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### Heat activation and inactivation of bacterial spores

*Is there an overlap?*

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# Heat Activation and Inactivation of Bacterial Spores: Is There an Overlap?

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**ABSTRACT** Heat activation at a sublethal temperature is widely applied to promote *Bacillus* species spore germination. This treatment also has the potential to be employed in food processing to eliminate undesired bacterial spores by enhancing their germination and then inactivating the less-heat-resistant germinated spores at a milder temperature. However, incorrect heat treatment could also generate heat damage in spores and lead to more heterogeneous spore germination. Here, the heat activation and heat damage profile of *Bacillus subtilis* spores was determined by testing spore germination and outgrowth at both population and single-spore levels. The heat treatments used were 40 to 80°C and for 0 to 300 min. The results were as follows. (i) Heat activation at 40 to 70°C promoted L-valine- and L-asparagine-glucose-fructose-potassium (AGFK)-induced germination in a time-dependent manner. (ii) The optimal heat activation temperatures for AGFK and L-valine germination via the GerB plus GerK or GerA germinant receptors were 65°C and 50 to 65°C, respectively. (iii) Heat inactivation of dormant spores appeared at 70°C, and the heat damage of molecules essential for germination and growth began at 70 and 65°C, respectively. (iv) Heat treatment at 75°C resulted in both activation of germination and damage to the germination apparatus, and 80°C treatment caused more pronounced heat damage. (v) For the spores that should withstand adverse environmental temperatures in nature, heat activation seemed functional for a subsequent optimal germination process, while heat damage affected both germination and outgrowth.

**IMPORTANCE** Bacterial spores are thermal-stress-resistant structures that can thus survive food preservation strategies and revive through the process of spore germination. The more heat resistant spores are, the more heterogeneous their germination upon the addition of germinants. Upon germination, spores can cause food spoilage and food intoxication. Here, we provide new information on both heat activation and inactivation regimes and their effects on the (heterogeneity of) spore germination.

**KEYWORDS** *Bacillus subtilis*, bacterial spores, heat activation, spore germination, germination heterogeneity

Applying mild heat for a short to moderate time (e.g., pasteurization treatment at 72°C for at least 15 s) is one of the conventional food processing procedures to extend shelf life in the food industry (1, 2). In this process, vegetative pathogens or spoilage organisms lose their viability, although bacterial spores survive. Not only that, heat treatment at sublethal temperatures (e.g., 60 to 75°C) can increase and synchronize the spore germination of *Bacillales* and *Clostridiales* (2–5). *Bacillus subtilis* spores have higher heat resistance than their vegetative forms due to the following factors (2–4). (i) The spore core has a low water content (25 to 45% wet weight) and a high level of dipicolinic acid (DPA) chelated to divalent cations, predominantly Ca<sup>2+</sup> (Ca<sup>2+</sup>-DPA) (25% of core dry weight). (ii) Spore DNA is saturated with α/β-type small acid-soluble protein (SASP). (iii) Spore cortex peptidoglycan, characterized by the muramic-δ-lactam moiety and low level of peptide cross-linking, also contributes to spore wet heat resistance. However, upon increasing the temperature above 80°C,

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spore damage and inactivation occur (6). Damage accumulated in spore molecules then results in more heterogeneous germination and slowed spore outgrowth (6). To analyze spore thermal physiology such that heat activation can be optimally applied for the stimulation of spore germination, it is necessary to have a detailed analysis of the effects of heat treatment, even at relatively mild temperatures.

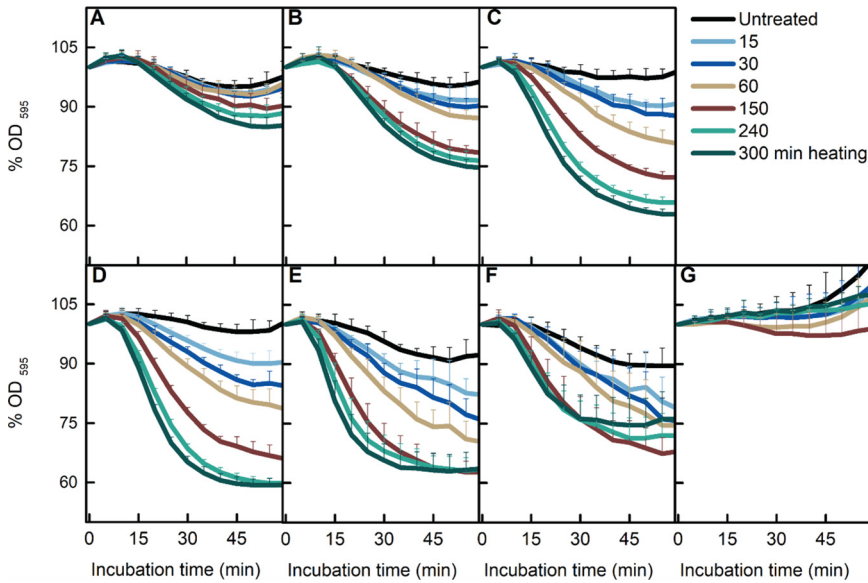
Heat activation studies have mainly focused on spores of members of the order *Bacillales* (5, 7–11). These studies have indicated that a sublethal heat treatment can reversibly activate spores' germinant receptor (GR)-dependent germination, which is induced by physiological small-molecule nutrients or hydrostatic pressures of ~150 MPa. In contrast, sublethal thermal treatment has no effect on GR-independent germination triggered by exogenous  $\text{Ca}^{2+}$ -DPA, dodecylamine, or hydrostatic pressures of ~550 MPa (4, 9, 12). In the heat activation process, temperature is not the only variable, as treatment time also affects outcomes. Luu et al. indicated that the best activation of *B. subtilis* spores was reached after 15 min at 75°C with L-valine-triggered germination via the GerA GR, whereas 4 h at 75°C was needed for optimal germination in L-asparagine, glucose, fructose, and potassium (AGFK)-triggered germination via the GerB and GerK GRs (9). When both temperature and time duration of heat treatment are considered, the following questions need to be addressed. (i) What is the best time-temperature combination for heat activation? (ii) When does heat activation shift to heat inactivation during long treatment times?

In the current paper, we employed *B. subtilis* strain PS832 spores to measure the time-temperature-activation/inactivation profile at sublethal temperatures. Spores were treated at 40 to 80°C for 15 to 300 min, followed by inducing germination by adding L-valine or AGFK. The spore viability after a variety of treatments was also tested to probe for heat inactivation. Spore germination and subsequent outgrowth and cell growth were monitored at the population level by microplate spectrophotometric analysis and at the single-cell level by phase-contrast microscopy.

## RESULTS

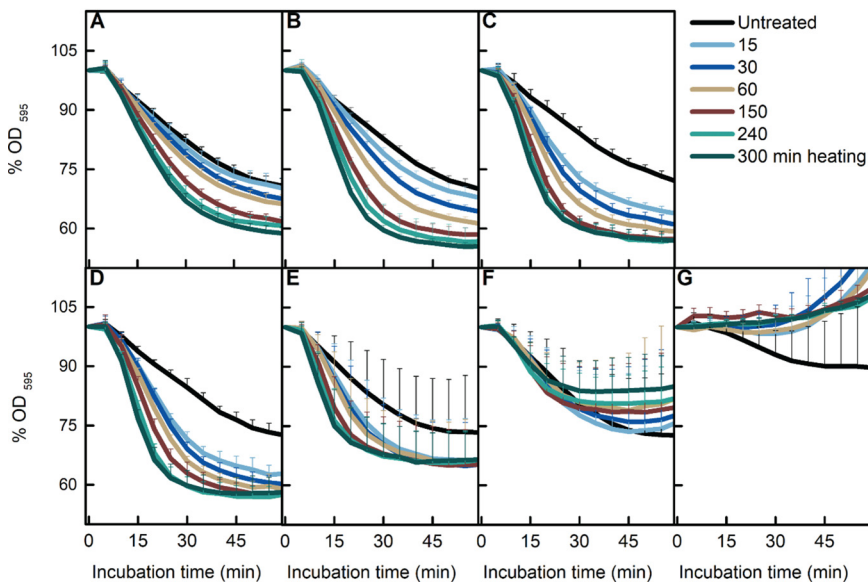
**Heat treatments at 40 to 70°C promote *B. subtilis* spore germination in a time-dependent manner.** Heat activation at sublethal temperatures is a common procedure to promote homogeneous spore germination in the laboratory. However, the temperatures applied differ among different laboratories, with 65, 70, 75, and 80°C used for *B. subtilis* spore heat activation (9, 13–15). In addition, as mentioned above, heat treatments at 75°C enhance AGFK-induced spore germination in a time-dependent manner (9). However, it is not clear whether different temperatures promote germination in a similar pattern. First, we focused on the four commonly used heat activation temperatures mentioned above. As shown by the results in Fig. 1D to G, with the exception of 80°C, the commonly used temperatures promoted AGFK-induced germination in a time-dependent positive manner. However, the 65°C treatment resulted in the largest decrease in the optical density at 595 nm ( $\text{OD}_{595}$ ), which represents the most complete germination. To test the germination promotion efficiency at lower temperatures, the same measurements were taken on spores treated at 40, 50, and 60°C. As shown by the results in Fig. 1A to C, the time-dependent positive correlation was observed again. According to the data presented here, the optimum condition of heat activation for AGFK-induced germination when completion of germination was monitored was 300 min at 65°C. Notably, the magnitude of the drop in the  $\text{OD}_{595}$  gradually decreased as the temperature was increased and was completely abolished at 80°C (Fig. 1G). These results suggested that the activation of germination by heat might have vanished at 80°C or been subsumed by damage at this temperature. Similar results, albeit with subtle differences, were observed for L-valine-induced germination (Fig. 2A to G). First, the optimal heat activation temperature for L-valine-induced germination was 50 to 65°C. Second, treatment at 75°C, while clearly promoting L-valine-induced germination, also decreased the magnitude of the maximum  $\text{OD}_{595}$  decrease. These results suggested there was a transition from only heat activation to heat activation plus damage to spores affecting germination at around 75°C.

**Heat damage accumulates at 70°C.** To determine the temperature at which heat damage occurs, we tested the viability of spores treated at 65 to 80°C for various times.

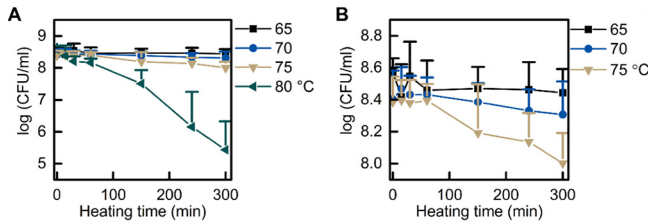


**FIG 1** Effect of heat treatment on AGFK-induced spore germination. *B. subtilis* PS832 spores were germinated at 37°C with (10 mM each) AGFK in HEPES buffer after heat treatments for various times (0, 15, 30, 60, 150, 240, and 300 min) at 40 (A), 50 (B), 60 (C), 65 (D), 70 (E), 75 (F), and 80°C (G). Spore germination was measured by the drop in the optical density due to the release of  $\text{Ca}^{2+}$ -DPA and water uptake. Values shown are the mean values and standard deviations of duplicate or triplicate measurements on at least two experiments.

Clearly, 80°C treatment led to significant killing of spores (Fig. 3). Careful analysis of the results of the other three temperatures also showed a small but significant decrease in spore viability for the spores treated for 300 min at 75°C (Fig. 3B). To test the effect of heat treatments on cell growth, we tracked the change in spore  $\text{OD}_{595}$  in LB medium supplemented with AGFK or L-valine. Thermal treatments of 60 and 70°C did not affect *B. subtilis* growth, whereas exposure to 75 and 80°C at longer heat treatment times (150, 240, and 300 min) decreased the yield of growing cells when LB medium was



**FIG 2** Effect of heat treatment on L-valine-induced spore germination. *B. subtilis* PS832 spores were germinated at 37°C with 10 mM L-valine in HEPES buffer after heat treatment for various times (0, 15, 30, 60, 150, 240, and 300 min) at 40 (A), 50 (B), 60 (C), 65 (D), 70 (E), 75 (F), or 80°C (G). Spore germination was measured by the drop in optical density due to the release of  $\text{Ca}^{2+}$ -DPA and water uptake. Values shown are the mean values and standard deviations of duplicate or triplicate measurements in two or three experiments.

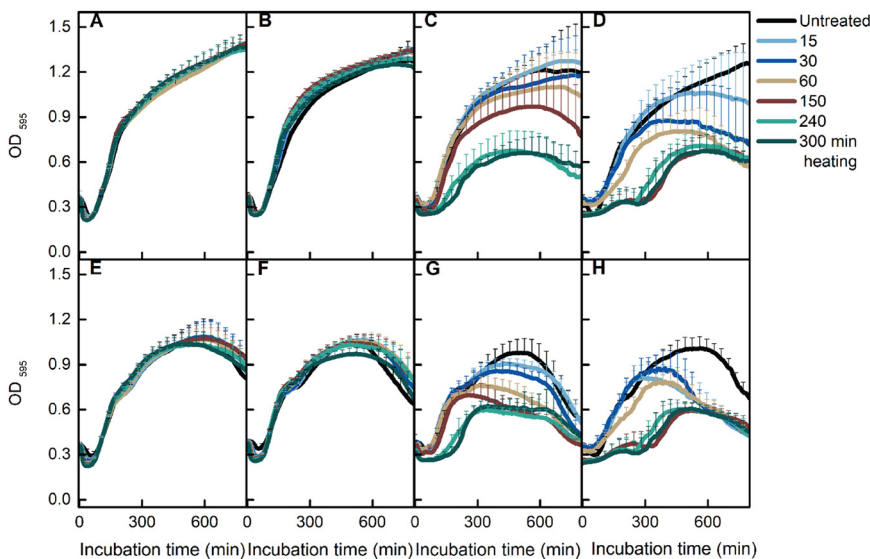


**FIG 3** Effect of heat treatment on spore viability. *B. subtilis* PS832 spores were incubated at 37°C on LB agar plates supplemented with (10 mM each) AGFK after heat treatment at 65, 70, 75, or 80°C for various times (0, 15, 30, 60, 150, 240, and 300 min). (A) Viability of spores pretreated at 65, 70, 75, and 80°C. (B) An enlarged portion of panel A (the x axis remains the same, but the y axis zooms in on the range between log 8 and log 8.8). Values shown are the mean values and standard deviations of triplicate measurements in two or three experiments.

supplemented with either L-valine or AGFK (Fig. 4A to H). These results suggested that both damage and inactivation of spores take place at 70 to 75°C.

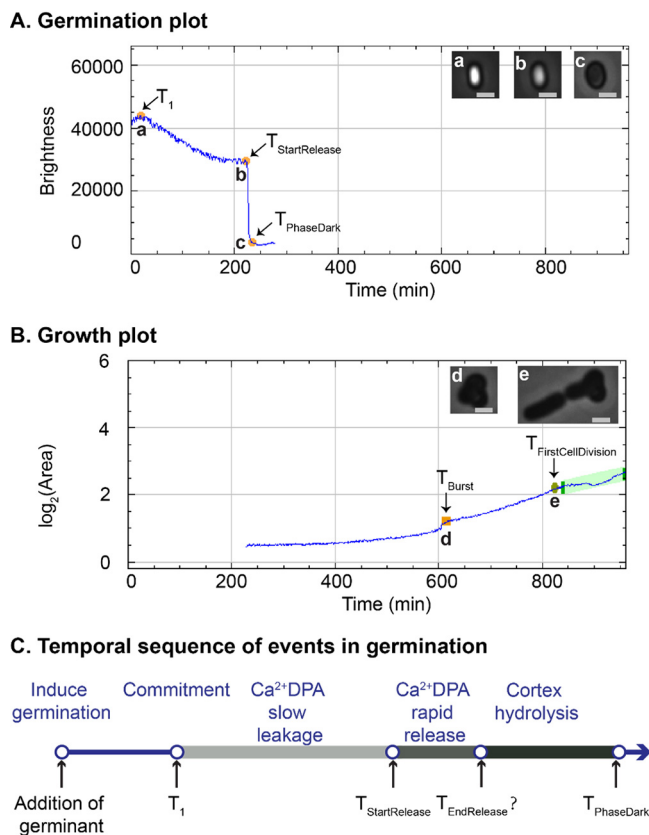
**Effects of heat treatment at the single-spore level.** Our population data indicated that treatments at 65 to 75°C resulted in a time-dependent positive enhancement of AGFK-induced spore germination at the population level, and detectable heat damage/inactivation were observed in spores treated at 70 to 75°C for long times. Considering that the population level data cannot provide a detailed view of what sublethal heat does to individual *B. subtilis* spores, we employed phase-contrast microscopy to track AGFK-induced spore germination, outgrowth, and cell growth after treatments at 65, 70, 75, and 80°C. By analyzing the time-lapse images, germination plots and growth plots of individual spores were created by SporeTrackerX (ImageJ macro) (Fig. 5). Based on the changes in plot profiles, germination, burst (the cell’s escape from the spore coat), and first cell division events were detected and marked for further analysis. The heat treatment durations at 65 to 70°C were 30, 150, and 300 min, while treatments for 15 and 60 min at 80°C were added because of a lack of sufficient germination events for quantitative analysis in the groups with prolonged heat treatment (Table 1).

**Damage to the germination apparatus occurs at 70°C.** As shown by the results in Fig. 5A, the spore germination plot can be divided into three segments, the first before



**FIG 4** Effect of heat treatment on spore germination and cell growth. *B. subtilis* PS832 spores were heated for various times (0, 15, 30, 60, 150, 240, and 300 min) at 65 (A and E), 70 (B and F), 75 (C and G), and 80°C (D and H). Spore germination and cell growth were monitored by the change of optical density in LB medium supplemented with (10 mM each) AGFK (A to D) or 10 mM L-valine (E to H). Values shown are the mean values and standard deviations of triplicate measurements in two or three experiments.





**FIG 5** Germination and growth plots of a single *B. subtilis* spore (treated at 80°C for 150 min), created by SporeTrackerX. (A) In the germination plot, two phases of brightness drop at different speeds were observed. The slow drop was considered the slow leakage of Ca<sup>2+</sup>-DPA. The fast drop was likely due to both the rapid Ca<sup>2+</sup>-DPA release and the subsequent cortex hydrolysis induced by the released Ca<sup>2+</sup>-DPA. The time of the start of rapid Ca<sup>2+</sup>-DPA release ( $T_{\text{StartRelease}}$ ), time of complete phase darkening ( $T_{\text{PhaseDark}}$ ), and time of the start of the slow drop in brightness ( $T_1$ ) were assessed during spore germination by SporeTrackerX. (B) The time of burst ( $T_{\text{Burst}}$ ) and time of first cell division ( $T_{\text{FirstCellDivision}}$ ) were determined, and the various events are marked in the growth plot. Micrographs of spores and cells at each time point are shown at the top right of panels A and B. The brightness display ranges for images a and b and images c to e are 0 to 40,000 and 0 to 25,000, respectively. Scale bar, 1  $\mu\text{m}$ . (C) The temporal sequence of events in germination, adapted from the work of Wang et al. (19). The top shows the temporal sequence of germination events, and the bottom indicates the time points detected in the current work as shown in panel A. For the rapid Ca<sup>2+</sup>-DPA release, we only estimated its initiation from the germination plot.

the drop in brightness, followed by a slow drop in brightness and then a fast drop in brightness. Wang et al. observed slow leakage of Ca<sup>2+</sup>-DPA in *B. subtilis* spore germination, along with a slow decrease in differential interference contrast image intensity, followed by rapid release of the remaining Ca<sup>2+</sup>-DPA and cortex hydrolysis during spore germination, and they further suggested that spores become committed to germinating at the starting point of the slow Ca<sup>2+</sup>-DPA leakage (16). We speculated that the slow decrease of spore brightness in the germination plot was due to the slow leakage of Ca<sup>2+</sup>-DPA, and time 1 ( $T_1$ ) was set as the time point when spore commitment occurred (Fig. 5C). The fast drop in brightness in our germination plot was attributed to the rapid release of Ca<sup>2+</sup>-DPA and cortex hydrolysis triggered by the release of Ca<sup>2+</sup>-DPA. We refer to the following parameters:  $\Delta T_{\text{SlowLeakage}}$  is the time between  $T_1$  and  $T_{\text{StartRelease}}$  and  $\Delta T_{\text{PhaseDarkening}}$  is the time between  $T_{\text{StartRelease}}$  and  $T_{\text{PhaseDark}}$ . Our live-imaging data showed that AGFK-induced germination was shortened by heat treatments at 65 to 75°C in a time-dependent positive manner (Fig. 6A to D). The  $T_{\text{PhaseDark}}$  values for untreated spores and those treated at 65 to 75°C for 300 min were 66.6, 21.1, 22.1, and 30.2 min, respectively. In addition, 65 to 75°C treatments shortened  $T_1$  and  $\Delta T_{\text{SlowLeakage}}$  in a time-dependent positive manner, and 70 to 75°C treatments prolonged the  $\Delta T_{\text{PhaseDarkening}}$  in a time-dependent positive manner (Table 1). Given the fact that the release of

**TABLE 1** Germination parameters of individual heat-treated *B. subtilis* PS832 spores in time-lapse images<sup>a</sup>

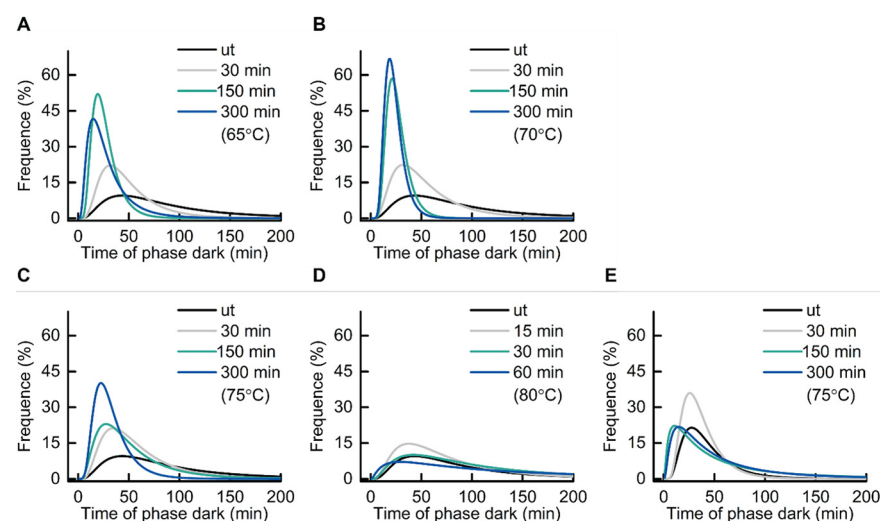
Nutrient(s), treatment		Time (min) of indicated event <sup>b</sup>									No. of germination events (% germination)
		$T_1$			$\Delta T_{\text{SlowLeakage}}$			$\Delta T_{\text{PhaseDarkening}}$			
Temp (°C)	Time (min)	Median	IQR	P value	Median	IQR	P value	Median	IQR	P value	
AGFK											
Untreated	12.0	4.7		40.0	65.1			12.1	3.9		184 (100)
65	30	9.0	3.2	< 0.01	15.0	29.1	< 0.01	11.0	3.0	< 0.01	216 (100)
	150	5.2	2.1	< 0.01	5.0	12.9	< 0.01	10.2	3.0	< 0.01	263 (100)
	300	4.0	0.0	< 0.01	3.2	10.4	< 0.01	11.1	4.0	< 0.01	231 (98.7)
70	30	10.3	7.0	< 0.01	19.1	36.4	< 0.01	11.1	2.4	< 0.01	204 (99.5)
	150	5.5	1.0	< 0.01	3.9	9.9	< 0.01	12.2	3.9	0.13	225 (99.6)
	300	4.0	2.3	< 0.01	3.0	6.9	< 0.01	14.1	4.3	< 0.01	213 (100)
75	30	8.0	2.0	< 0.01	23.1	40.0	< 0.01	13.1	3.8	< 0.01	211 (100)
	150	6.0	2.1	< 0.01	11.0	25.3	< 0.01	17.1	5.9	< 0.01	176 (90.7)
	300	3.0	1.0	< 0.01	4.0	10.0	< 0.01	19.2	9.4	< 0.01	126 (88.7)
80	15	7.0	4.1	< 0.01	29.1	56.0	0.02	14.2	5.4	< 0.01	231 (92)
	30	6.0	2.0	< 0.01	59.5	99.4	0.04	15.2	6.9	< 0.01	122 (77.2)
	60	8.0	1.7	< 0.01	66.0	304.1	< 0.01	16.2	9.0	< 0.01	153 (59.8)
	150	10.0	1.0	0.19	48.0	140.0	0.53	16.1	18.1	0.42	11 (12.4)
	300										0 (0)
L-Valine											
Untreated		7.5	1.0		11.1	22.0		13.3	5.1		112 (100)
75	30	7.0	1.0	< 0.01	10.1	15.8	0.84	12.1	3.5	< 0.01	99 (98)
	150	3.0	0.5	< 0.01	7.0	37.5	0.32	15.1	10.4	0.40	75 (87.2)
	300	3.0	1.0	< 0.01	0.1	17.0	0.02	19.3	10.0	< 0.01	61 (77.2)

<sup>a</sup>As described in the legend to Fig. 5, *B. subtilis* PS832 spores were treated with various temperature-time conditions, followed by being germinated and grown on a MOPS-agarose pad with (10 mM each) AGFK and 10 mM L-valine. Data were collected by SporeTrackerX (ImageJ Macro) as described in Materials and Methods. The significance of differences between heat-treated groups and the untreated group was measured by the Mann-Whitney test.

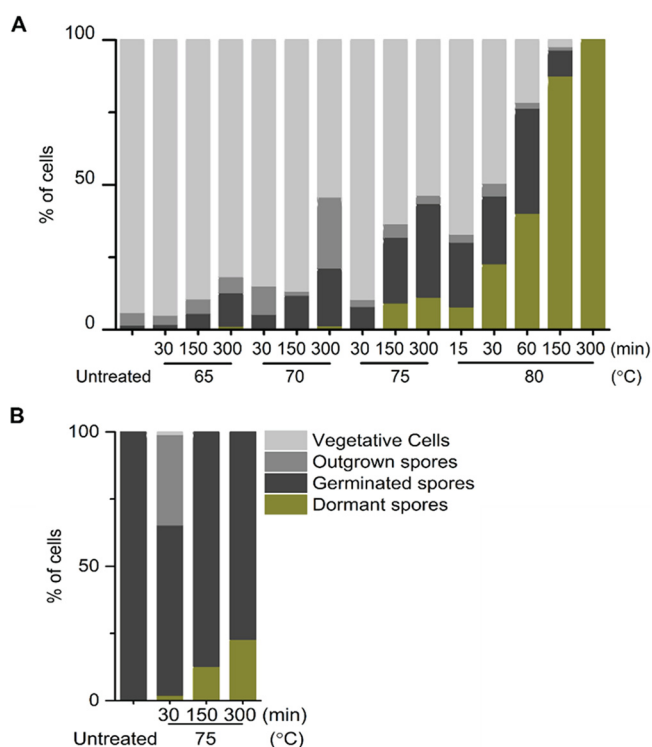
<sup>b</sup> $T_1$ , time when spore commitment occurred following exposure to germinant;  $\Delta T_{\text{SlowLeakage}}$ , time between  $T_1$  and  $T_{\text{StartRelease}}$  (start of rapid  $\text{Ca}^{2+}$ -DPA release);  $\Delta T_{\text{PhaseDarkening}}$ , time between  $T_{\text{StartRelease}}$  and  $T_{\text{PhaseDark}}$  (time of the appearance of the phase-dark spore); IQR, interquartile range.

$\text{Ca}^{2+}$ -DPA is via the SpoVA channel in the spore inner membrane and is slowed markedly when the cortex lytic enzyme CwlJ is absent, SpoVA proteins and cortex lytic enzymes are potential heat damage targets when the thermal treatment temperatures rise above 70°C (17).

**Treatment at 65°C can be sufficient to damage essential cell growth molecules stored in spores.** The accumulated heat damage, potentially in the germination apparatus,



**FIG 6** Frequency distribution of the times of appearance of phase dark spores ( $T_{\text{PhaseDark}}$ ). *B. subtilis* PS832 spores were preheated at 65, 70, 75, or 80°C for various times or not heated (ut, untreated). Subsequently, spores were immobilized on a MOPS-agarose pad at 37°C with (10 mM each) AGFK (A to D) or 10 mM L-valine (E) for 16 h of time-lapse imaging. The line profile is the log-normal distribution fit of the time to phase darkening. The numbers of germinated spores examined by microscopy are given in Table 1.



**FIG 7** Quantification of spores with different fates after 16 h of time-lapse tracking. *B. subtilis* PS832 spores in water were heated at 65, 70, 75, or 80°C for different times. Subsequently, spores were immobilized on a MOPS-agarose pad at 37°C with (10 mM each) AGFK (A) or 10 mM L-valine (B) for 16 h of time-lapse imaging. The numbers of individual spores examined by microscopy are given in Tables 1 and 2.

is likely responsible for the broad  $T_{\text{PhaseDark}}$  distribution of spores treated at 75 and 80°C, differing from the narrow distribution seen in longer treatment times at 70°C, indicative for heat-induced germination activation. Instead, at 80°C, the distribution at  $T_{\text{PhaseDark}}$  approached the  $T_{\text{PhaseDark}}$  distribution of untreated spores at all treatment times applied (Fig. 6C and D). In addition, decreased germination efficiency was observed in spores treated at 75 and 80°C, and this decline had a positive correlation with time/temperature (Table 1, Fig. 7). The subsequent outgrowth and cell growth of heat-treated spores was also monitored in our time-lapse imaging. We noticed a remarkable decrease (~41%) in the cell division events of spores treated at 70°C for 300 min compared to those of untreated spores, although 100% of spores of both groups germinated (Tables 1 and 2, Fig. 7). The decline of cell division events occurred in spores treated at 65°C for 150 min and increased when the treatment temperature and time increased (Tables 1 and 2, Fig. 7). These data suggest that 65°C was sufficient to damage molecules essential for growth that were stored in spores, although spores themselves have high heat resistance. We did not find time-/temperature-dependent effects of heat on outgrowth, except that an inverse correlation between the treatment and the burst time was observed in the 80°C groups.

**Treatment at 75°C affects L-valine- and AGFK-induced germination similarly.** At the population level, 75°C treatment promoted AGFK-induced germination in a time-dependent manner, which was different from the results with L-valine-triggered germination (Fig. 1F and 2F). To better understand this difference, L-valine-induced germination was tracked by phase-contrast microscopy after spore heating at 75°C. Unlike spores supplemented with AGFK and MOPS (morpholinepropanesulfonic acid) medium, no cell division events occurred under nutrient conditions with L-valine and MOPS. Similar to the cell growth in LB medium (Fig. 4), AGFK acted as nutritional assistance for cell growth. In both L-valine- and AGFK-induced germination, the germination efficiency decreased with increased heat treatment time. In addition,  $T_1$  and  $\Delta T_{\text{SlowLeakage}}$  were shortened in a time-dependent positive manner,



**TABLE 2** Outgrowth and growth parameters of individual heat-treated *B. subtilis* PS832 spores in time-lapse images<sup>a</sup>

Nutrient(s), treatment		Time (min) of indicated event <sup>b</sup>									No. (%) of indicated events	
		$\Delta T_{burst}$			$\Delta T_{FirstCellDivision}$			$T_{Generation}$				
Temp (°C)	Time (min)	Median	IQR	P value	Median	IQR	P value	Median	IQR	P value	Burst	First cell division
AGFK												
Untreated		219.8	106.5		192.0	81.0		250.6	91.4		159 (86.4)	172 (93.5)
65	30	204.8	89.5	0.13	163.0	58.5	< 0.01	210.5	94.1	< 0.01	175 (81.0)	205 (94.9)
	150	205.7	62.1	< 0.01	179.5	58.0	0.14	288.1	230.9	< 0.01	235 (89.4)	231 (87.8)
	300	239.3	118.7	0.14	167.0	62.0	< 0.01	218.8	88.5	< 0.01	196 (83.8)	189 (80.8)
70	30	229.8	145.5	0.23	179.0	85.3	0.64	241.8	66.7	0.67	183 (89.3)	173 (84.4)
	150	222.3	93.6	0.61	150.0	44.8	< 0.01	189.4	57.3	< 0.01	188 (83.2)	190 (84.1)
	300	287.8	145.4	< 0.01	242.5	87.5	< 0.01	371.6	149.2	< 0.01	163 (76.5)	117 (54.9)
75	30	197.9	118.0	0.02	186.0	55.0	0.37	206.9	58.9	< 0.01	186 (88.2)	185 (87.7)
	150	284.9	124.7	< 0.01	201.0	72.8	0.03	232.7	103.8	0.12	130 (67.0)	120 (61.9)
	300	269.8	102.6	< 0.01	177.5	54.0	0.29	183.0	60.6	< 0.01	74 (52.1)	75 (52.8)
80	15	234.8	93.9	0.05	211.0	73.8	< 0.01	293.2	216.2	< 0.01	162 (64.5)	162 (64.5)
	30	271.9	125.4	< 0.01	179.0	80.0	0.53	246.9	114.2	0.94	83 (52.5)	77 (48.7)
	60	307.0	196.3	< 0.01	206.0	96.5	0.03	204.1	61.0	< 0.01	54 (21.1)	53 (20.7)
	150	499.8	34.0	< 0.01	331.0	49.0	0.03	222.4	0.0	0.70	3 (3.4)	2 (2.2)
	300										1 (0.7)	0 (0)
L-Valine												
Untreated											0 (0)	0 (0)
75	30	584.8	261.0		519.9						35 (34.7)	1 (1.0)
	150										0 (0)	0 (0)
	300										0 (0)	0 (0)

<sup>a</sup>As described in the legend to Fig. 5, *B. subtilis* PS832 spores were treated with various temperature-time conditions, followed by being germinated and grown on a MOPS-agarose pad with (10 mM each) AGFK and 10 mM L-valine. Data were collected by SporeTrackerX (ImageJ Macro) as described in Materials and Methods. The significance of differences between heat-treated groups and the untreated group was measured by the Mann-Whitney test.

<sup>b</sup> $\Delta T_{burst}$ , time between  $T_{PhaseDark}$  (time of appearance of phase-dark spore) and  $T_{Burst}$  (time of escape of cell from spore coat);  $\Delta T_{FirstCellDivision}$ , time between  $T_{Burst}$  and  $T_{FirstCellDivision}$  (time of first cell division);  $T_{Generation}$ , time between the first division and the end of the linear part of the  $\log_2(\text{area})$ -versus-time plot; IQR, interquartile range.

and  $\Delta T_{PhaseDarkening}$  was prolonged in a time-dependent positive manner (Table 1). In other words, the 75°C heat treatment affected AGFK- and L-valine-induced germination in the same way. The detectable time-dependent positive correlation in AGFK-induced germination at the population level was due to the bigger differences in the germination parameters of different groups. However, it was not clear why AGFK-induced germination was more sensitive to the change in heat activation time.

## DISCUSSION

Heterogeneous spore germination in processed food is a major concern for food spoilage and foodborne disease. Promoting synchronous germination and then inactivating germinated spores and vegetative cells would extend food shelf life at minimal cost in the decontamination process (10). In the laboratory, spore germination heterogeneity can be stimulated by a heat activation procedure consisting of an exposure for a given time period to sublethal temperatures. However, with elevated temperatures, heat treatment could result in the accumulation of damage and further increases in the germination and outgrowth heterogeneity (18, 19). Thus, thermal treatment can in fact create increased difficulties in food processing. In the current work, we measured the spore germination and growth profiles after spore heat treatment at sublethal temperatures for a variety of treatment times. We hoped to determine the optimal heating time/temperature combination for optimal *B. subtilis* spore germination and outgrowth.

Our study showed that 50 to 65°C is the optimal heat activation temperature for *B. subtilis* PS832 spores obtained from cells sporulated in 2× SG medium (37) at 37°C. The 50 to 65°C treatment resulted in the largest decrease in OD<sub>595</sub> without any germination apparatus damage. Such damage, as well as damage to molecules essential for growth stored in the dormant spore, which is linked to increased levels of spore inactivation, starts accumulating after treatment at 70 to 75°C. Published differential scanning calorimetry (DSC) thermogram profiles of *B. subtilis* spores present a reversible endothermic peak at

around 60°C, which is followed by a second, irreversible endothermic peak initiated at around 70°C (20). It is suggested that the reversible endothermic peak is the outcome of heat activation, and the following irreversible peak is referred to as an inactivation peak. Our measured results fit the prediction from the *B. subtilis* thermogram profile. While we did not measure the heat activation/inactivation temperatures for other spore formers, it is likely that spores of other *Bacillus* species require similar heat activation/inactivation temperatures, based on their typical thermogram profiles. Indeed, the activation endothermic peaks are at 56°C for *Bacillus megaterium* spores and ~55°C for *Bacillus cereus* spores (21, 22). Notably, the heat activation temperature has a positive correlation with heat resistance. For instance, superdormant spores or very-heat-resistant spores require higher heat activation temperatures (5, 23, 24). It would be useful to investigate the optimal heat activation conditions for heat-resistant spores that are major troublemakers in the food industry (2).

Sublethal heat activation is reversible in a temperature-dependent manner (25). Various phenomena have been observed in heat-activated spores, including but not limited to some release of Ca<sup>2+</sup>-DPA, a loss of coat proteins and small acid-soluble proteins, a change in the ultrastructure of the spore coat, including the development of comblike striations on the coat, and reversible protein denaturation (13, 26–30). However, how heat activation works and why spores require 50 to 65°C to gain the optimal heat activation is still unclear. Studies have proposed that heat activation might promote germination by directly increasing the level of functional GRs and affecting the state of the spore inner membrane, which surrounds the GRs (9, 31). Our data support the notion that GRs' response to nutrient triggers has an important role in heat activation, considering the different germination behaviors in L-valine- and AGFK-induced germination processes seen previously and in the current work.

The exact mechanism of heat activation of spores remains unclear, as does the full mechanism of heat damage to spores. One study showed that the inner membrane of the spore was damaged in the spore heat inactivation process (32). Other studies also suggested that moist heat (e.g., 89°C for 2 h) killed spores by protein denaturation in *B. subtilis*, *B. cereus*, *B. megaterium*, and *Clostridium perfringens* spores (6, 19, 33). The current work shows that the effects of thermal treatment on spores are different in different growth stages, including germination, outgrowth, and subsequent vegetative cell growth. Further study should focus on the relation between the thermal properties of bacterial spores and their proteome-wide analysis. Such work might identify one or more macromolecules and/or processes key to the various thermal stress effects described in the current study.

## MATERIALS AND METHODS

**Strain used and spore preparation.** *B. subtilis* strain PS832, a prototrophic derivative of *B. subtilis* strain 168, was obtained from the laboratory of Peter Setlow. Spores were obtained from cells cultured at 37°C in 2× SG medium in Erlenmeyer flasks under continuous rotation at 200 rpm for 2 days, using the procedure detailed by Abhyankar et al. (34). Two-day sporulation cultures were harvested, followed by extensive washing with MilliQ water and further purification of phase-bright spores using HistoDenz density gradient centrifugation (35). Small volumes of aqueous spore suspensions (OD<sub>600</sub> of ~60, in MilliQ water) were dispensed, frozen, and stored at –80°C until use, after confirming the phase brightness of the spores (≥98%) by phase-contrast microscopy, as well as the absence of any visible debris.

**Heat treatment.** The heat treatment procedure was modified based on Luu's methods, in which spores were prepared at 37°C on 2× SG medium agar plates (9). Here, 200-μL amounts of aqueous spore suspensions (OD<sub>600</sub> of ~2, ~3 × 10<sup>8</sup>/ml) were incubated at 45, 50, 55, 60, 65, 70, 75, or 80°C for 0, 15, 30, 60, 150, 240, and 300 min, followed by cooling in a water-ice bath (≥15 min).

**Monitoring germination and growth at the population level.** The exchange of spore core Ca<sup>2+</sup>-DPA for water during germination results in a drop in phase brightness, such that spore germination can be monitored by following spores' optical density at 595 nm (OD<sub>595</sub>). Heat-treated aqueous spore suspensions (final OD<sub>595</sub> of ~1, 150 μL) were dispensed into 96-well microtiter plates and germinated (and grown) in either HEPES buffer (25 mM) or LB medium supplemented with either L-valine (10 mM) or AGFK (L-asparagine, glucose, fructose, and potassium chloride at 10 mM each). The OD<sub>595</sub> was measured every 5 min at 37°C from cultures that underwent continuous shaking in a Multiskan FC microplate photometer (Thermo Fisher Scientific). Data were collected from at least two independent experiments with at least two replicates for each individual experimental condition.

**Spore viability after heat treatment.** Heat-treated aqueous spore suspensions (OD<sub>595</sub> of ~2) were diluted 1/10 in MilliQ water at room temperature. Aliquots of further dilutions were plated on LB agar plates that were incubated at 37°C overnight, followed by incubation at room temperature until no further colonies appeared. Colonies were counted to determine spore viability in least two independent biological experiments, with at least two replicates for each heat treatment condition.

**Monitoring germination, outgrowth, and cell growth of individual spores.** Heat-treated PS832 spores (0.5  $\mu$ l, OD<sub>595</sub> of  $\sim$ 2) were immobilized on 1% agarose pads supplemented with MOPS minimal medium and additional nutrient germinants (10 mM valine or 10 mM each AGFK) in an air-containing chamber, as described elsewhere (36). Time-lapse phase-contrast images were captured by a phase-contrast microscope, which was coupled with a Nikon Ti microscope, a numeric aperture (NA) 1.45 Plan Apo  $\lambda$  100 $\times$  oil Ph3 DM objective, a C11440-22CU Hamamatsu ORCA flash 4.0 camera, and NIS Elements software. Spores from each heat treatment condition were imaged twice at 37°C to track spore germination, outgrowth, and cell growth in detail. For each imaging process, 6 to 9 fields of view were recorded in parallel once every 1 min for 16 h.

Images were analyzed by using the modified ImageJ macro SporeTrackerX, which runs with the assistance of the ImageJ plugin ObjectJ (13). SporeTrackerX is capable of assessing multiple germination, outgrowth, and cell growth events based on the drop in spore brightness, "jump-like" surface area increase, and cell surface area increase in phase-contrast time-lapse images (13, 36). Briefly, the spore for analysis was labeled in the first frame of the time-lapse images, and subsequently, the changes in brightness and the log<sub>2</sub>(area) of the spore over time were assessed, stored, and plotted by SporeTrackerX, as shown by the results in Fig. 1. The conspicuous fast drop is almost certainly due to the rapid Ca<sup>2+</sup>-DPA release and the subsequent cortex hydrolysis induced by the released Ca<sup>2+</sup>-DPA (Fig. 1A and C) (4). Both the time of the start of rapid Ca<sup>2+</sup>-DPA release ( $T_{\text{StartRelease}}$ ) and the time of the appearance of the phase-dark spore ( $T_{\text{PhaseDark}}$ ) were marked and stored for quantitative analysis. In addition, a slow decline in brightness occurred before the rapid drop, and the start time of the slow drop of brightness was stored as  $T_1$  (Fig. 5A and C). The slow drop is most likely due to the slow leakage of Ca<sup>2+</sup>-DPA (4). In the growth plot,  $T_{\text{Burst}}$  was defined as the time of escape of the cell from the spore coat,  $T_{\text{FirstCellDivision}}$  was the time of the first cell division, and the generation time ( $T_{\text{Generation}}$ ) was the time between the first division and the end of the linear part of the log<sub>2</sub>(area)-versus-time plot. (Fig. 5B). Based on the measurements described above, SporeTrackerX also calculated the following parameters:  $\Delta T_{\text{SlowLeakage}}$  is the time between  $T_1$  and  $T_{\text{StartRelease}}$ ;  $\Delta T_{\text{PhaseDarkening}}$  is the time between  $T_{\text{StartRelease}}$  and  $T_{\text{PhaseDark}}$ ;  $\Delta T_{\text{Burst}}$  is the time between  $T_{\text{PhaseDark}}$  and  $T_{\text{Burst}}$ ; and  $\Delta T_{\text{FirstCellDivision}}$  is the time between  $T_{\text{Burst}}$  and  $T_{\text{FirstCellDivision}}$ .

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