 0.5. Final concentrations of the germinant were 1mM L-alanine and 1mM inosine. Spores were added to the cuvette immediately before the start of the assay. Assays were performed for one hour with readings taken manually every five minutes. Included in the readings was a zero-time point, which is the OD at the moment the spores were added to the germinant. A blank, consisting of the above concentrations minus spores, was used to calibrate the OD₅₈₀ of the spectrometer prior to the assays. Germination assays were conducted using a Thermo Spectromic Genesys 20 spectrometer set to an OD₅₈₀.

Each assay was performed in duplicate on days one, three, five, seven, and 14. Final results are an average of assays on three independent sporulations performed in

duplicate. One exception is day 14, in which the results are the average of two independent sporulations. Germination data, presented as the percentage optical density loss, was calculated using the following formula:

$$((\text{OD}_{580} \text{ at } t_0 - \text{OD}_{580} \text{ at } t_x) / \text{OD}_{580} \text{ at } t_0) \times 100$$

3.2.3 Statistical Analysis

A two-way repeated measures ANOVA test was used to determine statistical relevancy of the germination data using the STAS program.

3.2.4 Spore Purification by Density Gradient

While troubleshooting this study, at times spores were purified by a nonionic gradient Nycodenz wash to segregate dormant *B. anthracis* spores from other cells and debris. This wash requires resuspension of spores in 200 μ L of a 20% weight/volume Nycodenz, which is then layered over 400 μ L of a 50% weight/volume Nycodenz. The solution would then be centrifuged, resulting in a pellet of dormant spores at the bottom of the tube with the cell debris and germinated spores caught between the two layers. The Nycodenz would then be removed and the spores washed three times with 1mL ddH₂O to remove any Nycodenz residue.

CHAPTER 4

RESULTS

4.1 Overview

I hypothesized that if *B. anthracis* spores were heat activated and stored at 4°C in water over 14 days, then I would see a decrease in germination rate and extent over time that correlated with a loss of activation. Therefore, I prepared three independent batches of *B. anthracis* spores by the exhaustion method and extensively water washed each batch. Next, I divided my spores into three groups: a control group (UH) that would never be heat activated, a group that would be heat activated on day one only (HA), and a group that was heat activated every day an assay was performed (Hn).

Germination assays based upon percentage of loss of OD₅₈₀ were performed on days one, three, five, seven, and 14 after spore preparation. Hydration is a critical activity in spore germination and reduces the spores' refractivity which is indicated by a reduction of OD (Figure 4-1).

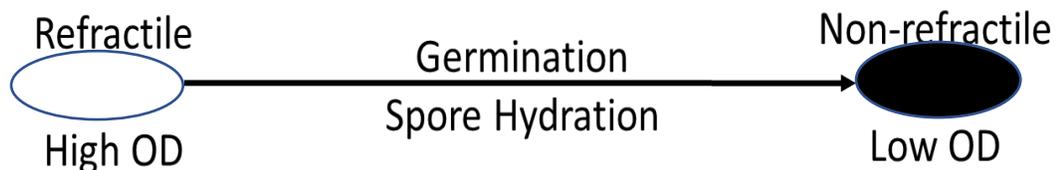


Figure 4-1. Representation of spore hydration during germination. A spore that undergoes germination becomes phase dark and no longer refracts as much light, resulting in a loss of OD. This OD loss can then be used to calculate germination.

A drop in the optical density demonstrates a spore has germinated and the rate and percentage of germination can then be calculated. Loss of OD, therefore, can be used as a measure to indicate germination. All spores were stored in water at 4°C when not in use.

4.2 Troubleshooting

This lab commonly studies *B. subtilis* and *B. anthracis*. Initial attempts to monitor activation were centered on *B. subtilis* PY79 using a 96-well plate reader. Over 60 assays were conducted with *B. subtilis* with no conclusive or constant germination data. The spores did not perform the same way twice, and germination data often showed uncharacteristic dips and peaks. In more than one instance, final percent loss of OD for UH spores was higher than HA spores. This data was contrary to preliminary data collected by a previous graduate student.

Next was an attempt to use *B. anthracis* in the 96-well plate reader using the germinants L-alanine and inosine. These assays were not completed because there were problems with the spore purification procedure. When the spores were run through a Nycodenz density gradient to segregate dormant spores from cell debris, the spores did not separate from the cell debris making it impossible to use them.

A third attempt, using non-gradient washed *B. anthracis* spores with the germinants L-alanine and inosine with OD₅₈₀ loss measured manually in a spectrometer gave encouraging results. This procedure was selected as the method of this study. Results presented for day one, three, five, and seven are the average of three separate experiments performed in duplicate on a given day. Germination data were also collected

on day 14 using two of the sporulations. Although this data is presented and discussed, the sample size may be too small to rely on.

4.3 Data

Spores were heat activated and germination was measured by loss of OD addressed in the methods. On day one of the experiment, HA and H1 spores germinated at nearly identical rates. Both samples had a final OD loss near 57% (HA 57.24%, H1 57.88%). Since the spores were heat activated on this day, a similar OD loss was expected. The UH spores had a much lower germination rate as indicated by an OD loss of only 34% (Figure 4-2). This too was expected as unheated spores are known to have a lower OD loss than heated spores (8, 9, 11). Day one behaved as anticipated.

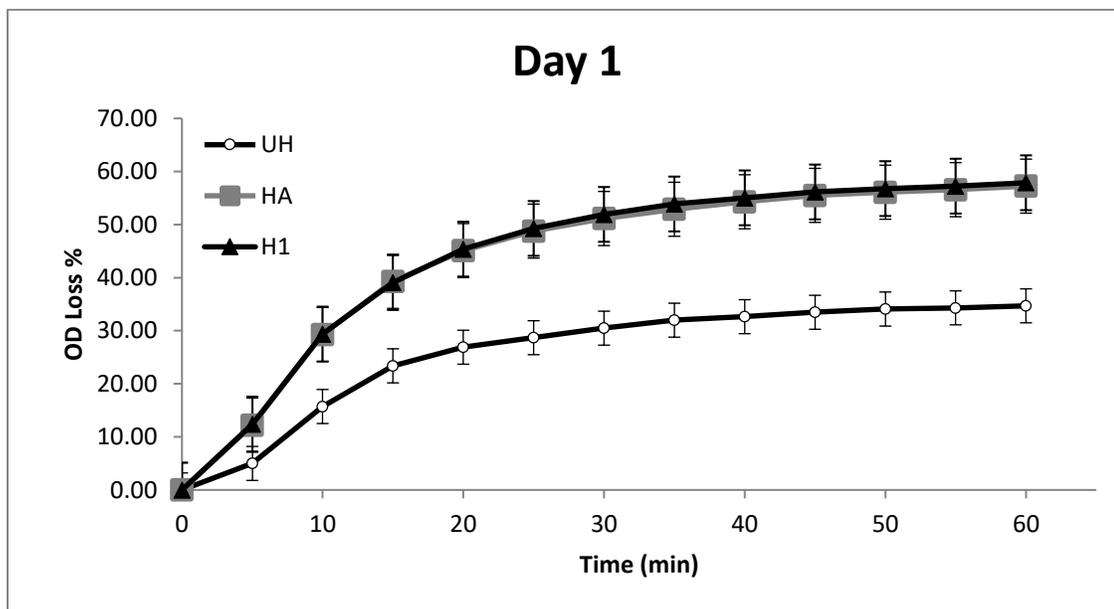


Figure 4-2. Germination represented by percentage OD₅₈₀ loss for unheated (UH), heat activated on day one only (HA), and heat activated on day one (H1). Samples set to an initial OD₅₈₀ of 0.5. Measurements collected every five minutes for one hour beginning immediately after exposure to germinant. Results are the average of three separate assays performed in duplicate. Error bars represent standard error of the mean.

The second assay was performed two days later on day three. The final OD losses of HA and H3 spores were similar at 54.36% and 55.11%, respectively. UH had an OD loss of 31.32% (Figure 4-3). Day three might be easily explained as a difference between *B. anthracis* vs. *B. cereus*. If the effects of heat activation are reversible in as little as three days, then the OD loss of the HA spores should have been closer to that of the UH spores. Therefore, we tested later time points.

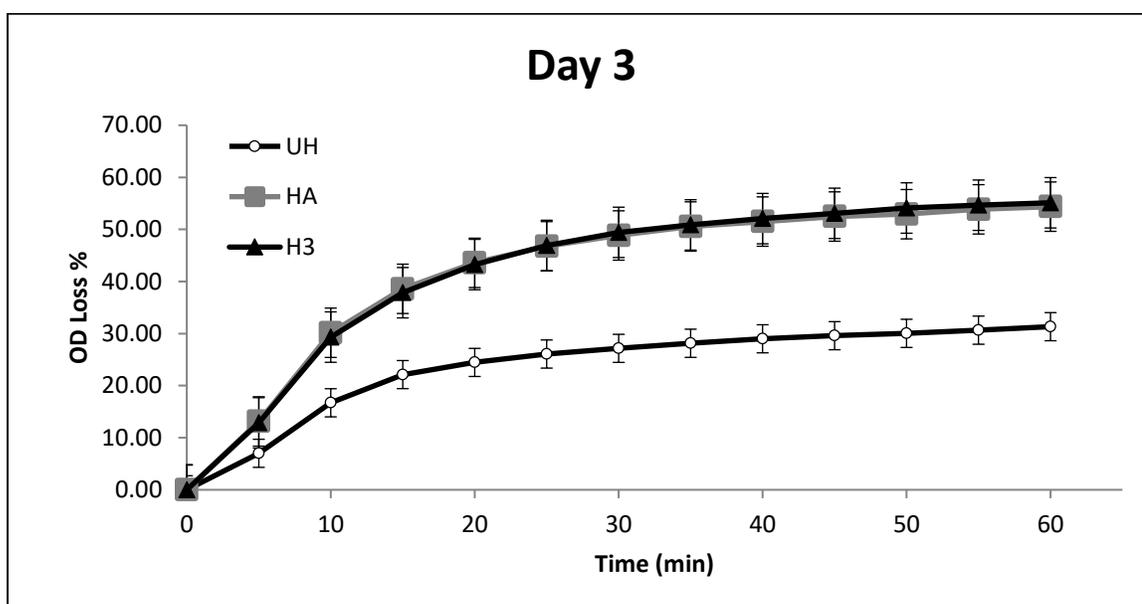


Figure 4-3. Germination represented by percentage OD₅₈₀ loss for unheated (UH), heat activated on day one only (HA), and heat activated on day three (H3). Samples set to an initial OD₅₈₀ of 0.5. Measurements collected every five minutes for one hour beginning immediately after exposure to germinant. Results are the average of three separate assays performed in duplicate. Error bars represent standard error of the mean.

On day five, the final OD loss of HA was 54.87% and that of H5 was 55.11%.

While the spores heat treated on day 5 had a slightly higher final loss, both heated suspensions had similar germination rates. The final OD loss for UH was 30.82% (Figure 4-4). A definite drop in the germination rate of HA was expected at this point but

was not observed in the data. HA still had a significantly higher loss of OD than UH, suggesting HA spores remained activated.

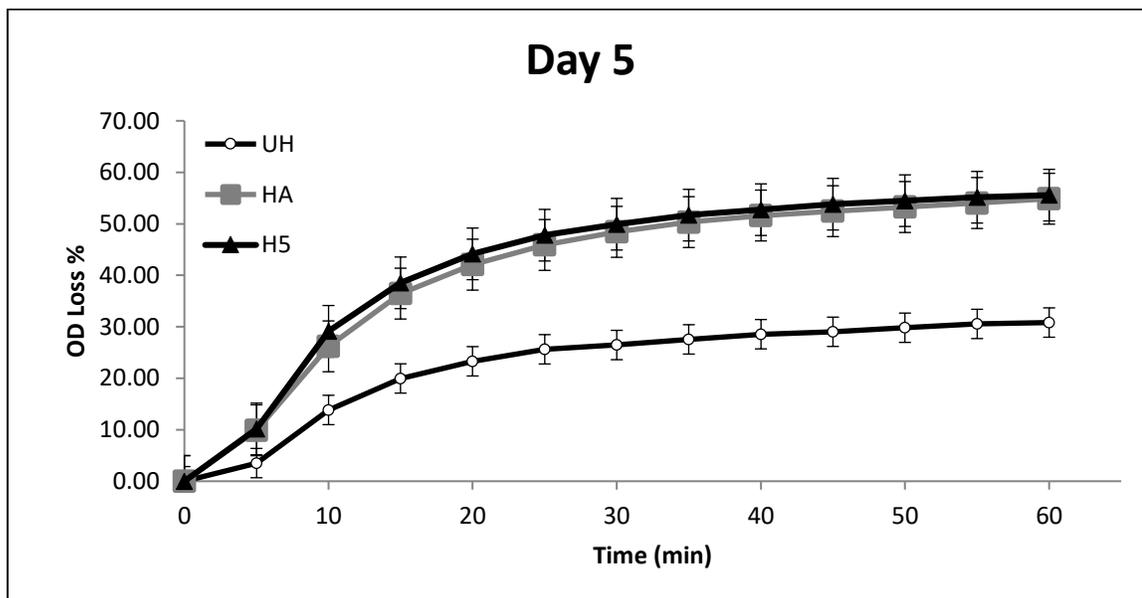


Figure 4-4. Germination represented by percentage OD₅₈₀ loss for unheated (UH), heat activated on day one only (HA), and heat activated on day five (H5). Samples set to an initial OD₅₈₀ of 0.5. Measurements collected every five minutes for one hour beginning immediately after exposure to germinant. Results are the average of three separate assays performed in duplicate. Error bars represent standard error of the mean.

On day seven, HA, H7, and UH had a final OD loss of 54.16%, 52.66%, and 27.49%, respectively (Figure 4-5). Again, HA possessed a much higher germination rate than UH. HA, the batch heated seven days prior, had a minor but higher germination rate than H7 over the course of the 60-minute assay. However, HA and H7 were trending toward each other at the end of 60 minutes, with the HA having an unexplained drop between 45 and 50 minutes. A review of the original data shows the drop occurs in the first assay performed during the third sporulation. It is unknown why this dip occurred. However, the assay suggests HA spores are not reverting to an inactivated state.

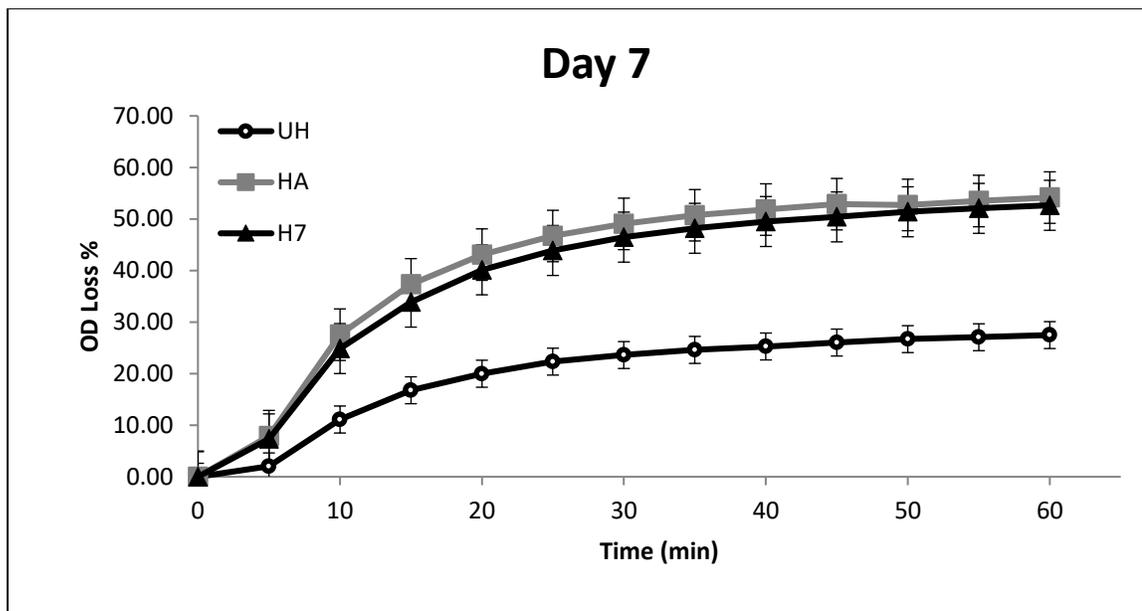


Figure 4-5. Germination represented by percentage OD₅₈₀ loss for unheated (UH), heat activated on day one only (HA), and heat activated on day seven (H7). Samples set to an initial OD₅₈₀ of 0.5. Measurements collected every five minutes for one hour beginning immediately after exposure to germinant. Results are the average of three separate assays performed in duplicate. Error bars represent standard error of the mean.

Some data for OD loss up to Day 14 was collected, but the presented data is the average of two assays, not three. The third sporulation was contaminated midway through the experiment. HA, H14, and UH had a final OD loss of 56.23%, 50.78%, and 36.28%, respectively (Figure 4-6). Even two full weeks after activation, the HA spores had the highest OD loss, and therefore the best germination rate. At this time, HA was expected to have some drop in OD loss, yet it remained in the same range as day one OD loss.

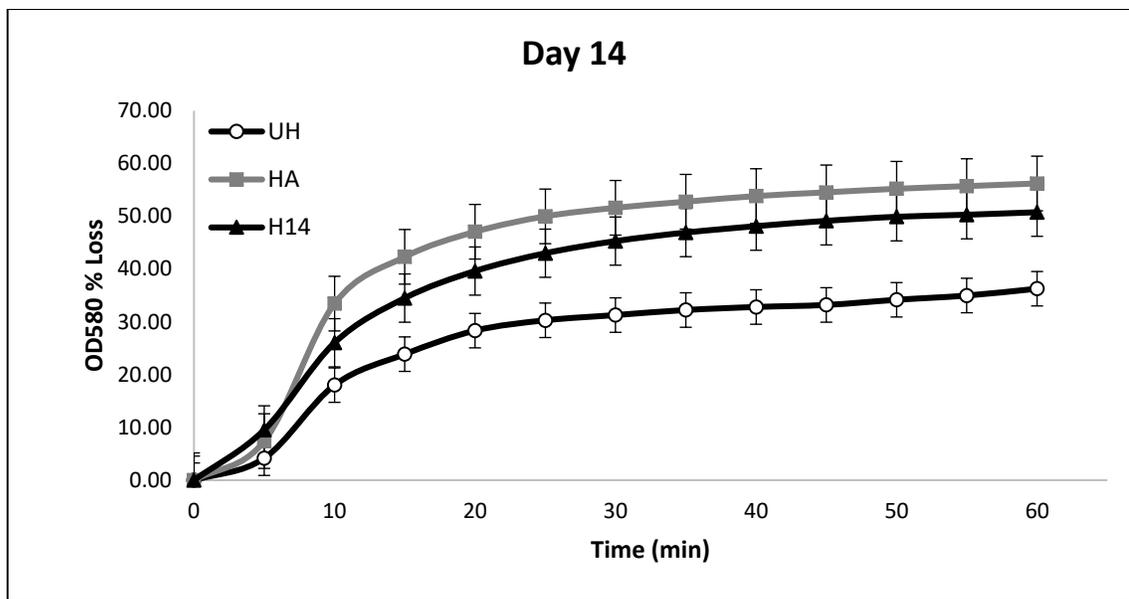


Figure 4-6. Germination represented by percentage OD₅₈₀ loss for unheated (UH), heat activated on day one only (HA), and heat activated on day 14 (H14). Samples set to an initial OD₅₈₀ of 0.5. Measurements collected every five minutes for one hour beginning immediately after exposure to germinant. Results are the average of two separate assays performed in duplicate. Error bars represent standard error of the mean.

A comparison of final OD loss from days one and seven was plotted on the same graph (Figure 4-7). All suspensions showed a decrease in germination on day seven when compared to day one. However, a two-way ANOVA test with replication ($p=0.05$) showed this decrease to be statically insignificant for all suspensions. Each group, when compared to itself on day one to day seven (UH1 to UH7, HA1 to HA7, H1 to H7), showed a significance (p-value) of 0.114. Because the significance is higher than the p-value of 0.05, there is no statistical difference between germination values of day one and day seven.

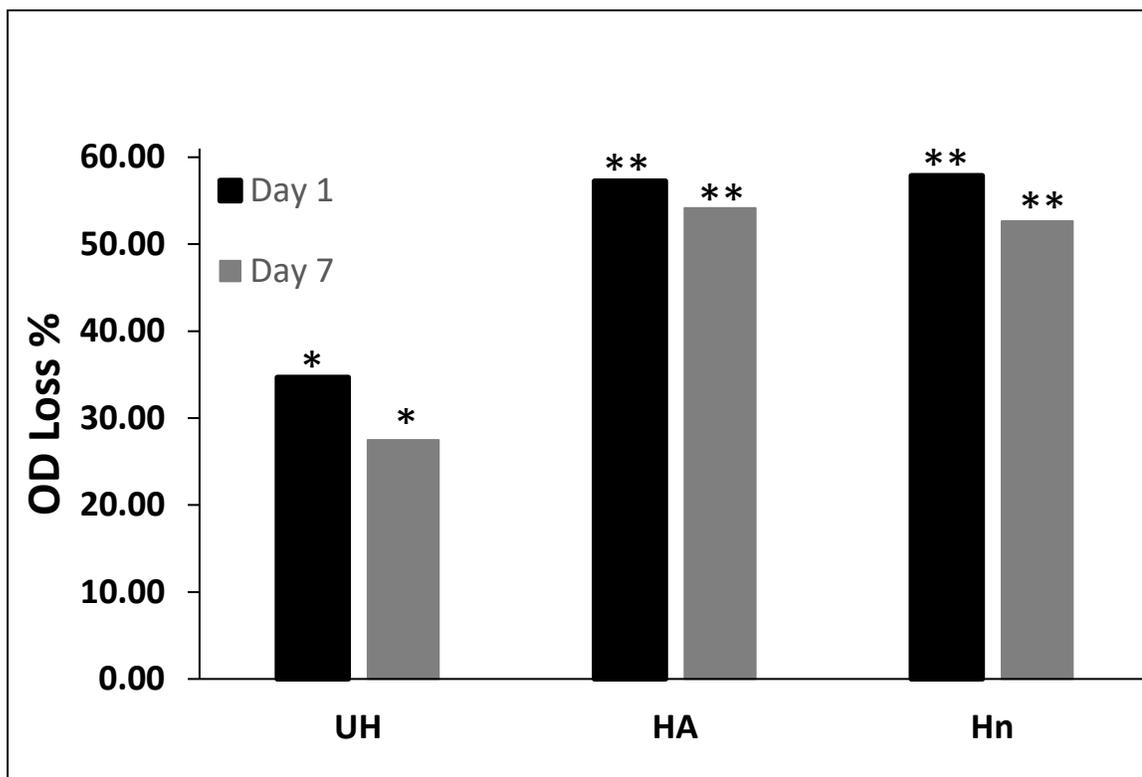


Figure 4-7. Comparison of final loss of OD₅₈₀ for days one and seven for all three groups: unheated (UH), heat activated on day one only (HA), and heat activated on days one and seven (Hn). Data that share the same number of * show no statistically significant difference from each other.

When spore samples heated prior to germination (HA and Hn) are compared to UH samples both on day one and day seven, the increase in OD loss (germination) was shown by the ANOVA test ($p = 0.05$) to be significant. A significance value of 0.029 was calculated when comparing UH to HA. UH compared to H7 had a significance of 0.036. Another ANOVA test ($p = 0.05$) was used to determine if there was a significant difference in germination between H7 and HA. A significance value of 0.878 was calculated indicating no difference in germination for spores heated on day seven and

those heated on day one. Taken together these data suggest that *B. anthracis* spores remain activated for seven days after heating.

CHAPTER 5

DISCUSSION

As expected, heat activated spores germinated to a greater extent than unheated spores. The results from day three begin to deviate from accepted knowledge that activated spores will revert to an inactive state. I expected the germination rate of HA spores to resemble that of UH spores, or at least have a significant reduction in germination. Surprisingly, spores heated on day one still retained their “activated” phenotype at day seven. This is evidenced by the loss of OD for HA on day seven and day one not being significantly different. The results of this study indicate that *B. anthracis* spores remain activated for seven days with a single heat activation. This is in contrast to a study on *B. cereus* which indicated a reversal of heat activation beginning as early as one day after heating (11).

Many studies have been performed on the impact of HA on spore germination. While the results of HA are well known, its mechanisms are not. Most studies agree the effects of HA are reversible over the course of several days (9, 11, 15). These studies on reversibility focused primarily on *B. cereus* and *B. subtilis*, with no studies on *B. anthracis* spores.

Some germination data on heated versus unheated spores were collected through 14 days. These data show activation is retained even after two weeks. The day 14 sample size was not large enough to attach statistical significance to it. Studies

performed in the lab that inspired this experiment also support retention of activation in *B. anthracis* spores through 14 days.

The spore coat appears to have a role in germination (21, 45, 46). A structural change to the *B. anthracis* spore coat during activation may play a role in the retention of HA. This has been demonstrated in other *Bacillus* species (15, 47). An early paper suggested heat activation of *B. cereus* under optimal conditions caused striations to spread through the spore coat. Spores with these striations retained their activation properties (24). More recent research on *B. cereus* and *B. subtilis* indicate at least some changes to the ultrastructure of the spore coat do not reverse when heated spores are cooled (15). However, this paper also noted many of the changes brought about by heating these spores do reverse (15).

It is noted that aged spores of *B. cereus* form striations like those found in HA spores (24). This suggests activation by heat may induce changes in spores similar to those found in aged spores. No age was given for when these striations form (11, 24), but activation by aging is irreversible (11).

Other studies have shown *B. subtilis* spores undergo an endothermic transition at 56°C. This transition changes the spores' appearance from "glasslike" to "rubberlike", a change that is not reversible (4). However, they did not measure germination rates. T_{lag} is also decreased by HA (8, 13, 14). Studying T_{lag} was not a focus of this experiment, but the data showed a higher germination rate over less time for activated suspensions.

Previous studies on *B. cereus* and *B. subtilis* showed the effects of HA are reversible. Most of these papers do not provide a time frame for this activation loss (4, 9, 11), but Keynan's work on *B. cereus* revealed inactivation in as little as one day (11).

My study showed no significant loss of activation in *B. anthracis* seven days after heating. These differing results could be explained in multiple ways. The species studied could be an explanation. This research used *B. anthracis* spores, while most others used *B. cereus* or *B. subtilis* (11). Temperature at which activated spores are stored is another important factor to study. In Keynan's study, spores stored at 28°C had a more severe reversal than those stored at 4°C, suggesting the temperature of storage could impact activation reversal. Other factors that could influence these results are sporulation media, the density of the initial spore stock, and activation conditions.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

A knowledge of the length of time *B. anthracis* spores maintain their “activated” phenotype can impact decontamination procedures that incorporate a germination step. The effects of a single HA on germination of *B. anthracis* were definitely retained through seven days, and were indicated out to 14 days. Individuals involved in the creation of decontamination strategies that include germination of *B. anthracis* spores may find this information on HA useful to their procedures.

Many studies on the germination of *B. cereus* and *B. subtilis* have found HA to be reversible, but do not give a time frame for inactivation. Therefore, the activation retention observed in this study may be unique to *B. anthracis*. Ascertaining the time needed for a reversal, if any, in *B. anthracis* is an avenue of future study.

Other factors besides species could play a role in the retention of HA. Future directions for this field of study include examining the claims that HA does not reverse if the spores are activated optimally (37). Studies can also be performed to determine if HA impacts the germination of *B. anthracis* over an extended period of time, even up to several months after activation. Research into the impacts that spore preparation (e.g. freeze-dried vs wet stored) has on the loss of heat activation properties needs to be conducted.

A more pressing matter is determining if the temperature of storage affects the reversal time of *B. anthracis*. Keynan's paper demonstrated a loss of HA in just one day at 4°C, but also showed a larger drop in spores stored at 28°C (11). It could be that storage at 4°C caused *B. anthracis* to revert so slowly that no change was detected by this experiment.

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